Handbook of nutrition in heart health

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Wageningen Academic
Publishers

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Human Health Handbooks no. 14

ISSN 2212-375X

Wageningen Academic
P u b l i s h e r s

Wageningen Academic Publishers
P.O. Box 220
6700 AE Wageningen
The Netherlands
www.WageningenAcademic.com
copyright@WageningenAcademic.com

EAN: 9789086863082
e-EAN: 9789086868537
ISBN: 978-90-8686-308-2
e-ISBN: 978-90-8686-853-7
DOI: 10.3920/ 978-90-8686-853-7

First published, 2017

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Preface

Cardiovascular disease (CVD) includes a variety of heart and vascular conditions: hypertensive heart disease, stroke, and ischemic heart disease. Causation includes diet, tobacco, drugs of abuse, alcohol and lack of exercise. This book's experts review the validity of various dietary approaches in prevention and treatment of CVD for promotion of heart health. Although CVD mortality is declining in the developed countries it remains the primary cause of death worldwide. In the USA, CVD affects primarily older adults with 70% of those 60-80-years-old and 85% of older people. Therefore what dietary factors accelerate or delay CVD? Which are the healthful dietary factors readily available to people to prevent CVD? Some risk factors, age, gender and family history cannot be changed. Which other lifestyle approaches, nutritional and dietary extract supplementation in older adults alter or prevent heart disease?

Section 1 'Vitamins and minerals in heart health'

Vitamins and minerals are widely used as supplements. For various reasons the elderly may have low intakes or absorption, or may be taking them for other reasons in large amounts. The book reviews the role of antioxidant vitamins in reducing myocardial infarct in patients being treated by surgery. A specific set of antioxidant vitamins E and D and carotenoids, precursors of vitamin A are described for heart health. The importance of Vitamin D in heart health led to reviews of its role alone on CVD as well as specifically on heart failure. The genomic and nongenomic controls of vitamin D were researched relative to the heart function. Finally a broad intake of vitamins on CVD was summarized. Clearly vitamins can play a role in heart health.

Section 2 'Nutrition and nutrition counseling in heart function and growth'

Many people work with the elderly and especially those with or at risk of CVD, using diet, food and nutrition. Therefore the role of foods groups, something within the control of the patient, is described specifically for heart health. The role of food in the diet in modifying systemic and neural inflammation in obesity and metabolic syndrome is presented. This is critical to understanding CVD as these conditions are major contributors to heart disease. To assess change and determine nutritional needs the heart disease burden needs to be evaluated. This was reviewed through modeling studies. Finally this section concludes with a discussion of copper in the diet and health of the heart.

Section 3 'Dietary supplements, herbs and foods in health'

This is the major and most diverse section of the book including causes of CVD. Taurine is a small molecule that can be in the diet and its role in heart disease is reviewed. The additional chapter focuses on another cause of CVD looking at environmental causes. Then bioactive nutrients describe the mechanisms of actions of nutrients by reviewing the potential impact on CVD risk factors. Similarly, dietary considerations of nutrients on cardiometabolic risk are important in senior citizens. Phytosterol, another dietary material, is described and its role in coronary artery disease. Small molecules with dietary importance but not vitamins or minerals can play a real role in CVD induction or prevention. Therefore, fatty acids in the diet are described for heart disease and function. Additionally green leaves in the diet are described in a model for the heart. Then scientists describe a variety of herbs and functional foods yielding materials acting on the heart. Salt is a key dietary material. However, the epidemiology of the role of different levels of salt intake on heart health and at high levels affects heart disease. Finally, a second review by resveratrol on a major precursor on CVD is described in inducing metabolic syndrome in the obese.

Section 4 'Protein and energy in heart health'

Clearly calories and protein intake can be important for the heart and body functions. These can be subject to change especially in lower functioning bodies during CVD and older age. A major source of protein and calories in seniors include dairy products. Therefore their roles in cardio-metabolic risk factors important in CVD and heart health are reviewed. Another chapter updates the role of red wine, its resveratrol via hormesis on protection of the heart and its function, modeling what may be occurring with consumption of other fruits and their non-nutritive constituents.

Section 5 'Microbes in heart health'

Microflora play important roles in the metabolism of non-nutrients in the gastrointestinal tract. They appear to alter the function of the heart in some cases including via absorption of nutrients, nutraceuticals and macronutrients. Therefore a major review chapter asks the question if probiotics and prebiotics contribute to heart health. Another set of authors found and described their role in heart function. Finally a specific set of Indian fungi were reviewed for their unique role in heart health.

In summary, nutrients, nutraceuticals, macronutrients and gastrointestinal microbes modified by prebiotics and probiotics play important roles in heart health and disease.

Vitamins and minerals in heart health

1. The effectiveness of antioxidant vitamins in reducing myocardial infarct size in patients subjected to percutaneous coronary angioplasty

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Abstract

Acute myocardial infarction (AMI) is the leading cause of mortality worldwide. Reperfusion therapy with systemic thrombolysis and percutaneous coronary angioplasty (PCA), have decreased the risk of mortality. These procedures have been aimed to recover the blood flow in the cardiac zones affected by the occlusion of a branch of the coronary artery. However, damage is generated in the heart tissue, known as myocardial reperfusion injury (MRI), an event associated with increased oxidative stress. Reactive oxygen species are able to trigger cell death pathways, and also myocardial structural and functional impairment. Studies on animal models of AMI suggest that lethal reperfusion accounts for up to 50% of the final size of a myocardial infarct, a part of the damage likely to be prevented. In clinical trials exogenous antioxidant vitamin therapy has been used during reperfusion in patients with ST-segment-elevation myocardial infarction subjected to PCA, showing encouraging results in preventing MRI. Nevertheless, further studies are still lacking to elucidate the mechanism accounting for this cardioprotective effect.

Keywords: myocardial reperfusion injury, oxidative stress, vitamin C

Key facts

- Cardiovascular diseases correspond to 1/3 of all deaths worldwide by non-communicable diseases in 2012, and include ischemic heart disease, stroke, arterial hypertension, peripheral artery disease, among others.
- Ischemic heart disease remaining as the principal cause of death over the past decade, and in 2012 was estimated in 7.4 million (13.2%) of total deaths in worldwide.
- Ischemic heart disease includes to myocardial infarction and angina. Myocardial infarction occurs when there is a partial or complete occlusion of coronary arteries by atherosclerotic plaques and circulating thrombus.
- Oxidative stress corresponding to imbalance between oxidative and antioxidant factors, with overproduction of reactive oxygen and nitrogen species and decreased levels of antioxidant defenses, causing oxidative cell damage.
- An antioxidant is a molecule with the ability to inhibit the oxidation of other molecules, preventing loss of electrons and formation of free radicals.

Summary points

- Reperfusion therapy by coronary angioplasty or systemic thrombolysis is the treatment of choice for acute myocardial infarction, reducing early mortality. However, this procedure paradoxically causes myocardial reperfusion injury (MRI).
- MRI occurs when blood flow is restored in an occluded (ischemic) area of the coronary arteries, causing cell death and structural and functional damage to the myocardium.
- Oxidative stress is a central mediator in MRI, causing direct and indirect cellular damage.
- Antioxidants administered exogenously have shown cardioprotective effects against MRI in experimental myocardial ischemia-reperfusion models and in some clinical trials.
- Patients with ST-segment-elevation myocardial infarction subjected to percutaneous coronary angioplasty treated with high doses of vitamin C infusion before or at the onset of reperfusion have shown beneficial effects.

Abbreviations

AMI	Acute myocardial infarction
ATP	Adenosine triphosphate
CK-MB	Creatine kinase-MB
LMRI	Lethal myocardial reperfusion injury
METC	Mitochondrial electron transport chain
MIR	Myocardial ischemia reperfusion
MPTP	Mitochondrial permeability transition pore
MRI	Myocardial reperfusion injury
NO	Nitric oxide
NOX	NADPH oxidase
8-OHdG	8-hydroxy-2-deoxyguanosine
PCA	Percutaneous coronary angioplasty
PCI	Percutaneous coronary intervention
ROS	Reactive oxygen species
STEMI	ST-segment-elevation myocardial infarction
TMPG	TIMI myocardial perfusion grade
XO	Xanthine oxidase

1.1 Introduction

Over the last 3 decades, mortality from acute STEMI has decreased due to the successful early reperfusion therapy by primary PCI or thrombolysis (Moran *et al.*, 2014; Roe *et al.*, 2010; White and Chew, 2008). However, according to the World Health Organization, AMI remains the leading cause of mortality worldwide. A timely and complete reperfusion is the most effective way to limit infarct size, but reperfusion also adds an additional reperfusion injury, contributing to increase the infarct size and reducing the beneficial effects of reperfusion therapy. This a phenomenon – called myocardial reperfusion injury – has been extensively studied in MRI experimental models for several years (Hausenloy and Yellon, 2013; Ibanez *et al.*, 2015). The MRI causes four types of cardiac dysfunction, being reversible the first two and irreversible the others: (1) reperfusion-induced arrhythmias; (2) myocardial stunning; (3) microvascular obstruction or no-reflow phenomenon; and (4) LMRI. LMRI is the most important because it may account for up to 50% of the myocardial infarct final size, as shown in both MRI experimental models and patients with STEMI applying therapeutic interventions solely at the onset of myocardial reperfusion, being a damage that can be prevented (Hausenloy and Yellon, 2013). Limit infarct size during reperfusion is important for the long-term prognosis of post-AMI patients, as these often develop heart failure and left ventricular adverse remodeling in proportion of the infarct size and cardiac dysfunction following myocardial infarction (Garcia-Dorado *et al.*, 2014; Gaudron *et al.*, 1993). The most important mediator of the MRI is oxidative stress, which has been proposed as a pharmacologic target for an exogenous antioxidant cardioprotective therapy. Administration of exogenous antioxidants, including vitamins, have been used to prevent the MRI in clinical trials

with STEMI patients subjected to PCI to reduce infarct size and improve clinical end-points, and the evidence shows that some of them significantly reduced oxidative stress and myocardial damage as well as improved cardiac function and clinical outcomes (Ekelof *et al.*, 2014). In the following paragraphs, we describe the pathophysiological mechanisms involved in MRI and the role of oxidative stress, together with highlight the main clinical findings of the use of antioxidant vitamins in patients with STEMI subjected to PCI.

1.2 Role of oxidative stress in myocardial ischemia-reperfusion injury

When an acute occlusion in the coronary artery occurs, the blood flow to myocardial tissue decreases, depriving cardiac cells from oxygen and nutrients, and causing a state of prolonged ischemia. The Figure 1.1 shows the main metabolic and biochemical changes within the cardiomyocyte as a consequence of ischemia.

The lack of oxygen affects the process of mitochondrial respiration, thus declining production of ATP levels, leading to significant cell death by necrosis in cardiomyocytes. In addition, the absence of oxygen causes a switch in glycolytic pathway to anaerobic respiration with intracellular accumulation of lactic acid and decrease in intracellular pH (Ambrosio *et al.*, 1987; Luna-Ortiz *et al.*, 2011; Raedschelders *et al.*, 2012). The latter increases the Na^+ influx through the Na^+/H^+ exchanger, while the ATP depletion stops Na^+ efflux through Na^+/K^+-ATPase. This intracellular Na^+ accumulation activates Na^+/Ca^{2+} exchangers in the reverse direction, leading to cytosolic

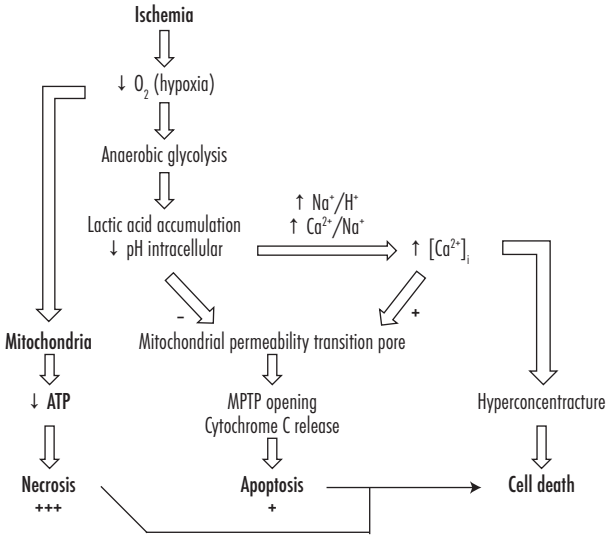

Figure 1.1. Metabolic and biochemical changes in the cardiomyocyte and cell death pathways during myocardial ischemia. It is noted that necrosis contributes more than apoptosis to the death of cardiomyocytes during ischemia in acute myocardial infarction.

Ca^{2+} overload (Avkiran and Marber, 2002; Hausenloy and Yellon, 2013), where the sarcoplasmic reticulum is unable of uptaking Ca^{2+} from the cytosol because the sarco(endo)plasmic reticulum Ca^{2+}-ATPase transporter needs ATP to function (Rossi and Dirksen, 2006). These high levels of intracellular Ca^{2+} induce cell hypercontracture (Luna-Ortiz *et al.*, 2011) and MPTP opening, a protein complex of the mitochondrial inner membrane, thus collapsing the mitochondrial membrane potential, producing mitochondrial matrix swelling, and allowing the release of cytochrome c into the cytosol that leads to cell death by apoptosis (Ong *et al.*, 2015; Raedschelders *et al.*, 2012). However, this is attenuated by acidic intracellular pH because it exerts an inhibitory effect on the MPTP opening (Bernardi *et al.*, 1992; Hausenloy and Yellon, 2013; Raedschelders *et al.*, 2012).

The coronary revascularization post-myocardial ischemia rapidly increases the level of tissue oxygenation, which triggers a series of mechanisms producing LMRI. The most important mediators of this process are shown in the Figure 1.2 and are described below.

1.2.1 Oxidative stress

During the first minutes of the onset of myocardial reperfusion, a burst of ROS occurs, in accordance with several experiments demonstrating direct measurements of free radicals in isolated hearts and *in vivo* MIR models (Grill *et al.*, 1992; Zweier *et al.*, 1987). The potential enzymatic sources

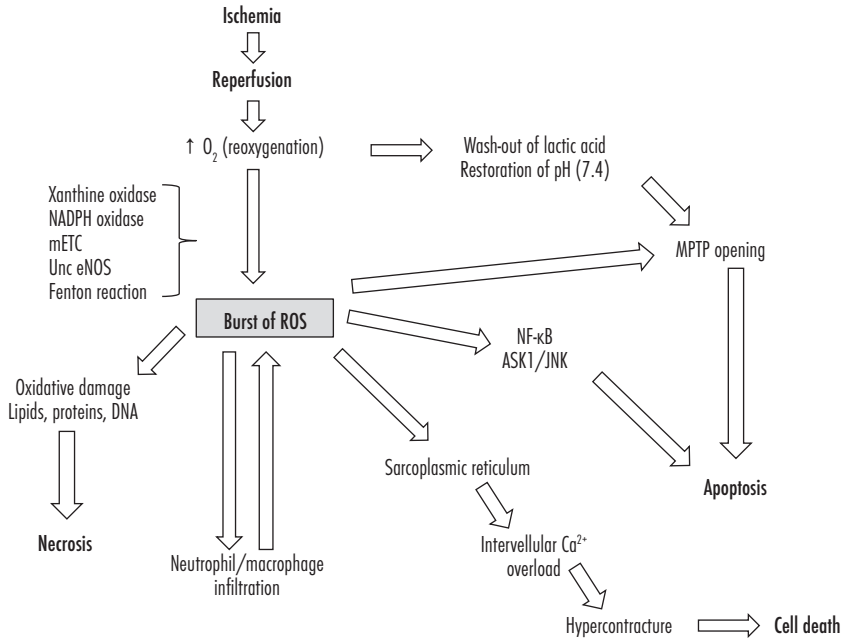

Figure 1.2. Pathophysiological mechanism of the myocardial reperfusion injury and role of the oxidative stress as the main mediator.

of ROS production in cardiac tissue exposed to ischemia-reperfusion are XO in endothelial cells, NOX in neutrophils, METC, uncoupled nitric oxide synthase, and hydroxyl radical from hydrogen peroxide plus Fe^{2+}, known as Fenton reaction (Granger and Kvietys, 2015; Raedschelders *et al.*, 2012). XO is an isoform of xanthine oxidoreductase enzyme; XO activation and ATP catabolism to hypoxanthine occurs in ischemic period, generating high levels of superoxide and hydrogen peroxide together with uric acid from oxygen and accumulated hypoxanthine (or xanthine), when blood flow is restored (Makoto and Takashi, 2007; Raedschelders *et al.*, 2012). NOX is a superoxide-producing enzyme, present mainly in immune system cells, also in cardiac cells, and its inducible isoform NOX-2 is localized in cell membrane. The important role of the NOX family in MRI has been shown in experimental studies where NOX-isoform specific knockout mice have significantly reduced infarct sizes compared to wild type controls, confirming these results in buffer perfused Langendorff models (Braunersreuther *et al.*, 2013). During ischemia, uncoupled oxidative phosphorylation in mitochondria occurs due to lack of oxygen as electron acceptor, but high levels of oxygen in reperfusion, and reactivation of the Krebs cycle, increase the leak of superoxide anion at the level of complex I and III in the METC, where electron transport is stopped because of the lack of cytochrome c and cardiolipin (Raedschelders *et al.*, 2012). NOS is an NO-producing enzyme, a potent vasodilator, that could be uncoupled during ischemia-reperfusion due to oxidation of tetrahydrobiopterin cofactor, generating superoxide instead of NO (Granger and Kvietys, 2015; Raedschelders *et al.*, 2012). Finally, the iron that participates in the Fenton reaction comes from intracellular ferritin of cardiac cells due to acid pH during ischemia and superoxide anion, together with the efflux into the extracellular space by necrosis (Biemond *et al.*, 1984; Chevion *et al.*, 1993; Funk *et al.*, 1985).

The burst of ROS at the onset of myocardial reperfusion overwhelms the endogenous antioxidant defenses (superoxide dismutase, catalase, glutathione peroxidase, etc.), causing free radical propagation reactions with direct damage to cellular biomolecules, as lipid peroxidation, protein oxidation/nitration, and DNA damage (Avery, 2011; Raedschelders *et al.*, 2012), and can induce redox-sensitive intracellular pathways as nuclear factor kappa B and apoptosis signal-regulating kinase 1/c-Jun N-terminal kinases, which are related with apoptosis in this context (Gloire *et al.*, 2006; Sinha *et al.*, 2013). Furthermore, high levels of ROS actively induce MPTP opening, and intracellular Ca^{2+} overload due to direct damage on sarcoplasmic reticulum, leading to hypercontracture and cell death (Hausenloy and Yellon, 2013; Raedschelders *et al.*, 2012).

1.2.2 Intracellular pH

The intracellular acidic pH generated in ischemia returns to physiological values during myocardial reperfusion due to a wash out of lactic acid from intracellular (Ambrosio *et al.*, 1987), leading to MPTP opening because inhibitory effect of acidic pH is no longer present. Simulated ischemia-reperfusion conditions in cultured neonatal rat cardiac myocytes, demonstrated that when intracellular acidic pH increases to 7.4 occurs hypercontracture and cell death. In addition, free Ca^{2+} increases during simulated ischemia as well as in simulated reperfusion (Bond *et al.*, 1993). Other *in vitro* model of ischemia-reperfusion in cultured cardiac myocytes and perfused papillary muscles demonstrated that inhibition of Na^+/H^+ exchanger delayed the increase of

intracellular pH after reperfusion and prevented reperfusion-induced cell death, but did not reduce the increase in intracellular free Ca^{2+} (Lemasters *et al.*, 1996). By contrast, reperfusion with inhibition of Na^+/Ca^{2+} exchanger decreases intracellular free Ca^{2+} but does not reduce cell death. These results suggest that acidotic pH is generally protective in ischemia-reperfusion, and Na^+/H^+ exchanger contributes to reperfusion washout effect on intracellular acidic pH, leading to a Ca^{2+}-independent lethal reperfusion injury in cardiomyocytes.

1.2.3 MPTP opening

Within the first few minutes of myocardial reperfusion, MPTP opening occurs together with the burst of oxidative stress and intracellular pH normalization, these two factors being the main contributors (Kim *et al.*, 2006; Seidlmayer *et al.*, 2015). On the other hand, Ca^{2+} overload seems not to be a causative factor in ischemia-reperfusion model. In adult rat myocytes, both cytosolic and mitochondrial Ca^{2+} increases during ischemia but decreases to basal levels in the first minutes of reperfusion. Ca^{2+} overload occurred late in both compartments, event that was prevented by MPTP inhibitors. Besides, intramitochondrial Ca^{2+} chelation did not prevent cell death after reperfusion. Thus, Ca^{2+} overload appears to be the consequence of bioenergetic failure after MPTP opening (Kim *et al.*, 2006). Another study showed that, at the onset of reperfusion, there is a transient increase in cytosolic Ca^{2+} levels together with a simultaneous transient sarcoplasmic reticulum Ca^{2+} depletion (Valverde *et al.*, 2010), corroborating the latter. The MPTP is a potential pharmacological target to prevent LMRI, and experimental studies with MPTP inhibitors (such as cyclosporin A), at the onset of myocardial reperfusion, has been reported to reduce myocardial infarction size by 40-50% (Argaud *et al.*, 2005; Skyschally *et al.*, 2010).

1.2.4 Inflammation

Ischemia is associated with slow infiltration of neutrophils, but recruitment toward the necrotic zone is favored after reperfusion by increased ROS exacerbation that triggers up-regulation of adhesion molecules (P-selectin, CD11/CD18, ICAM-1) in cardiomyocytes, with cytokines (TNFα, IL-1, IL-6, IL-8, NAP-1, PAF, MIP-2) and complement which are released from ischemic-reperfused myocardium. Neutrophils adhesion to coronary vascular endothelium occurs rapidly (within minutes) after onset of reperfusion, with abundant accumulation into the infarct zone during the following 6 hours. Neutrophils release more than 20 different proteolytic enzymes (hydrolases, metalloproteinases, and proteases) and are a major ROS source by generating superoxide anions through NOX, positioning them as important contributors to MRI (Vinten-Johansen, 2004).

1.3 Antioxidant vitamins use to prevent myocardial reperfusion injury in patients subjected to percutaneous coronary angioplasty

According to the evidence shown in experimental models, oxidative stress plays a central role in MRI during the first minutes and triggers mechanism of cell death that extend over time. The

use of antioxidants *in vitro*, *ex-vivo* and *in vivo* MIR models have demonstrated beneficial effects (Gao *et al.*, 2002; Grill *et al.*, 1992; Guaiquil *et al.*, 2004; Onogi *et al.*, 2006; Peng *et al.*, 2011), delivering a positive background to be proposed as adjunctive therapy in clinical practice. In that sense, the protocols of some clinical trials consider the use of antioxidant vitamins (mainly vitamin C) during reperfusion in patients with STEMI subjected to PCA, and the results are mentioned below.

It has recently been published a randomized, double-blind, placebo-controlled trial (Valls *et al.*, 2016) conducted in 53 either-sex patients with diagnosis of STEMI with indication for primary PCA, with their first myocardial infarction, from three clinical centers of the public health system, and high levels of ascorbate (320 mmol/l) was administered through an infusion, given prior the restoration of the coronary flow and up to 2 hours, which was then followed by oral treatment with vitamin C (500 mg/12 h) plus vitamin E (400 IU/day) for 84 days. The mean plasma ascorbate levels (mmol/l), immediately after the onset of reperfusion, for the control group were 0.03±0.04, while in the treated group were 9.79±3.87, declining to 1.79±1.51 at 6-8 hours after reperfusion. The left ventricular ejection fraction (determinated by using cardiac magnetic resonance) of the treated group was significantly higher (33%) than of the control group on day 84. Also, this was accompanied by an improvement in the microvascular dysfunction (TMPG of 2-3), after PCA, 95% of the patients in the treated group and 79% in the control group. In the biochemical parameters, there was a direct correlation between plasma antioxidant capacity (assessed by ferric reducing ability of the plasma assay) and the vitamin C levels following the onset of reperfusion. No significant differences were observed between the groups for the myocardial damage biomarker CK-MB, at baseline or at 6-8 hours after PCA. However, the treated group shows a significant decrease in the erythrocyte GSH levels at 6-8 hours after PCA, and a significant increase of the lipid peroxidation biomarker 8-isoprostane immediately after reperfusion. This clinical trial data obtained indicate that supraphysiological plasma levels of ascorbate protect against MRI in patients with STEMI subjected to PCA, although further studies are required to elucidate its mechanism of action against oxidative challenge that occurs at the beginning of reperfusion.

It is important to note that vitamin C (ascorbic acid or ascorbate) is a potent water soluble antioxidant in humans, which cannot be endogenously synthesized (Nishikimi and Yagi, 1996) and must be incorporated through vegetables and fruits (Haytowitz, 1995). Vitamin C is an electron donor and is oxidized to dehydroascorbate when acting as a reducing agent, returning to reduced form when it is used by the cell (Padayatty *et al.*, 2003). The administration by infusion of vitamin C can achieve supraphysiological plasma concentrations, as the oral administration in a dose range of 200 to 2500 mg/day producing a steady-state plasma concentration approximately by 80 µmol/l (0.08 mmol/l) due to apparent saturation of tissue uptake and in less degree by function of oral bioavailability and renal excretion (Graumlich *et al.*, 1997). This is necessary to abrogate oxidative stress-dependent processes in the first minutes of myocardial reperfusion because plasma levels of ascorbate about 10 mmol/l are capable to prevent chemical reaction of NO and superoxide anion (Jackson *et al.*, 1998), otherwise resulting in a highly peroxidant pathway.

In another clinical trial with 21 patients with AMI subjected to PCA, the treated group with the administration of vitamin C orally (2.0 g) followed by a constant infusion (20 mg/min), before reperfusion, had no differences in the levels of urinary 8-epi-prostaglandin F2α, a biomarker of oxidative stress *in vivo* measured by enzyme immunoassay, after PCA with respect to control group, whose marker levels were elevated following reperfusion. Thus, vitamin C fails to suppress the increase of the oxidative stress marker (Guan *et al.*, 1999). However, a prospective, single-center, randomized study with 56 enrolled patients, with clinically stable class I or II effort angina pectoris, subjected to elective PCI, compared the administration 1 g vitamin C infusion (16.6 mg/min), 1 hour before of intervention, versus placebo, and the results showed that 79% of the treated group achieved complete microcirculatory reperfusion (TMPG=3) vs 39% of the placebo group. Also, plasma levels of 8-OHdG and 8-iso-PGF2α were significantly reduced in vitamin C-treated group with respect to control group, indicating that vitamin C infusion improved the impaired microcirculatory reperfusion and oxidative stress markers levels in patients with angina subjected to elective PCI (Basili *et al.*, 2010).

In 2014, a prospective, single-center, randomized, placebo-controlled trial with 532 patients undergoing elective PCI demonstrated that administration of 3 g vitamin C infusion within 6 hours before the PCI, reduced the incidence of MRI defined by plasma levels of troponin I and CK-MB (measured by radioimmunoassay), compared to control group. Also, the biomarker of oxidative stress 8-OHdG was significantly lower in the vitamin C-treated group than in the control group, corroborating the beneficial effects of vitamin C against MRI and the underlying oxidative stress (Wang *et al.*, 2014).

It is important to note that antioxidant vitamins have been used in other clinical trials with patients diagnosed with AMI in order to improve clinical outcomes of interest, but those trials were not included because the administration protocol was different (not in reperfusion) or did not have PCA indication (Rodrigo *et al.*, 2013).

1.4 Conclusions and perspectives

This chapter focused on emphasizing the MRI as a current global clinical problem, describing in detail their pathophysiological bases pointing to oxidative stress as a central mediator, and allowing justify the use of antioxidant vitamins in both experimental models and clinical trials performed in patients with STEMI subject to PCA, to prevent this damage and improve long-term clinical prognosis.

There are many clinical studies that can be done to prove the effectiveness of the infusion of vitamin C in high doses during reperfusion to prevent MRI, and that will allow to determine more clearly its mechanism of action. Nevertheless, available data has shown encouraging results that allow further progress in the investigation of cardioprotective adjuvant therapy with antioxidants vitamins during PCA. In addition, this strategy has advantages in terms of costs, risks and benefits that potentially will certainly help millions of people worldwide.

Acknowledgments

This study is supported by FONDEF ID15I10285.

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2. The role of carotenoids, vitamin E and vitamin D in cardiovascular health

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Abstract

Globally cardiovascular disease (CVD) is the leading cause of death. Nutrition plays a central role in the prevention of many non-communicable diseases such as CVD, diabetes and cancer. A diet abundant in micronutrients from fruit and vegetables has shown to be an important role player in the prevention of CVD because of their anti-inflammatory properties. Carotenoids are the yellow, red and orange colour pigments in fruit and vegetables and possess certain biochemical properties due to their chemical structure. The most well-known carotenoids in the human diet include α- and β-carotene, β-cryptoxanthin, lutein, lycopene and xeaxanthin. Vitamin E is the collective name for tocopherols and tocotrienols which are fat soluble vitamins displaying potent antioxidant activity through their lipoperoxyl radical-scavenging characteristics. Vitamin E consists of eight lipophillic molecules which include α-, β-, γ- and δ-tocopherols and tocotrienols. Vitamin D is a fat-soluble vitamin manufactured by the skin which functions as a steroid hormone. The liver derived precursor of vitamin D, 7-dehydrocholesterol is converted in the upper layer of the skin by UVB radiation to pre-vitamin D where it is spontaneously converted to cholecalciferol or vitamin D3 (25(OH)D). Observational studies have shown that carotenoids, vitamin E and vitamin D can reduce the risk for CVD through their various effects on blood lipids as well as their anti-oxidant and anti-inflammatory properties. Randomised controlled trials (RCTs) e.g. dietary intake and supplementation studies with carotenoids, vitamin E and vitamin D showed diverse results. This article aims to summarise results from observational studies and RCTs to create a more holistic picture of the role of carotenoids, vitamin E and vitamin D in the prevention of CVD.

Keywords: inflammation, carotenes, tocopherol, tocotrienol, cholecalciferol

Key facts

- Globally cardiovascular disease (CVD) is the leading cause of death.
- Chemicals in fruit and vegetables such as carotenoids, vitamin E and vitamin D have been shown to reduce the risk for CVD.
- Carotenoids are the yellow, orange and red colour pigments in fruit and vegetables and possess anti-inflammatory properties. Carotenoids such as α- and β-carotene plus β-cryptoxanthin are vitamin A precursors.
- Vitamin E is a fat soluble anti-oxidant vitamin which protects cell membranes against oxidation and show strong anti-inflammatory characteristics. Vitamin E exists as tocopherols and tocotrienols.
- Vitamin D deficiency is a global phenomenon and linked to an increased risk of death from CVD. It is a fat-soluble vitamin manufactured by the skin and functions as a steroid hormone.

Summary points

- Chronic low grade inflammation is a key role player in the development of CVD.
- Selected nutrients from fruit and vegetables have anti-inflammatory properties which assist in reducing the risk for diseases of lifestyle such as CVD.
- Carotenoids have been associated with lower prevalence of CVD and are inversely associated with reduced levels of inflammatory biomarkers such as C-reactive protein (CRP).
- A combination rather than single tocopherols and tocotrienols assist in reducing CRP and thereby reduce the risk for CVD.
- Vitamin D deficiency is closely associated with death from CVD which could be addressed with widespread supplementation programmes.

Abbreviations

25(OH)D	25-hydroxyvitamin D
CLGI	Chronic low grade inflammation
CHD	Coronary heart disease
CRP	C-reactive protein
CVD	Cardiovascular disease
HDL-C	High-density lipoprotein cholesterol
HF	Heart failure
HOMA-IR	Homeostasis model assessment of insulin resistance
hs-CRP	High sensitivity C-reactive protein
IL	Interleukin
LDL-C	Low-density lipoprotein cholesterol
LURIC	Ludwigshafen risk and cardiovascular health
MI	Myocardial infarction
MONICA/KORA	Monitoring Trends and Determinants on Cardiovascular Diseases/ Cooperative Research in the Region of Augsburg
NHANES	National Health and Nutrition Examination Survey
RCT	Randomised controlled trial
TNF-α	Tumour necrosis factor alpha
TRF	Tocotrienol-rich fraction

2.1 Introduction

According to WHO, CVD is the leading cause of death worldwide. Mortality due to CVD substantially increased from 28.2% in 2000 to 31.4% in 2012 (WHO, 2013). Underlying contributors to CVD include overweight/obesity, hypertension, hyperglycaemia and hyperlipidaemia with inflammation as a common denominator. The role of inflammation in CVD, especially CLGI has been acknowledged over the past 20 years. Inflammation is a double-edged sword since it is a protective component in a range of complex biological responses to pathogens and cellular injury. Conversely a chronic low grade inflammatory state predisposes the human body to a number of degenerative diseases such as diabetes, CVD and cancer. CLGI is characterised by the secretion of systemic inflammatory markers such as pro-inflammatory cytokines, acute phase proteins and chemokines. CRP is an acute phase protein synthesised by the liver. Elevated levels of CRP reflect continuing systemic inflammation which in turn is associated with CVD and mortality.

Dietary micronutrient intake is considered an important component influencing the inflammatory environment during all stages of the inflammatory process. Amongst these, micronutrients derived from fruit and vegetables that showed encouraging results combatting the impact of CLGI on cardiovascular health include carotenoids, vitamin E as well as vitamin D. In large scale cross-sectional studies (Fung *et al.*, 2005; Lopez-Garcia *et al.*, 2004; Nettleton *et al.*, 2006; Panagiotakos *et al.*, 2006) micronutrients derived from fruit and vegetable were favourably

associated with inflammatory markers and concomitant effects on CVD risk (Liu *et al.*, 2000; Macready *et al.*, 2014; Ndanuko *et al.*, 2016; Wang *et al.*, 2014b).

2.2 Carotenoids

Carotenoids are the orange, yellow and red colour pigments found in fruit and vegetables of which α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin, and lycopene are the most abundant in the Western diet. Alpha- and β-carotene as well as β-cryptoxanthin serve as precursors of vitamin A.

2.2.1 Carotenoids and CVD risk

A number of epidemiological studies indicated associations between the risk of CVD or atherosclerosis. In the study of D'Odorico *et al.* (2000) data of 392 individuals who participated in the Bruneck Study was analysed to assess the relationship between plasma carotenoids and atherosclerosis of the carotid and femoral arteries. Alpha- and β-carotene plasma concentrations were significantly negatively associated with the prevalence of atherosclerosis in both arteries (P<0.004) as well as with the five year incidence of atherosclerotic lacerations in the carotid arteries (P<0.04) after adjustment for traditional CVD risk factors. The risk for atherosclerosis also decreased gradually with increasing levels of plasma α- and β-carotene (P<0.004) concentrations. The Kuopio Ischaemic Heart Disease Risk Factor Study followed 840 middle aged Finnish men over a seven year period. After adjusting for confounding factors such as age, body mass index, systolic blood pressure, smoking, physical activity and serum LDL-C, family history of CHD maximum intima media thickness was negatively associated with lycopene (P=0.005), α-carotene (P=0.002) and β-carotene (P=0.019), respectively (Karppi *et al.*, 2013). Other observational studies which also showed inverse associations between blood carotenoid concentrations and risk for CVD include Rissanen *et al.* (2000, 2003), Klipstein-Grobusch *et al.* (2000), Gey *et al.* (1993), and Sesso *et al.* (2004).

2.2.2 Carotenoids and inflammation

The putative anti-inflammatory effect of carotenoids has been examined in a number of human studies. Data from observational studies (Table 2.1) have shown consistent negative associations between blood CRP and carotenoid levels when carotenoids were consumed from fruit and vegetable sources. McGeoghegan *et al.* (2016) reported that among 1,531 individuals who participated in the UK's National Diet and Nutrition Survey that a dietary pattern low in chips, sugar and white bread but rich in fruit and vegetables was inversely related to serum CRP and positively to plasma carotenoids. This dietary pattern was also associated with a reduced odds ratio for diabetes. In the Aberdeen Prospective Osteoporosis Screening Study on data obtained from 1, 064 post-menopausal women, hs-CRP, IL-6, serum amyloid A and E selectin concentrations decreased prominently across increasing quintiles of serum carotenoids (Wood *et al.*, 2014). The authors concluded that a dietary pattern high in fruit and vegetables with the addition of fish,

Table 2.1. Observational studies on C-reactive protein (CRP) and carotenoids.

Reference	Country	Study design	Number of participants	Types of participants	Inflammatory markers studied	Specimen	Carotenoids studied	Outcome
Wood *et al.* (2014)	UK	cross-sectional	1,064	post-menopausal women	hs-CRP, IL-6, serum amyloid A, E selectin	serum	alpha and beta-carotene, beta-cryptoxanthin, lutein/zeaxanthin, lycopene	significant negative association between carotenoid component score on IL-6, CRP and E-selectin
Ford *et al.* (2003)	USA (NHANES)	cross-sectional	14,519	non-institutionalised men and women ≥20 years	CRP	serum	alpha and beta-carotene, cryptoxanthin, lutein/ zeaxanthin, lycopene	significantly inversely associated with CRP levels
Holt *et al.* (2009)	USA	cross-sectional	285	adolescent boys and girls	CRP, IL-6, TNF-a, 15-keto-dihydro-PGF2a	serum	beta-carotene	IL-6, TNF-a significantly inversely associated with beta-carotene
Valtueña *et al.* (2007)	Italy	cross-sectional	247	healthy adults	hs-CRP	serum	plasma beta-carotene	significant inverse relationship between beta-carotene and CRP across quartiles of beta-carotene
Hu *et al.* (2004)	USA MacArthurStudies of Successful Aging	prospective cohort	672	adults >65 years	CRP, IL-6	serum	serum beta-carotene	beta-carotene inversely associated with CRP and IL-6
Beydoun *et al.* (2012)	USA	cross-sectional (NHANES)	1,786	12-19-years-old adolescents	CRP	serum	serum total carotenoids (α- and β-carotene, lycopene, lutein+zeaxanthin, and β-cryptoxanthin)	total carotenoids inversely related to CRP
Wang *et al.* (2014b)		cross-sectional (NHANES 2003-2006)	2,856	apparently healthy men and women	CRP	serum	serum α-carotene, *cis*- and *trans*-β-carotene, β-cryptoxanthin, lutein+zeaxanthin and lycopene	after adjustment for covariates and exposure total serum carotenoids showed significant inverse associations with CRP

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Table 2.1. Continued.

Reference	Country	Study design	Number of participants	Types of participants	Inflammatory markers studied	Specimen	Carotenoids studied	Outcome
McGeoghegan *et al.* (2016)	UK	cross-sectional (National Diet and Nutrition Survey rolling programme, UK)	1,531	men and women (19-65 years)	CRP	serum	plasma total carotenoids	significant inverse association between dietary carotenoid intake and CRP
Boosalis *et al.* (1996)	USA	longitudinal epidemiologic study	85	women 77-99 y	CRP	serum	plasma α- and β-carotene, lycopene, lutein+zeaxanthin, β-cryptoxanthin and total carotenoids	significant inverse relationship between CRP, alpha and beta-carotene, lycopene and total carotenoids
Kritchevsky *et al.* (2000)	USA	NHANES III	4,557	non-smoking 25-55 y	CRP, fibrinogen and white blood cell count	serum	serum α- and β-carotene, lycopene, lutein+zeaxanthin and β-cryptoxanthin	adjusted concentrations of all carotenoids were significantly lower in those with CRP levels above 0.88 mg/dl
Wang *et al.* (2008a)	USA	Women's Health Study	2,895	women free of CVD and cancer with a 3.2% prevalence of daibetes, 35.1% hypercholesterolaemic and 34.6% hypertension	CRP	plasma	plasma α- and β-carotene, lycopene, lutein+zeaxanthin and β-cryptoxanthin	significant inverse association between β-carotene and CRP
Erlinger *et al.* (2001)	USA	NHANES III	14,470	current smokers, ex-smokers, and never smokers aged 18 years or older	CRP and white blood cells	serum	serum β-carotene	strong inverse association between β-carotene and CRP as well as white blood cell count

yoghurt, pulses, rice, pasta and wine in combination with an increased serum carotenoid level was associated with lower levels of inflammatory markers which in turn is suggestive of a lower risk for CVD.

Wang *et al.* (2008a) studied the cross-sectional association between plasma carotenoids and CRP in 2,895 middle aged women initially free of CVD and cancer who participated in the Women's Health Study. The authors reported for each 2 mg/l increase in CRP plasma β-carotene levels decreased by 1.3%. A marginally significant negative association was observed between α-carotene and CRP. Other carotenoids such as β-cryptoxanthin, lutein and xeaxanthin were not meaningfully associated with CRP. Studies from the NHANES series illustrated consistent negative associations between blood carotenoids and inflammatory markers. The analysis of Kritchevsky *et al.* (2000) which included 4,557 non-smoking individuals aged 25-55 years illustrated significantly lower levels of α- and β-carotene, β-cryptoxanthin, lycopene and lutein/xeaxanthin with CRP levels higher than 0.88 mg/dl. β-cryptoxanthin levels tended to decrease with an increase in fibrinogen while β-carotene levels were meaningfully lower in individuals with white blood cell counts higher than 7.85×10^9/l. Erlinger *et al.* (2001) reported significant inverse associations between serum β-carotene concentrations and CRP among 14,470 smokers, non-smokers as well as ex-smokers. A strong inverse association was indicated between white blood cell count and serum β-carotene across the study population. Similar results between CRP and carotenoids from other NHANES publications were reported (Beydoun *et al.*, 2012; Ford *et al.*, 2003; Wang *et al.*, 2014b)

Results from dietary manipulated clinical intervention trials as well as carotenoid dietary supplement trials showed mixed results. Biddle *et al.* (2015) investigated in a parallel RCT the effect of a low sodium vegetable juice on CRP levels in a group (n=22 in control group, n=18 in intervention group) of patients with HF. The drink provided 29.4 mg lycopene per day and was consumed over a 30 day period. Blood lycopene levels increased significantly in the intervention group, while a significant negative association between lycopene and CRP was reported in women (n=10) but not in men (n=8). In a RCT by Watzl *et al.* (2005) on 63 non-smoking men the effects of low, medium and high intakes of fruit and vegetables on non-specific markers of inflammation were studied. After a four week run-in period where subjects consumed two servings of vegetables and fruit per day they were arbitrarily assigned to consume either two, five or eight servings of carotenoid rich fruit and vegetables per day over a four week period. The highest intake of fruit and vegetables significantly increased plasma carotenoid levels compared to the lowest intake towards the end of the study. CRP was also significantly reduced in the subjects who consumed eight servings per day of fruit and vegetables compared to those consuming only two servings per day.

In terms of dietary supplementation, Scheurig *et al.* (2008) examined the relationship between CRP and the intake of micronutrient supplements in the MONICA/KORA Augsburg population study which included 2,045 women and 2,172 men, aged 25-74 years. Intake of supplemental carotenoids was not significantly associated with levels of CRP in both men and women. Church *et al.* (2003) performed a post hoc subgroup analysis of a six month, randomized, double-blind,

placebo-controlled trial on the effects of the use of a multivitamin on CRP levels in 87 apparently healthy individuals. CRP levels were meaningfully lower in the multivitamin compared to the placebo group after six months. The reduction was most apparent in individuals with increased baseline CRP levels. No association between supplementary β-carotene levels and CRP was found. The supplement provided 1,500 μg of vitamin A in the form of β-carotene (3,000 μg).

In summary, cross-sectional epidemiological trials have consistently shown significant negative associations between blood levels of carotenoids and CRP. The majority of RCTs with fruit and vegetables showed the same trend while trials which supplemented either multivitamins and/or carotenoids did not show significant associations between blood CRP and carotenoid levels.

2.3 Vitamin E

Vitamin E is the collective name for tocopherols and tocotrienols which are fat soluble vitamins displaying potent antioxidant activity through their lipoperoxyl radical-scavenging characteristics. Vitamin E consists of eight lipophillic molecules which include α-, β-, γ- and δ-tocopherols and tocotrienols. Tocopherol protects cell membranes from oxidation and is the main form in which vitamin E exist in food. Tocotrienols may protect against stroke and decrease platelet aggregation as well as demonstrate anti-inflammatory effects (Sen *et al.*, 2007). Tocotrienols are however much less prevalent than tocopherols in food sources. Plant seeds and their oils such as sunflower, peanuts and almonds are the richest food sources of α-tocopherol, while γ-tocopherol is mostly found in sesame, pumpkin and flaxseeds as well as soybean and corn oils (USDA, 2015). To the contrary tocotrienols are less commonly found and is most prominent in palm oil, rice bran oil, wheat germ, barley, saw palmetto, annatto and oils thereof. Alpha-tocopherol is the foremost form of vitamin E retained in human plasma.

2.3.1 Vitamin E status

Low levels of vitamin E and vitamin E deficiency are thought to be uncommon and limited to individuals with poor intestinal absorption. It was never really considered a health problem until more current research revealed a different picture. A systematic review published by Péter *et al.* (2016) reported that only 21% of the global population displays blood α-tocopherol concentration above 30 μmol/l which is recommended as the desirable level for favourable effects on human health. 13% of the global population displays blood levels below the functional deficiency threshold concentration of 12 μmol/l. These low levels seem to be most evident in infants and children. This notion is confirmed by at least three clinical trials, two in the United States and one in Korea where clinical deficient vitamin E concentrations (<12 μmol/l) in blood were observed among preschool children. Kim *et al.* (2006) reported a vitamin E deficiency of 69% among Latino immigrant children aged four to eight years residing in Nebraska. In addition Drewel *et al.* (2006) reported that 68% of children from different ethnical backgrounds between the age of two and five years residing in Lincoln Nebraska displayed vitamin E concentrations <7

μmol/l while two thirds of apparently healthy children in the Korean study aged two to six years old displayed vitamin E concentrations less than 12 μmol/l (Giraud *et al.*, 2008).

2.3.2 Vitamin E and cardiovascular disease

Until recently, research on vitamin E focussed mostly on α-tocopherol since it is the most abundant form found in human blood; dietary deficiency thereof is associated with peripheral neuropathy and ataxia (Hammans and Kennedy, 1998). To a large extent α-tocopherol supplementation failed to show significant clinical benefit in prevention of CVD. More recent research suggests rather a combination of the different components of the vitamin E family in human health. In a recent meta-analysis Li *et al.* (2016) reported results on α-tocopherol and circulating tocopherols and the risk of coronary artery disease in 24 case-control and nested case-control studies. Results illustrated that circulating levels of tocopherols were significantly (P<0.01) lower in patients with coronary artery disease than in controls. In contrast circulating α-tocopherol was not significantly associated with the risk for coronary artery disease. The prospective cohort study of Wright *et al.* (2006) among 29 092 Finnish male smokers aged 50-69 years showed a significant inverse relationship after a 19 year follow-up between higher quintiles of serum α-tocopherol and total as well as cause-specific mortality (relative risk for CVD = 0.81; 95% CI: 0.75, 0.88). The greatest risk reduction was seen with increasing α-tocopherol concentrations up to 30-32.5 μmol/l. In the Japan Collaborative Cohort Study for evaluation of cancer risk, data of 39,242 participants (age range, 40-79 years) was analysed. During the 13 year follow-up 530 stroke deaths and 211 deaths from CVD were reported. Among women serum α-tocopherol concentrations were negatively associated with total stroke mortality and haemorrhagic stroke mortality while serum γ-tocopherol was inversely associated with ischemic stroke mortality in men (Nagao *et al.*, 2012). In the Physicians' Health Study (Hak *et al.*, 2003, 2004) no association was reported between α- and γ-tocopherol and the risk of ischemic stroke or MI among male physicians. The physicians with higher plasma levels of γ-tocopherol tended to have an increased risk of MI (P<0.01).

Evidence on the effect of tocotrienols on cardiovascular health is mostly described in randomised controlled clinical trials. Hyperlipidaemia is a known risk factor for CVD. Qureshi *et al.* (1991) studied the effects of a TRF palm oil supplement (200 mg/day) over eight weeks in hypercholestrolaemic patients. Significant decreases were observed from baseline in total cholesterol (-15%), LDL-C (-8%), apoB (-10%), thromboxane (-25%) and platelet factor 4 (-16%). In a small study on 81 chronic haemodialysis patients, subjects received a TRF supplement containing 180 mg tocotrienols over 16 weeks. In comparison to baseline, triglyceride levels reduced significantly (P<0.05) at 12 weeks while HDL-C increased significantly (P<0.05) at weeks 12 and 16 (Daud *et al.*, 2013). Chin *et al.* (2011) administered a 160 mg/day tocotrienol rich fraction supplement over a 6 month period in 62 individuals recruited from two age groups (35-49 years and >50 years) to study the effect on blood lipids and oxidative stress. HDL-C as well as plasma ratio of HDL-C to total cholesterol significantly increased in both groups after six months. Additional studies which reported similar positive effects of TRF supplementation on lipids and cardiovascular health include those of Qureshi *et al.* (1995), Heng *et al.* (2013), Rasool *et al.* (2006) and Qureshi *et al.* (2002). In contrast the study of Mensink *et al.* (1999) which studied

the effect of a tocotrienol rich concentrate on blood lipid levels and platelet function in men with an increased risk for heart disease reported no significant effects on the selected measurements. Twenty male participants received a combination supplement providing 140 mg tocotrienols and 80 mg α-tocopherol per day over a six week period. The control group (n=20) was supplemented with 80 mg α-tocopherol. No significant differences were reported for HDL-C, triglycerides, lipoprotein(a) and lipid peroxide concentrations.

Other mechanisms such as inhibition of hydroxymethylglutaryl-CoA reductase, reduction of the expression of adhesion molecules as well as monocyte endothelial cell adhesion have also been described as protective effects of tocotrienols against CVD (Ahsan *et al.*, 2014).

2.3.3 Vitamin E and inflammation

Tocopherols display anti-inflammatory effects by inhibiting cyclooxygenase-2 and 5-lipoxygenase mediated eicosanoid activity, suppression of nuclear factor kappa B signalling pathways (Jiang, 2014) as well as reducing pro-inflammatory cytokines such as IL-1β with which CRP production is interrelated (Saboori *et al.*, 2015). Saboori *et al.* (2015) conducted a systematic review and meta-analysis studying high dose RCTs which investigated the effect of α- and γ-tocopherol supplementation on serum CRP levels. Doses varied between 100-500 mg/day. Pooled analysis indicated a significant reduction in CRP (P<0.001) in supplemented individuals while subgroup analysis indicated a magnified decrease in CRP in participants with baseline levels less than 3 mg/l compared to individuals with baseline levels above 3 mg/l. There seemed to be a greater decrease in CRP with α-tocopherol versus γ-tocopherol supplementation. It was also reported that serum CRP level reduction was greater in studies longer than six weeks. In a seven year dietary intake study (Helmersson *et al.*, 2009) among people 70 years and older in Sweden, α-tocopherol intake from fruit and vegetable sources was negatively associated with hs-CRP and IL-6. In a cross-sectional study, Van Herpen-Broekmans *et al.* (2004) measured among other nutrients serum carotenoids and α-tocopherol concentrations in 379 individuals of the general Dutch population to determine the effect on inflammation. Inflammatory markers such as hs-CRP, fibrinogen and leukocytes were measured. B-carotene was significantly negatively associated with CRP (P=0.0003) and leukocytes (P=0.007) while α-tocopherol was positively (P=0.02) associated with CRP. No associations were indicated for fibrinogen.

According to Ahsan *et al.* (2014) α-, γ-, and δ-tocotrienols have been associated with suppression of potent pro-inflammatory signalling of NF-κB, TNF-α, interleukins-1,-6 and -8, nitric oxide synthase as well as cyclooxygenase-2 activity *in vitro* and *in vivo*. Human studies on the effect of tocotrienols and the focus on inflammation linked with CVD are limited. With regards to *in vitro* studies Muid *et al.* (2016) compared among others the effect of various concentrations of tocotrienol isomers (0.3-10 μM) on the expression of IL-6, TNF-α as well as NF-κB in human umbilical vein endothelial cells. It appeared that δ-tocotrienol was the most effective in inhibiting IL-6 and NF-κB while γ-tocotrienol seem to be the second most effective over all ranges of concentrations. Alpha- and β-tocotrienols showed superior inhibition of IL-6 expression at 10 μM but tended to augment IL-6 at lower concentrations. In randomised controlled clinical

trials Haghighat *et al.* (2014) studied the effect of 200 mg/day tocotrienol enriched canola oil on microalbuminuria and inflammation on 44 type 2 diabetic subjects over an eight week period. Urine microalbumin levels ($P<0.003$) as well as hs-CRP ($P<0.048$) levels were significantly lower in the tocotrienol group compared to the control group which received un-enriched canola oil at the end of the study. In the study of Irandoost *et al.* (2013) over eight weeks, 44 overweight or obese women followed a weight loss diet of which one group were supplemented with tocotrienols in the form of grape seed oil while the control group received sunflower oil with negligible levels of tocotrienols. Subjects who received the grape seed oil presented with significant lower levels of insulin resistance (HOMA-IR) scores as well as serum hs-CRP and TNF-α levels compared to the sunflower oil group. Qureshi *et al.* (2015) investigated the impact of various doses of δ-tocotrienol supplementation on inflammatory markers and oxidative stress in 31 patients with hypercholesterolaemia. Doses ranged between 125-750 mg/day δ-tocotrienols and were combined with the American Heart Association Step-1 diet over 30 weeks. A dose of 250 mg/day δ-tocotrienol seemed to be most effective and compared to baseline significantly reduced CRP (40%) as well as inflammatory plasma cytokines such as resistin, IL-1 and IL-12 which were reduced by 15-17%.

In summary, research has shown that α-tocopherol in isolation doesn't hold any additional benefit to reduce the risk for CVD. However, observational studies provide a fairly consistent picture of the anti-inflammatory properties of vitamin E with concomitant effects on CVD. With supplementation studies vitamin E seems to have a more outspoken effect when supplemented in individuals with elevated CRP levels.

2.4 Vitamin D status

Vitamin D is a fat-soluble vitamin manufactured by the skin which functions as a steroid hormone. The liver derived precursor of vitamin D, 7-dehydrocholesterol is converted in the upper layer of the skin by UVB radiation to pre-vitamin D where it is spontaneously converted to cholecalciferol or vitamin D3 (25(OH)D). Vitamin D2 or ergocalciferol is a plant-derived form of vitamin D and mainly found in portabella mushrooms (USDA, 2015). The majority of 25(OH)D found in the human body is derived from skin exposure to sunlight (Lugg *et al.*, 2015); food sources include egg yolks, fish liver oils, fortified dairy products and cereals (USDA, 2015).

Globally vitamin D status seems to be problematic with a high prevalence especially among women from the Middle East/Africa and Asia/Pacific region where vitamin D levels are strongly associated with clothing covering large parts of the body (Van Schoor and Lips, 2011). North Americans present with the highest vitamin D levels most probably due routine fortification of foods. Scandinavian countries tend to display higher vitamin D levels due to the regular use of cod liver oil and fatty fish consumption compared to countries in southern Europe. New-borns as well as elderly institutionalised individuals seem to be at higher risk for vitamin D deficiency (Hilger *et al.*, 2014). The Institute of Medicine considers a serum vitamin D level higher than 50 nmol/l adequate for bone and overall health in healthy individuals (Ross *et al.*, 2011).

2.4.1 Vitamin D and cardiovascular disease outcomes

An increasing number of research studies (Alkerwi *et al.*, 2015; Cigolini *et al.*, 2006; Dobnig *et al.*, 2008; Ginde *et al.*, 2009; Giovannucci *et al.*, 2008; Kilkkinen *et al.*, 2009; Li *et al.*, 2016; Messenger *et al.*, 2012; Pilz *et al.*, 2009; Poole *et al.*, 2006; Schöttker *et al.*, 2013; Wang *et al.*, 2008b) indicate either a relationship between CVD risks, outcomes, or mortality and sub-optimal vitamin D levels. In a recent meta-analysis by Chowdhury *et al.* (2014) data of 19 primary prevention cohort studies representing 80, 662 participants, illustrated risk ratios for death from CVD of people with mean 25(OH)D values (median 51.8 nmol/l; interquartile range 43.8-60.8 nmol/l), after adjusting for potential risk factors and comparing bottom vs top thirds of baseline serum 25(OH)D levels to be 1.35. In secondary prevention cohort studies (n=10) representing 20,987 participants the risk ratio for death from CVD was 1.60 with similar mean baseline values of 25(OH)D. In a meta-analysis of prospective studies by Wang *et al.* (2012) data from 65,994 participants showed that when comparing the lowest (20 nmol/l) to the highest (60 nmol/l) circulating 25(OH)D categories the relative risk for total CVD was 1.52, for CVD mortality 1.42, for CHD 1.38 and for stroke 1.64.

The prospective study of Anderson *et al.* (2010) which analysed data of 41, 497 subjects from the Intermountain database reported highly significant increases ($P<0.0001$) in the prevalence of diabetes, hypertension, hyperlipidaemia, and peripheral vascular disease with 25(OH)D levels below 75 nmol/l. In those without risk factors for heart disease these 25(OH)D levels were also significantly ($P<0.0001$) associated with coronary artery disease, MI, HF and stroke as well as with the incidence of HF, coronary artery disease/MI (all $P<0.0001$), and stroke ($P=0.003$). In terms of hypertension data from 12, 644 people older than 20 years in the NHANES III displayed significantly lower mean systolic (3.0±0.7 mm Hg; $P=0.0004$) and diastolic blood pressure (1.6±0.6 mm Hg; $P=0.011$) across increasing quintiles of serum 25(OH)D concentrations after adjustment for age, gender, ethnicity and physical activity for participants in the highest quintile (vitamin D > 85.7 nmol/l). Even after adjusting for body mass index the association remained significant ($P<0.05$) for systolic blood pressure while the association also seemed to be stronger in participants older than 50 years (Scragg *et al.*, 2007). The cross-sectional study of Bhandari *et al.* (2011) studied the prevalence of hypertension in adults older than 18 years within a large ethnic diverse population. Participants were categorised into quartiles of 25(OH)D levels namely ideal (≥100 nmol/l), adequate (75-98 nmol/l), deficient (38-73 nmol/l), and severely deficient (<38 nmol/l). After adjusting for age, gender, race and renal insufficiency the prevalence of hypertension in the population was 24%. Rates of hypertension were 52, 41, 27, and 20% in the severely deficient, deficient, adequate and ideal quartiles respectively ($P<0.001$). Odds ratios were 2.7 (1.4-5.2), 2.0 (1.5-2.6) and 1.3 (1.2-1.6) severely deficient, deficient and adequate quartiles respectively compared to the ideal group.

It seems that vitamin D may play an important role in HF however, studies on 25(OH)D levels in HF patients are limited. In a study by Gotsman *et al.* (2012) vitamin D levels of 3, 009 HF patients were compared to 46, 825 people in a control group. HF patients displayed significantly ($P<0.00001$) lower vitamin D levels compared to the control group. There was also a significant

difference (P<0.00001) in the percentage patients with HF (28%) with vitamin D deficiency (<25 nmol/l) compared to the control group (22%). The groups were followed up after 518 days and regression analysis illustrated that vitamin D deficiency was an independent predictor of increased mortality in patients with HF (hazard ratio 1.52) vs a hazard ratio of 1.91 in the control group. Data from 3,299 participants from the LURIC study (Pilz *et al.*, 2008) routinely referred for coronary angiography showed that during a 7.7 year follow-up 116 patients died due to HF and 188 from sudden cardiac death (SCD). In patients with severe 25(OH)D deficiency (≤25 nmol/l) the hazard ratio for death due to HF was 2.84 (1.20-6.74) and for SCD 5.05 (2.13-11.97) compared to patients within the optimal range of 25(OH)D (≥75 nmol/l) after adjustments for cardiovascular risk factors.

Vitamin D also seems to influence lipoprotein levels. Results from the 2001-2006 NHANES reported among 4,632 subjects >20 years that lower vitamin D levels were significantly associated with adverse lipid levels such as lower HDL-C as well as a higher LDL-C to HDL-C ratio and triglyceride levels in abdominally obese patients (Vogt *et al.*, 2016). The Jackson Heart Study investigated the relationship between 25(OH)D concentration and CVD risk factors which included among other HDL-C levels. Data from 4, 010 individuals displayed a mean 25(OH) D level of 36±16.8 nmol/l of which 80% of participants were vitamin D deficient (<50 nmol/l), 17.7% had insufficient levels (50-75 nmol/l) with only 2.3% displaying optimal (>75 nmol/l) 25(OH)D levels. Although LDL-C was not different across the 25(OH)D categories a significant positive association (P<0.05) between 25(OH)D and HDL-C levels was indicated. In a paediatric study by Kelishadi *et al.* (2014) among 1, 095 children in the Middle East and North Africa (CASPIAN III study) where cardiometabolic risk factors were associated with vitamin D levels the median 25(OH)D concentration in boys was 31.8 nmol/l and in girls 33.0 nmol/l. An overall vitamin D deficiency of 40% was reported and 39% of children displayed insufficient levels, no difference existed between boys and girls. A weak significant negative association was indicated between LDL-C and 25(OH)D while a significant positive association was indicated with HDL-C.

2.4.2 Vitamin D and inflammation

Variable results have been indicated in observational studies in the relationship between vitamin D and inflammation. RCTs with vitamin D supplementation have shown mixed results with some indicating no effect on inflammatory markers while others showed an inverse relationship. Data from the NHANES (2001-2006) presented by Amer and Qayyum (2012) reported an inverse association between CRP and serum 25(OH)D levels below 53 nmol/l. In the group of 15,167 participants the median serum concentrations of 25(OH)D and CRP were 53 nmol/l and (interquartile range 15 to 27) and 0.21 mg/dl (interquartile range 0.08 to 0.5) respectively. They have showed a 0.29 mg/dl increase for each 25 nmol/l decrease in 25(OH)D when serum 25(OH)D concentrations were below 53 nmol/l. In contrast an increase in CRP of 0.06 mg/dl was indicated for each 25 nmol/l increment increase in 25(OH)D when serum levels were above 53 nmol/l after adjusting for traditional CVD risk factors such as hypertension, hyperlipidaemia, body mass index, serum glucose, smoking, etc. In the LURIC study (O'Hartaigh *et al.*, 2013) data from 3 299 subjects was analysed to determine the relationship between glycaemic status

and serum 25(OH)D concentrations. Glycosylated haemoglobin as well as HOMA-IR indices reduced significantly with increasing serum 25(OH)D levels ($P<0.001$) while hs-CRP and IL-6 concentrations were significantly lower in the highest 25(OH)D quartile compared to the other quartiles. Other studies that also showed significant associations between 25(OH)D and inflammatory markers include Khan *et al.* (2016); Peterson and Heffernan (2008); Fornari *et al.* (2015); Murr *et al.* (2012). In contrast the study of Michos *et al.* (2009) failed to show any association between 25(OH)D and CRP in 650 participants of Amish people who participated in the Amish Family Calcification Study. More than 38% of subjects presented with 25(OH) D levels below 50 nmol/l and 48% with 25(OH)D levels between 53 and 75 nmol/l. 25(OH)D levels were mostly related to season, age, body mass index and parathyroid hormone levels. In a cross-sectional study (Ewers *et al.*, 2008) with 161 renal-transplant patients the study aimed to assess whether vitamin D states and obesity could be associated with CRP serum concentrations. Median serum 25(OH)D and CRP levels were 50 nmol/l and 0.17 mg/dl, respectively. 83% of the study population presented with 25(OH)D levels below 75 nmol/l. No significant relationship between 25(OH)D concentrations and CRP could be found. Body mass index was significantly positively associated with CRP concentrations while lean body mass was significantly negatively associated with CRP. Other observational studies which failed to show associations between 25(OH)D and CRP include Shin *et al.* (2016) and Azizieh *et al.* (2016).

Results from meta-analyses and systematic reviews which included RCTs and investigated the effect of 25(OH)D supplementation on inflammatory biomarkers seem to be inconsistent. The meta-analysis of Chen *et al.* (2014) included ten RCTs representing the data of 924 participants with a variety of ailments. Supplemental 25(OH)D doses varied between 10 and 180 μg per day and trials were between 8 and 48 weeks in time. Overall a significant inverse association ($P<0.01$) was observed between vitamin D supplementation and circulating hs-CRP when compared with controls. During subgroup analysis 25(OH)D supplementation lead to significantly higher reductions in hs-CRP concentrations (0.22 mg/dl) among participants with baseline hs-CRP levels above 0.5 mg/dl. In addition more prominent reductions in hs-CRP (0.17 mg/dl) were seen with 25(OH)D doses <100 μg per day compared to higher doses. In the study of Jamka *et al.* (2016) data from 1, 955 individuals participating in 13 RCTs were included in this review. The study aimed to examine the effect of vitamin D supplementation on CRP, TNF-α and IL-6 in obese and/or overweight individuals. Studies were between 4 and 156 weeks while 25(OH) D dosages varied between 18 and 180 μg per day. Mean serum 25(OH)D concentrations ranged between 30.00 and 81.50 nmol/l and mean plasma CRP, TNF-α and IL-6 between 0.03 and 0.74 mg/dl, 1.53 to 9.30 pg/ml and 1.00 and 8.90 pg/ml, respectively. No significant association could be indicated between 25(OH)D supplementation and CRP ($P=0.15$) or TNF-α ($P=0.31$) or IL-6 ($P=0.71$).

To summarise, sub-optimal vitamin D levels seem to be a global phenomenon. Several large primary and secondary observational studies showed associations between 25(OH)D levels and CVD as well death from CVD. Vitamin D deficiency also showed to play an important role in the outcome of HF as well as to influence lipoprotein levels. In RCTs and observational studies the effect of 25(OH)D supplementation on inflammatory biomarkers seems to be less clear.

2.5 Concluding remarks

In general, when considering epidemiological evidence, carotenoids, vitamin E and vitamin D seem to have favourable effects on CVD. However, evidence from supplementation studies and dietary intervention trials seem to deliver inconsistent results. The lack of consistency can be ascribed to a number of factors. Dietary supplements in general provide substantially higher doses of micronutrients than normally found in foods. Some RCTs administered mega-doses of supplemented nutrients compared to observational studies where no supplementation was consumed. Mega-doses may indeed have pro-oxidative effects. Bioavailability of nutrients also differs depending on the food matrix in which it appears as well as the form in which dietary supplements (emulsion vs soft gel capsules vs tablets vs synthetic forms) are ingested. Background diet need to be compensated for since interactions between nutrients need to be accounted for. Supplementation studies are normally of shorter duration and smaller sample sizes are utilised.

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3. Vitamin D and cardiovascular disease

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Abstract

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality and has a significant impact on health care systems. Changing lifestyle and increasing environmental pollution are giving fuel to its incidence. Besides these, there are certain emerging conditions which also have been proposed to increase the risk of CVD and vitamin D deficiency is one of them. In recent years, the scientists have put all their efforts in exploring the relation between vitamin D and CVD. Formation of atherosclerotic plaque and several other processes including endothelial dysfunction, cardiomyocyte proliferation, inflammation, immune activation, hormonal imbalance, dyslipidemia, platelet activation, thrombus formation, oxidative stress, altered matrix metabolism contribute to development of CVD. Vitamin D has been shown to play a role in the pathogenesis of CVD by affecting all these processes. This chapter is an attempt to summarize the available information related to vitamin D and its possible role in CVD. In the first half, this chapter gives an overview of vitamin D, its biological functions, routine requirements, and definition of vitamin D deficiency. In the second half, it covers various mechanisms proposed to be altered by vitamin D deficiency which are likely to be involved in the causation of CVD. At the end, the link between vitamin D and various CVD related conditions and cardiovascular events is discussed.

Keywords: vitamin D, vitamin D deficiency, cardiovascular disease, association

Key facts

- Vitamin D works in our body like a steroid hormone rather than a vitamin.
- Vitamin D deficiency has been found to be associated with certain heart diseases inducing conditions like hypertension, obesity, diabetes mellitus, hyperlipidemia and different cardiovascular complications like coronary artery disease, myocardial infarction, heart failure, stroke, peripheral artery disease.
- Sedentary and indoor lifestyles are associated with vitamin D deficiency and are risk factors for the cardiovascular complications
- Low vitamin D levels increase the risk of cardiovascular disease (CVD) probably by upregulating the renin-angiotensin-aldosterone system, promoting inflammation, thrombosis, atherosclerosis, cardiomyocyte proliferation and endothelial dysfunction.
- Basic research, clinical and observational studies propose a causal relationship between vitamin D deficiency and CVD however, some of the randomized controlled trials, follow-up studies and meta-analyses have failed to confirm the same.

Summary points

- Vitamin D is a multitasking hormone.
- Interestingly, it is synthesized by skin in the presence of sunlight.
- Unfortunately, due to modernization and indoor lifestyle deficiency of vitamin D has become a common phenomenon.
- This has resulted in the development of several ailments in the body.
- The cardiovascular system is also to some extent under its control hence there is increased risk of development of CVD.
- Though there is a link between vitamin D and CVD its causal association is not yet confirmed.

Abbreviations

AF	Atrial fibrillation
CAD	Coronary artery disease
CKD	Chronic kidney disease
CVD	Cardiovascular disease
CVS	Cardiovascular system
DM	Diabetes mellitus
HDL	High-density lipoprotein
HF	Heart failure
HTN	Hypertension
LVH	Left ventricular hypertrophy
MI	Myocardial infarction
MMP	Matrix metalloproteinase
NHANES	National Health and Nutrition Examination Survey
NCEP	National Cholesterol Education Program
PAD	Peripheral artery disease
PTH	Parathyroid hormone
rNCEP	Revised National Cholesterol Education Program
RAAS	Renin-angiotensin-aldosterone system
RCT	Randomized controlled trial
TNF	Tumour necrosis factor
UVB	Ultraviolet B
VCAM	Vascular cell adhesion molecule
VDR	Vitamin D receptor
Vitamin D	1,25-dihydroxyvitamin D/1,25-$[OH]_2$ D

3.1 Introduction

Vitamin D was discovered by American researchers Elmer McCollum and Marguerite Davis in 1914 and in 1922. It was described by them as a substance capable of curing rickets (Holick, 1994; Wolf, 2004). Since then vitamin D is primarily considered as an essential molecule required for calcium metabolism, skeletal growth and development and its deficiency may lead to bone disorders like rickets in children and osteomalacia in adults. In the recent years however, enough data have accumulated to suggest that vitamin D might also play a role in various extra-skeletal ailments including autoimmune disorders, infections, cancer, neuropsychological manifestations and CVD. CVD is the commonest cause of mortality and has a significant impact on health care systems and the economy of several countries, with >23 million individuals expected to succumb to CVD annually by 2030 (Mathers and Loncar, 2006).

Extensive research and trials related to vitamin D and CVD, carried out in the last decade suggest that vitamin D exerts its effects on CVS via different mechanisms and its deficiency in patients

with CVD is associated with increased mortality and morbidity. The aim of this chapter is to assess the association between Vitamin D and CVD. The emphasis will be on vitamin D, its metabolism, functions and various effects on CVS. The chapter will also scrutinize the current information available in the form of studies and trials related to vitamin D and CVD with an intention to understand the association between the two and any therapeutic role of vitamin D in prevention of a cardiovascular catastrophe.

3.2 Vitamin D

In the beginning of 20th century McCollum and his co-workers showed in their experiment that diet containing cod liver oil can cure rickets in rats. Since this anti-rickets factor was present in foodstuff, they thought it to be a vitamin similar to other newly discovered vitamins (vitamin A, B and C) in that era. Hence, they named it 'vitamin D' (McCollum *et al.*, 1922). The Nobel laureate Adolf Windaus however, proved that the factor is actually a pro-vitamin, rather a precursor steroid hormone and is involved in the formation of the calcitriol (Windaus, 1931; Windaus and Hess, 1926).

Subsequently vitamin D was characterized as a group of fat-soluble secosteroids which are primarily responsible for bone and calcium metabolism. Though 5 forms of vitamin D (D1 to D5) are known, only vitamin D2 and D3 are clinically relevant and the term 'vitamin D' generally denotes both D2 and D3 or any compound with biological activity of 1,25-dihydroxyvitamin D and collectively called calciferol (Table 3.1). Plants and invertebrates synthesize vitamin D2, also known as ergocalciferol. It is produced by UVB irradiation of ergosterol. Humans consume this form of vitamin in diet, fortified products or as supplements. Vitamin D3 or Cholecalciferol is primarily of vertebrate origin and synthesized in human skin after exposure of 7-dehydrocholesterol to UVB (Holick, 2007). Its dietary sources are limited as beneficial amounts

Table 3.1. Types of vitamin D and their significance.

Type	Chemical name	Significance
vitamin D1	mixture of ergocalciferol and lumisterol	since it is a mixture of compounds the term is no longer used
vitamin D2	ergocalciferol/calciferol: made from ultraviolet irradiation of ergosterol or pre-vitamin D2	found in invertebrates, fungus, and plants
vitamin D3	cholecalciferol: made from 7-dehydrocholesterol or pre-vitamin D3	clinically most important form of vitamin D
vitamin D4	22,23-dihydroergocalciferol: vitamin D2 without 22,23 double bond	found in certain mushroom species
vitamin D5	sitocalciferol: made from 7-dehydrositosterol	antitumor activity

are found only in fish oils, fortified food products or in vitamin supplements. Skin synthesis of vitamin D3 is therefore important and comprises 80%-90% of total vitamin D (Pilz *et al.*, 2009).

3.2.1 Metabolism of vitamin D

The inactive precursors produced in skin or obtained from diet reach to circulation and then to liver via vitamin D binding proteins. In the liver they undergo 25-hydroxylation by cytochrome P450 enzymes (i.e. CYP2R1) and get converted into 25-hydroxyvitamin D [25(OH)D] or calcidiol. This is then converted into 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] or calcitriol by 1α-hydroxylase (another cytochrome P450 enzyme; CYP27B1) in the proximal convoluted tubules of kidney. Although $1,25(OH)_2D$ is considered to be the active form of vitamin D, its level in the serum does not correlate with overall vitamin D status. Rather, levels of 25(OH)D are clinically more relevant (GMosekilde, 2008). The $1,25(OH)_2D$ destined for excretion is finally metabolized in the kidneys, where it is transformed into 1,24,25-dihydorxyvitamin D [$1,24,25(OH)_2D3$] and finally into calcitroic acid, which is then excreted through urine (Figure 3.1) (Christakos *et al.*, 2010).

The active form of vitamin D ($1,25(OH)_2D$) crosses the cell membrane and cytoplasm and reaches the nucleus where it binds to VDRs to perform its endocrine functions. This conjugated vitamin D with its receptor forms a heterodimer complex with retinoic acid receptors and functions as a nuclear transcription factor, inducing protein synthesis and altering gene function (Holick,

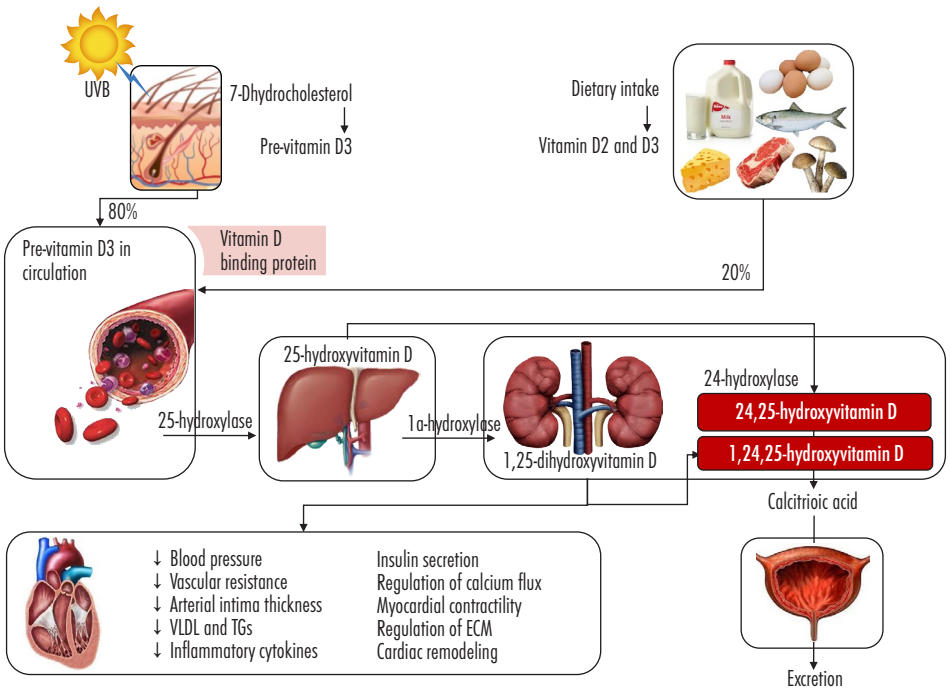

Figure 3.1. Metabolism of vitamin D (ECM = extracellular matrix).

2007). VDR regulates hundreds of genes i.e. nearly 3% of human genome which are involved in regulatory processes including cell proliferation and differentiation, apoptosis, oxidative stress, membrane transport, matrix homeostasis, tissue mineralization and cell adhesion (Pilz *et al.*, 2013a). It also downregulates some genes, including those for PTH and CYP27B1 (Bouillon *et al.*, 2008). Hence, it is tempting to speculate that vitamin D deficiency may have widespread adverse health consequences.

VDRs are present on a large variety of cell types, including osteoblasts, immunologic cells, nerve cells, pancreatic beta cells, enterocytes, parathyroid gland cells, distal renal tubule cells, vascular endothelial cells, myocytes and cardiac muscle cells (Holick, 2007; Lee *et al.* 2008; Nibbelink *et al.*, 2007). Hence, VDR is expressed in cells throughout the vascular system (Danik and Manson, 2012). All these cells also have capability to produce 1α-hydroxylase, which converts 25(OH)D to $1,25(OH)_2D$, the natural ligand of the VDR.

Vitamin D has been shown to inhibit vascular smooth muscle cell proliferation, regulate RAAS, decrease thrombus formation, and exhibit anti-inflammatory properties. Vitamin D deficiency reduces the absorption of dietary calcium to only 10-15% and that of phosphorus to 60% of total absorbable intake. If the deficiency is corrected it can increase the intestinal absorption of calcium by 30-40% and that of phosphorus to up to 80% (Holick, 2010).

Vitamin D inhibits the release of calcitonin and PTH from the thyroid and parathyroid glands respectively. The action of these two hormones and vitamin D is to regulate the intestinal absorption and renal reabsorption of calcium and phosphate metabolism. In the presence of low blood calcium, vitamin D and PTH act together to mobilize calcium from the skeleton through stimulating osteoclastogenesis and both act together to increase distal renal tubule reabsorption of calcium. PTH through its receptors on the osteoblasts stimulate the formation of osteoclasts which dissolve the bone matrix causing release of calcium into extracellular space. The secondary hyperparathyroidism therefore may also result in osteopenia and osteoporosis thereby increasing risk of fracture (Beveridge and Witham, 2013; DeLuca, 2004; Holick, 2007; Jones *et al.*, 1998)

3.2.2 Sources of vitamin D, normal serum levels and recommended daily intake

Besides sunlight, the dietary sources of vitamin D include oily fish such as salmon, mackerel, and herring and oils from fish e.g. cod liver oil. Their vitamin D content on an average is 500-1000 IU in 100 g (3.5 ounces). Minor quantity of vitamin D is also present in beef liver, milk, ricotta cheese, egg, mushroom, etc. In addition, food products like milk, juices, breads, yogurts, and cheese may be available as fortified with vitamin D. Vitamin D supplements are also available in various amounts i.e. 400, 1000, 2,000, 4,000, 5,000 and 50,000 IU vitamin D3 (Holick and Chen, 2008).

Vitamin D status is classified according to the serum levels of 25(OH)D. In the literature different optimal cut-off values and target ranges for vitamin D have been mentioned. However, based on beneficial effects, the Institute of Medicine of the National Academies (USA) recently proposed

that the concentrations of 50 nmol/l (20 ng/ml) are sufficient to meet the vitamin D requirements in 97.5% of the general population. It has been estimated that for every 100 IU of vitamin D ingested, the blood level of 25(OH) vitamin D increases by 1 ng/ml (2.5 nmol/l) and a daily intake of 600-800 IU is sufficient to meet the requirement. But, under conditions of low sunlight exposure e.g. during the winter in Europe, vitamin D intake of 800 IU or more is required per day to achieve 25(OH)D concentrations of 50 nmol/l (20 ng/ml) (Pilz *et al.*, 2016; Ross *et al.*, 2011; Vanga *et al.*, 2010).

3.2.3 Vitamin D deficiency

Since humans can synthesize vitamin D directly from sunlight exposure, ideally dietary requirement should be minimal. However, in most parts of the world, humans expose <5% of their skin to direct sunlight that frequently leads to vitamin D deficiency (Vanga *et al.*, 2010). The effectiveness of vitamin D synthesis also depends on the intensity of exposed sunlight hence in winters vitamin D synthesis may be slow. Furthermore, skin pigmentation also acts as a barrier to synthesis of vitamin D. Other risk factors affecting levels of skin derived vitamin D include latitude, cultural habits e.g. dressing style, application of sunscreen, sedentary and indoor lifestyle, age i.e. children have higher levels compared to adults and sex i.e. females have low levels due to greater storage in fat cells and genetic factors associated with vitamin D metabolism (Table 3.2) (Edwards *et al.*, 2014).

Vitamin D deficiency is pandemic and widely prevalent irrespective of age, gender, race and geography. It is estimated that 30 to 50% of the world's population has either vitamin D deficiency or insufficiency (Holick, 2010; Pilz *et al.*, 2013a). There is a controversy about the definition of vitamin D deficiency. The Endocrine Society Clinical Practice Guideline defines vitamin D deficiency as 25(OH)D level <50 nmol/l (20 ng/ml) and insufficiency as 52.5 to 72.5 nmol/l (Holick *et al.* 2011). The International Osteoporosis Foundation labels vitamin D deficiency

Table 3.2. Major risk factors for vitamin D deficiency.

Aging	Physical inactivity
Reduced dietary intake	Genetic factors
Vegetarian	Malabsorption
Increased distance from the equator	Renal disease
Winter seasons	Liver disease
Darkly pigmented skin	Certain medications
Institutionalized/housebound	• glucocorticoids
Sunscreens and cover-up clothing	• antirejection medications
Air pollution	• human immunodeficiency virus medications
Smoking	• certain antiepileptic drugs
Obesity	

as 25(OH)D level <25 nmol/l and insufficiency as <50 nmol/l with a target level of 75 nmol/l (Dawson-Hughes 2004). The guidelines from the Institute of Medicine of the National Academies (USA) propose 25(OH)D serum levels of 50 nmol/l (20 ng/ml) as sufficient (Vanga *et al.*, 2010). Hence different reference values have been suggested by different bodies and there is a lack of consensus. Furthermore, inherent fluctuations with season, diet, supplements and variability between the different assays used to measure serum 25(OH)D make the assessment more complicated (Lai *et al.*, 2012).

Deficiency of vitamin D is primarily responsible for bone abnormalities. In children, it is present as skeletal deformities in the form of bowing of legs, widened epiphyseal plates at costochondral junctions and at the end of the long bones, frontal bossing of the skull, craniotabes and a delay in tooth eruption (Holick, 2010). In adults, there are no obvious skeletal deformities as the epiphyseal plates are closed with enough mineral in the long bones. However, there exists a heavily innervated unmineralized matrix underneath the periosteal membrane which gets swollen and pushed upwards. The sensory nerve fibres present get stimulated leading to throbbing aching bone pain and tenderness. These patients may be misdiagnosed as having arthritis, myopathy or chronic fatigue syndrome. Though x-ray may show looser's zones as a clue to disease, diagnosis often comes from serum biochemistry. Decreased serum phosphate, elevated alkaline phosphatase with low 25(OH)D levels (may be normal sometimes e.g. in chronic renal failure) provide useful confirmatory evidence of vitamin D deficiency.

There is a long list of observational studies relating vitamin D deficiency and various disorders i.e. DM, metabolic syndrome and obesity (Skaaby, 2015). It might also be associated with certain malignancies e.g. breast, colorectal, prostate and lung cancer, etc. (Abbas *et al.*, 2009; Ahonen *et al.*, 2000; Kilkkinen *et al.*, 2008; Ma *et al.*, 2011). Respiratory infections, particularly mycobacterial, are seen more frequently in vitamin D deficient individuals (Charan *et al.*, 2012; Ginde *et al.*, 2009a). Other associated conditions include diseases of the liver e.g. cirrhosis, viral hepatitis, fatty liver; digestive system e.g. inflammatory bowel disease and celiac disease; central nervous system e.g. Alzheimer's disease, Parkinson's disease, multiple sclerosis and depression and; kidney e.g. CKD (Annweiler *et al.*, 2012; Iruzubieta *et al.*, 2014; Mpandzou *et al.*, 2016; Nigwekar *et al.*, 2014; Pappa *et al.*, 2008).

3.3 Vitamin D and cardiovascular diseases

In the early 1980s Robert Scragg hypothesized that the increase in CVD in winters might be because of low 25(OH)D levels as a result of reduced sunlight intensity and exposure during that period. This was followed by a spurt of publications relating vitamin D and CVD. The accumulated evidence so far suggests an inverse relation between vitamin D and risk of CVD (Liu *et al.*, 2016; Modarresi-Ghazani *et al.*, 2016; Mozos and Marginean, 2015; Pérez-Hernández *et al.*, 2016; Pilz *et al.*, 2016; Wang, 2016). There are several epidemiologic, ecologic and laboratory reports and cross-sectional studies on various CVD associated with vitamin D deficiency e.g. MI, LVH and hypertensive vascular disease, PAD, CAD, HF, arrhythmias and stroke (Brøndum-Jacobsen *et al.*,

2013; Chen *et al.*, 2015; Demir *et al.*, 2014; Melamed *et al.*, 2008a; Meredith and McManus, 2013; Scragg *et al.*, 2007; Siadat *et al.*, 2012). In the next section, we review effects of vitamin D on CVS and alterations in physiological and metabolic functions of the heart in vitamin D deficiency. We also compare a few important studies on the role of vitamin D deficiency in CVD to reach a conclusion whether the association between two is by chance or causal.

3.3.1 Physiologic and pathologic effects of vitamin D on CVS

Renin-angiotensin-aldosterone system

RAAS is a hormone system that regulates arterial blood pressure and maintains extracellular fluid volume. A decrease in the plasma sodium concentration results in reduction of blood flow to the kidneys. In such situation the juxtaglomerular cells in the kidneys convert prorenin into renin which is released directly into the circulation. Plasma renin then converts angiotensinogen into angiotensin I which is finally converted into angiotensin II by the angiotensin-converting enzyme. Angiotensin II is a potent vasoconstrictor and increases blood pressure by constricting arterioles (Figure 3.2). It also stimulates secretion of aldosterone hormone from the adrenal cortex which is responsible for reabsorption of sodium and excretion of potassium from the tubular epithelial cells of the kidneys (Lavie *et al.*, 2011).

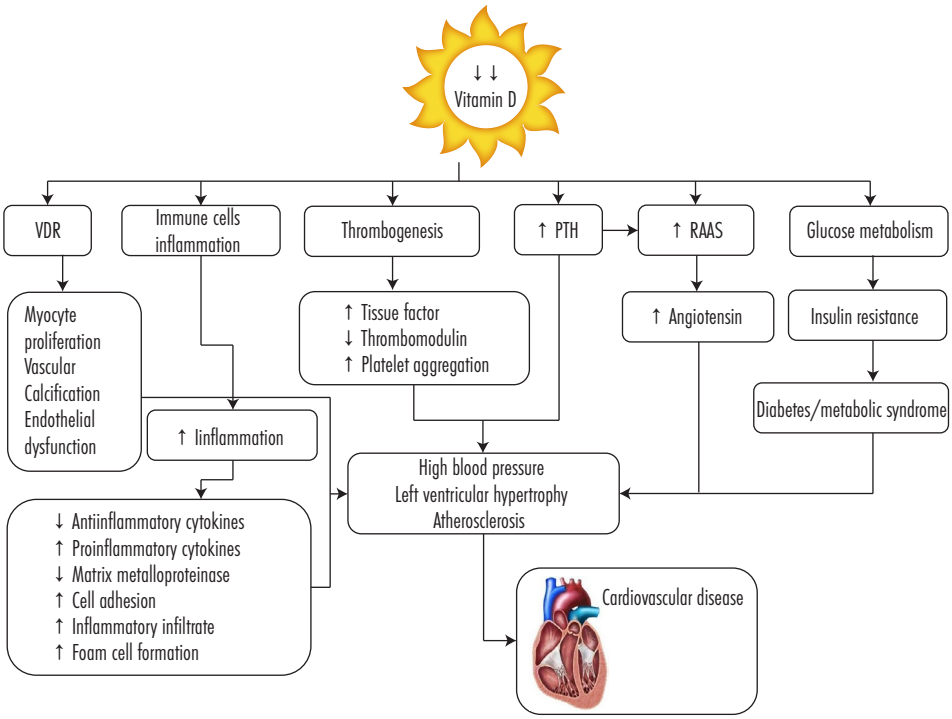

Figure 3.2. Mechanism and effects of vitamin D deficiency on cardiovascular disease (VDR = vitamin D receptor, PTH = parathryroid hormone, RAAS = renin-angiotensin-aldosterone system).

Vitamin D acts as a negative regulator of RAAS and inhibits the activity of cAMP response element in the renin gene promotor. In case of Vitamin D deficiency there is overproduction of renin and angiotensin II, thereby leading to HTN, LVH, increased water intake and sodium absorption (Beveridge and Witham, 2013).

Immune cells and inflammation

Vitamin D supresses the development and responses of Th1 and Th17 cells and promotes T regulatory and Th2 cells (Pilz *et al.*, 2013a). It also downregulates the expression of proinflammatory cytokines like TNF, IFNγ, IL-6, IL-1, IL-2, IL-8 and upregulates anti-inflammatory cytokines IL-4, IL-5 and IL-10, MMP-2 and MMP-9. It reduces expression of toll like receptors, adhesion molecules, smooth muscle proliferation and inhibits formation of foam cells from macrophages. All these observations suggest that vitamin D deficiency may play an active role in the pathogenesis of atherosclerosis, vascular calcification, aneurysm formation and other inflammatory vascular disorders (Norman and Powell, 2014; Rahman *et al.*, 2007; Riek *et al.*, 2013; Takeda *et al.*, 2010).

Thrombosis

Vitamin D downregulates tissue factor, plasminogen activator thrombospondin-1 and upregulates expression of antithrombin and thrombomodulin. It attenuates platelet activation and decreases fibrinolysis. This pattern of effects of vitamin D is consistent with its antithrombotic role (Norman and Powell, 2014). Further, in patients with vitamin D deficiency, the combined effect of elevated TNF-α and IL-6 levels and increased release of adhesion molecules has been found to increase mean platelet volume which is a predicting marker of future CVD (Cumhur *et al.*, 2014).

Vascular calcification

A mechanism similar to that for bone formation is involved in vascular calcification (Demer and Tintut, 2008). Several proteins associated with bone mineralization i.e. osteocalcin, osteoprotegerin, matrix Gla protein have been identified in vascular calcification (Hruska *et al.*, 2005). Vitamin D actions on mineral metabolism (increasing phosphate and calcium levels) and osteoblastic gene expression and modulation of inflammation may reduce vascular calcification (Razzaque, 2011). Vitamin D deficiency therefore is associated with increased vascular calcification. However, observational studies have found an inverse relation between vascular calcification and Vitamin D (Watson *et al.*, 1997).

Endothelial dysfunction

The association between vitamin D and endothelial dysfunction has been shown in both observational and interventional studies (Caprio *et al.*, 2012; Tarcin *et al.*, 2009). Proinflammatory and prothrombotic state and increased arterial stiffness lead to endothelial dysfunction and promote atherosclerosis. In brief, during endothelial stress there is upregulation of VDR and release of inflammatory cytokines which induce local production of vitamin D. Vitamin D

increases endothelial nitric oxide. Apart from its vasodilatory effects, nitric oxide is a potent inhibitor of platelet and leukocyte aggregation and adhesion which play important role in early atherosclerosis development. Vitamin D also protects endothelial cells from oxidative stress by counteracting superoxide anion generation, thus suppressing reactive oxygen species and counteracting apoptosis (Andrukhova *et al.*, 2014). Vitamin D deficiency also increases expression of nuclear factor κB and endothelial inflammation resulting in disturbance in flow mediated dilation (Zhang *et al.*, 2012).

Cardiomyocyte hypertrophy

Since cardiomyocytes express VDR, vitamin D has direct effect on regulation of myocyte growth. In case of deficiency there is maladaptive cardiac remodeling resulting in progressive myocyte hypertrophy and interstitial fibrosis (Norman and Powell, 2014; Pilz *et al.*, 2010a). It also interacts with caveolin-3 in t-tubules to modulate rate and magnitude of cardiomyocyte sarcomere contraction, helps in cardiomyocyte relaxation and improves coronary perfusion during diastole (Pilz *et al.*, 2010a; Zhao and Simpson, 2010).

PTH secreted by the parathyroid glands increases serum calcium by promoting calcium resorption from bone, kidney and intestine. It converts 25(OH)D to its active metabolite 1,25-$(OH)_2$ D by activation of the enzyme 1α-hydroxylase. Though calcium directly acts on parathyroid gland and induces feedback inhibition of PTH release, vitamin D also has an inhibitory effect on the parathyroid gland. Hence, PTH partially mediates the effects of vitamin D deficiency on the CVS. Observational studies suggest that elevated parathyroid levels also contribute to LVH as seen in patients of primary hyperparathyroidism. This could be an indirect mechanism related to vitamin D as vitamin D deficiency leads to elevated PTH (Hagström *et al.*, 2009; Pilz *et al.*, 2013a; Saleh *et al.*, 2003; Van Ballegooijen *et al.*, 2013a,b).

Glucose metabolism

Vitamin D deficiency predisposes to glucose intolerance and altered insulin secretion (Khan *et al.*, 2013; Tuomainen *et al.*, 2015). This effect on insulin secretion is via different pathways. First, it has direct action on pancreas since the Islet cells have receptors for vitamin D which stimulate β cells to release insulin (Borissova *et al.*, 2003; Pitocco *et al.*, 2006). Second, it influences β cell insulin secretion by causing increase in intracellular calcium through non-selective voltage dependent calcium channels (Sergeev and Rhoten, 1995). Other mechanisms of actions of vitamin D include activation of protein biosynthesis in pancreatic islets, regulation of PTH and immunomodulatory functions (Borissova *et al.*, 2003; Palomer *et al.*, 2008). In case of vitamin D deficiency an increase in insulin resistance has been observed in some of the studies (Pham *et al.*, 2012; Von Hurst *et al.*, 2010; Wallace *et al.*, 2016). However, some of the recent studies observed no effect of vitamin D supplementation on glucose concentrations and insulin level and propose that link between vitamin deficiency and insulin resistance is coincidental (Al-Shoumer and Al-Essa, 2015; Jamka *et al.*, 2015).

3.4 Cardiovascular diseases

In various clinical, population based, cross sectional, observational and ecological studies and randomized trials vitamin D deficiency has been linked to various cardiovascular risk factors i.e. HTN, obesity, hyperlipidemia, DM, metabolic syndrome, and CKD as well as cardiovascular events i.e. CAD and MI, arrhythmias, HF, stroke, PAD.

3.4.1 Cardiovascular risk factors

Arterial hypertension

While several cross-sectional and prospective studies and meta-analysis of observational studies demonstrate an inverse relationship between vitamin D and HTN (Burgaz *et al.*, 2011; Pilz and Tomaschitz, 2010; Sabanavagam *et al.*, 2011; Scragg *et al.*, 2007, 2010), some prospective studies show either no major effects or low to moderate effect on lowering systolic blood pressure only (Forman *et al.*, 2013; Jorde *et al.*, 2010; Margolis *et al.*, 2012). The reasons for such variable results may be many, e.g. selection of study subjects (prehypertensive, or hypertensive), vitamin D dosage, follow up duration and elimination of potential confounders such as use of anti-hypertensive and non-study related supplementation. Also the reported variations in blood pressure due to different vitamin D status in most of the studies were minor hence, of questionable clinical relevance (Arora *et al.*, 2015; Kienreich *et al.*, 2013b).

The proposed mechanisms for HTN are RAAS activity, PTH and the effect on endothelial function. The RAAS regulates electrolyte levels and plasma volume which in turn affects blood pressure. Inappropriate activation of the RAAS contributes to HTN and overall cardiovascular risk. Vitamin D keeps a check on PTH release and deficiency leads to defective feedback and overproduction of PTH which contributes to HTN, myocardial hypertrophy and ventricular arrhythmias by raising intracellular calcium (Pilz *et al.*, 2010b). Vitamin D improves endothelial function by increasing nitric oxide synthesis and reducing cyclooxygenase-1, oxidative stress and apoptosis. The endothelial dysfunction in vitamin D deficiency therefore may contribute to blood pressure (Carvalho and Sposito, 2015; Pérez-Hernández *et al.*, 2016).

Obesity

Obesity and higher BMI reduce vitamin D levels (Earthman *et al.*, 2012). The increased adipose tissue in obesity entraps more and more fat soluble vitamin D leading to its lower circulating levels (Wortsman *et al.*, 2000). Hence, obesity may be regarded as causal risk factor for vitamin D deficiency. Also proposed is that obese individuals tend to avoid exposure of their skin to sun in public areas, may be due to social physique anxiety, though this hypothesis has not been widely accepted. Other factors likely to contribute to low vitamin D include: lower dietary intake, impaired 25-hydroxylation and 1α-hydroxylation, imbalance of release of adipokines and inflammatory cytokines. Surprisingly, vitamin D supplementation in obese individuals does not

show any benefits which means vitamin D status has no effect on obesity (Kienreich *et al.*, 2013a; Mehmood and Papandreou, 2016; Vanlint, 2013).

Hyperlipidemia

Few observational studies have found low levels of HDL, higher triglycerides and apolipoprotein E levels and hypercholesterolemia in vitamin D deficient individuals (Jaimungal *et al.*, 2011; Jorde and Grimnes, 2011; Skaaby *et al.*, 2012a). There is a marginal increase in low-density lipoprotein cholesterol but the levels of HDL cholesterol and apoliprotein A-1 are reduced (Pilz *et al.*, 2013a). The vitamin D increases serum calcium by increasing calcium absorption from the intestine. This calcium decreases serum triglycerides by reducing hepatic triglyceride formation and secretion. Since vitamin D also suppresses PTH levels it causes increased peripheral removal of triglycerides. These triglyceride lowering mechanisms are severely affected in vitamin D deficiency. In addition, insulin resistance and reduced expression of very low-density lipoprotein cholesterol receptors in certain cells might also be responsible for hypertriglyceridemia in vitamin D deficiency (Chaudhuri *et al.*, 2013; Choi *et al.*, 2011; Ginsberg *et al.*, 2005; Kohno *et al.*, 1997; Lacour *et al.*, 1982). Though RCTs have shown similar results but they also failed to reveal any beneficial effects of vitamin D supplementation on lipid profile (Ponda *et al.*, 2012; Zittermann *et al.*, 2011).

Diabetes mellitus

Several studies suggest a link between low vitamin D levels, disturbances in glucose metabolism and higher risk of developing type II DM. Vitamin D deficiency causes disturbances in glucose homeostasis, β cell function and dysregulated calcium homeostasis which play crucial role in insulin synthesis and secretion. Also there is lack of stimulus to osteocalcin release resulting in insulin resistance (Pilz *et al.*, 2013b; Wolden-Kirk *et al.*, 2011; Wu *et al.*, 2012). These facts unfortunately have not been supported by many randomized trials as there is hardly any benefit of vitamin D supplementation in improving glycaemia or insulin resistance. In a RCT conducted by Davidson *et al.* in individuals with prediabetes and vitamin D deficiency, high dose vitamin D therapy (mean weekly dose of 88,865 IU) vs placebo was offered to participants. After one year of follow up no difference could be seen in plasma glucose, insulin secretion and sensitivity or development of diabetes in the treated group compared to those who received placebo therapy (Davidson *et al.*, 2013).

Metabolic syndrome

Metabolic syndrome is a group of medical conditions, which if present in an individual increase twofold risk of CVD. The NCEP defines the metabolic syndrome as having 3 or more of the following 5 cardiovascular risk factors: (1) central obesity (waist circumference: men >102 cm; women >88 cm); (2) elevated triglycerides (≥150 mg/dl); (3) diminished HDL cholesterol (men <40 mg/dl; women <50 mg/dl); (4) systemic HTN (≥130/≥85 mm Hg); and (5) elevated fasting glucose (≥110 mg/dl). In 2004, this NCEP definition was revised (rNCEP) by lowering the threshold for fasting blood glucose to ≥100 mg/dl in concordance with American Diabetes

Association criteria for impaired fasting blood glucose. Thresholds for central obesity were also lowered from strictly >102 cm in men and 88 cm in women to greater than or equal to these values. Finally, the rNCEP definition includes patients being treated for dyslipidemia, hyperglycemia, or systemic HTN (Grundy *et al.*, 2004; Moebus *et al.*, 2006; Mottillo *et al.*, 2010). The prevalence of metabolic syndrome is about 10%-30% and is likely to be even higher in older age group (Han and Lean, 2016). It is anticipated that the prevalence of the metabolic syndrome and CVD will increase in near future. The only relief is that the components of metabolic syndrome are reversible and 5%-10% of weight reduction by diet and exercise, substantially lowers all metabolic syndrome components, including CVD. As mentioned above, vitamin D deficiency is associated with most of the components of metabolic syndrome hence its screening and treatment might be a useful tool to reduce the CVD risk.

Chronic kidney disease

Deficiency of vitamin D is widely prevalent in patients of CKD and these patients frequently receive vitamin D therapy. The reasons for deficiency are impaired synthesis of vitamin D by the skin, limited sunlight exposure during morbidity, malnutrition, loss of vitamin D metabolites due to frequent dialysis and decreased hydroxylase activity due to progressive renal failure (De Boer *et al.*, 2012; Drechsler *et al.*, 2011; Kienreich *et al.*, 2013a; Pilz *et al.*, 2013a). These patients have higher mortality rate and one of the most common cause is cardiovascular complication (Pilz *et al.*, 2011). Role of vitamin D therapy either by natural route or in the form of supplementation reduces the PTH levels which is another risk factor for CVD (Hagström *et al.*, 2009). Duranton *et al* in a systemic review and meta-analysis observed 37% reduction in cardiovascular mortality after active treatment of CKD patients with vitamin D (Duranton *et al.*, 2013). However, active vitamin D therapy also carries the risk of side effects i.e. hypercalcemia or hyperphosphatemia besides added cost over natural vitamin D therapy. Hence active vitamin D treatment is used in those cases only where even after correction of vitamin D deficiency PTH keeps on rising and remains consistently high above the normal range (KDIGO, 2009).

3.4.2 Cardiovascular events

Coronary artery disease, myocardial infarction, arrhythmias and heart failure

Vitamin D protects vessel walls against damage caused by inflammation. It down-regulates plaque-destabilizing enzymes like MMP-9 and diminishes proliferative effects of RAAS on vascular smooth muscle cells (Pérez-Hernández *et al.*, 2016). Its deficiency therefore is associated with severity of coronary artery stiffness and stenosis (Chen *et al.*, 2014). Vitamin D is considered a prognostic factor for acute MI and CAD (Karur *et al.*, 2014; Khalili *et al.*, 2012; Lee *et al.*, 2011). Studies suggest that vitamin D therapy may be helpful in acute coronary syndromes as it reduces VCAM-1, MMP-1 and P-selectin (CD62P) expression and regulates platelet aggregation (Stach *et al.*, 2011). According to a meta-analysis, in vitamin D deficiency the risk of CAD is 35% higher and it is a potential risk factor for death (Brewer *et al.*, 2011; Brøndum-Jacobsen *et al.*, 2012; Vacek *et al.*, 2012). Not only vitamin D but even the VDRs have a cardioprotective role. Ding et al in their

study on apolipoprotein E deficient mice showed that VDR deficiency promotes development of atherosclerosis and decreases the stability of atherosclerotic plaque via different mechanisms i.e. cell proliferation and differentiation, apoptosis, oxidative stress, membrane transport, matrix homeostasis, and cell adhesion (Ding *et al.*, 2015). Observational studies also propose a graded relationship between vitamin D deficiency and the risk of CVD particularly in older individuals (Ginde *et al.*, 2009b; Skaaby *et al.*, 2012; Wang *et al.*, 2012; Zittermann *et al.*, 2012). There are few negative studies also which oppose this association. In a report from NHANES of 13,331 participants no such association was found (Melamed *et al.*, 2008b). Another population based study on 9,146 younger adults (age range 30-71 years) noticed significant associations with all-cause mortality but not CVD associated mortality (Skaaby *et al.* 2012b). In a nonrandomized prospective Framingham Offspring Study, participants without a prior CVD and low vitamin D had a higher incidence of MI, coronary insufficiency, and HF though the association was noted only in hypertensive individuals (Wang *et al.*, 2008). In an Indian study also, an association of MI and vitamin D deficiency was found (Roy *et al.*, 2015). Overall, it seems that many confounding factors, e.g. age, gender, HTN, hyperlipidemia, DM, etc., are likely to impact this association (Chowdhury *et al.*, 2014). It is possible rather than a direct cause of fatality vitamin D deficiency could be an indirect risk factor causing fatal outcomes by virtue of its immune modulating and inflammatory effects. Further investigations related to supplementation with vitamin D, optimal dose and duration after giving due consideration to confounding factors may give a definite clue for an exact association.

Arrhythmia is the malfunction of heart's electrical system leading to irregular, too fast or too slow heartbeat. The prevalence of AF is about 2% in the world population. Genetics and molecular pathways are deeply involved in the development of this disease and it has multifaceted implications in health and quality of life (Balouch *et al.*, 2014; Zoni-Berisso *et al.*, 2014). Connection between AF and vitamin D was first described by Kessel in 1990 though the association has not been established till date (Kessel, 1990). Probably, it occurs via effect of vitamin D on smooth muscle proliferation and RAAS since angiotensin levels are raised in AF (Cardus *et al.*, 2006). Angiotensin II promotes development and maintains AF by increasing the spontaneous release of calcium from sarcoplasmic reticulum and triggering fibroblast proliferation (Demir *et al.*, 2014). In a few studies low levels of vitamin D have been observed in patients with AF (Chen *et al.*, 2014; Demir *et al.*, 2014). Surprisingly, high levels of vitamin D (>100 ng/ml) have also been found to be associated with AF (Menezes *et al.*, 2013). Few studies on the other hand have found no correlation between them (Qayyum *et al.*, 2012; Rienstra *et al.*, 2011).

CAD, previous MI, AF, HTN, valvular heart disease, cardiomyopathy are the common causes of HF. It represents 1-2% among the health problems (McMurray and Pfeffer, 2005). The vitamin D related mechanisms involved in protection against HF include: effects on myocardial contractile function, regulation of natriuretic hormone secretion, effects on extracellular matrix remodeling, reduced LVH, and the regulation of inflammatory cytokines (Tishkoff *et al.*, 2008; Weishaar *et al.*, 1990). Indirectly vitamin D can also affect cardiac function by altering PTH and serum calcium levels (Vanga *et al.*, 2010). The supporting evidence in favor of vitamin D and HF association include frequent osteoporosis, osteopenia and low vitamin D levels in patients with congestive

HF and higher frequency of HF in African-Americans (Shane *et al.*, 1997; Vaccarino *et al.*, 2002). Vitamin D deficiency and hyperparathyroidism are more common in African-Americans and about 30% of these women remain vitamin D deficient even after oral supplementation (Bahrami *et al.*, 2008; Nesby-O'Dell *et al.*, 2002). Protective benefits of vitamin D supplementation against HF have also been demonstrated in older population and infants (Ford *et al.*, 2014; Shedeed, 2012). Few other studies, however, are inconclusive. Further studies thus need to be performed before drawing conclusions on the role of vitamin D in HF (Petrone *et al.*, 2013; Wannamethee *et al.*, 2014).

Stroke

Epidemiological studies suggest a possibility of association between vitamin D deficiency and stroke (Kienreich *et al.*, 2013b; Mozos *et al.*, 2015). RCTs however, do not prove that vitamin D reduces stroke incidence hence, there is no definite recommendation for vitamin D supplementation for the prevention and treatment of stroke. Further evaluation of preventive, and therapeutic role of vitamin D in these patients is still required (Mozos *et al.*, 2015).

Peripheral arterial disease

Vitamin D deficiency is frequent in patients with occlusive and aneurysmatic arterial disease. The relationship between low vitamin D status and arterial disease if any, is possibly due to a disturbed adaptive immune response and an inflammatory milieu, promoting vascular dysfunction (McDermott *et al.*, 2014). So far very few studies have assessed the effects of vitamin D on vascular function and their results are contradictory. Reis *et al.* (2008) in a cross-sectional study, based on the NHANES, investigating racial differences in vitamin D levels and the incidence of PAD between black and white populations observed higher risk of vitamin D deficiency in Afro-Caribbean populations. They also noticed significantly lower levels of vitamin D in blacks than in whites and higher prevalence of PAD in black adults compared to that in whites (Reis *et al.*, 2008). Furthermore, amputation rates have been found to be higher in patients of PAD with vitamin D deficiency (Gaddipati *et al.*, 2011). Though, the studies propose role of vitamin D deficiency in PAD, these patients generally have impaired motility thereby are relatively less exposed to sunlight. Hence, it is important to consider that even the vitamin D deficiency could be a potential risk factor for PAD. The studies are not sufficient to conclude a definitive role of vitamin D deficiency in the pathogenesis of PAD.

3.5 Why conflicting results?

Till date, the data regarding the causal link between vitamin D deficiency and CVD are mixed, conflicting and ambiguous. There are multiple reasons for such results i.e. study-design related factors; variations in definitions of vitamin D deficiency; confounding factors including age, body mass index, medication, diet, sunlight exposure, physical activity and concomitant intake of calcium; biases due to different diseases; heterogeneity of vitamin D doses, compounds

and therapy duration; differences of absorption and metabolism among individuals; genetic differences in the VDR; inappropriate follow up time or lack of a control group with normal vitamin D levels; lack of standardization of 25-hydroxyvitamin D assay; and different ethnic populations (Theodoratou *et al.*, 2014). It is still not clear whether vitamin D supplementation is required only when vitamin D levels are low? Which type of vitamin D or vitamin D analogue should be given for optimal effects? In fact, consumption of high amounts of vitamin D may interfere with the regulation of phosphate metabolism hence, there is a need of markers for phosphate homeostasis particularly during vitamin D therapy. Due to all these issues, so far it remains uncertain whether the association between vitamin deficiency and CVD is causal or just a bystander. We expect that outcomes of the VITAL prevention trial and J-DAVID trial will provide answers to unsolved queries.

3.6 Conclusions

Vitamin D plays a crucial role in multiple metabolic pathways particularly related to bone and calcium homeostasis. In recent years it has been hypothesized that the vitamin D is also involved in homeostasis of CVS and its deficiency increases the risk of one or more CVD. To support this hypothesis numerous studies have been carried out, many of which propose that deficiency of vitamin D predisposes to CVD by acting on several physiological mechanisms which regulate CVS. Vitamin D has been shown to reduce activity of the RAAS and lower the blood pressure. Further it has an anti-inflammatory, antiproliferative, antihypertrophic, antifibrotic, antidiabetic and antithrombotic effects, all of which are important to maintain normal physiology of CVS. From literature review it appears that there is high prevalence of vitamin D deficiency amongst those who are prone to CVD, i.e. the aging population and those preferring indoor lifestyle. However, currently available evidences neither prove a causal association of vitamin D deficiency and CVD nor support beneficial or harmful effects of vitamin D supplementation. Also, there are several gaps in the knowledge that need to be investigated in future like cardiovascular effects of vitamin D therapy in patients with overt vitamin D deficiency, actual levels of vitamin D which are associated with risk of CVD and required optimal doses of vitamin D therapy. We also need to know more about autocrine and paracrine influence of vitamin D on CVS both at cellular and molecular level specially the control of inflammatory pathway and local calcification. Bioactivity of vitamin D within the vascular tissue and its own availability to tissue and autoregulation is another aspect which needs to be explored either by proteomic or metabolomic approaches. Hence, with such a hazy depiction, it may be too early to consider a causal association between CVD and vitamin D deficiency.

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4. Vitamins and coronary artery disease

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Abstract

Cardiovascular diseases are the leading causes of mortality and morbidity in developed and developing countries all over the world. Atherosclerosis is the most important preparation factor in the development of coronary ischemia. It is known that vitamins play an important role in the regulation of numerous pathological events such as endothelial damage, reactive oxygen species due to oxidative damage, platelet dysfunction, platelet aggregation, leukocyte adhesion and vasoconstriction that are among the most important pathogenetic mechanisms in the development of acute cardiac ischemic diseases. Acute coronary vascular events can be prevented by adequate intake of these vitamins, regulation of nutrition to meet the need for vitamins, or exogenous supplementation to supplement daily needs.

Keywords: vitamins, acute coronary diseases

Key facts

- Acute coronary syndromes are the leading causes of death worldwide
- Inadequate blood levels of certain vitamins have effects on the development of atherosclerosis, an important pathogenetic mechanism in the pathogenesis of acute coronary syndromes.
- The vitamins A, E, and C with antioxidant effects may prevent the development of acute coronary syndromes by inhibiting the production of oxidative stress-related reactive oxygen species that play a role in endothelial dysfunction.
- Vitamin D may regulate endothelial and muscle cell proliferation and platelet function, that is, atherothrombosis.
- Inadequate group B vitamins (folic acid, vitamin B6 and vitamin B12) lead to hyperhomocysteinemia, a risk factor for endothelial damage and cardiovascular diseases.

Summary points

- Vitamin D deficiency has an important effect on the development of atherosclerosis and acute coronary syndromes. The prevention of acute cardiac ischemic events through the maintenance of the optimal blood levels with vitamin D supplementation has been supported by scientific researches.
- Although low level of vitamin E is a preparative and risk increasing factor for coronary atherosclerosis and acute coronary syndromes, provision of vitamin E supplements more than needed may lead to some health problems as well.
- Adequate vitamin C intake at a level that meets daily needs may be protective from ischemic cardiac diseases. Although the fact that insufficient vitamin C levels may have adverse effects on ischemic coronary diseases and cardiovascular events are supported by studies, large randomized controlled trials that result in that these diseases can be prevented through the intake of high doses of vitamin C as a supportive means are inadequate.
- Keeping the vitamin E at optimal level is important for cardiovascular health.
- Through keeping the folic acid, B12 and B6 vitamins at optimal levels, endothelial damage and acute ischemic cardiac events can be prevented. There are no large-scale randomized controlled trials supporting that acute cardiac ischemic diseases can be prevented with high dose B group vitamin supplementation.

Abbreviations

COX	Cyclooxygenase
CRP	C reactive protein
IL	Interleukin
LDL	Low-density lipoprotein
NO	Nitric oxide
ST	S-T segment in electrocardiogram
STEMI	ST elevation myocardial infarction
VCAM-1	Vascular cell adhesion molecule-1

4.1 Introduction

The rates of mortality and morbidity due to secondary diseases caused by malnutrition are increasing day by day, particularly in developing countries. Among these diseases, coronary artery disease is still the leading cause of death in developed and developing countries despite all the improvements in treatment and interventional procedures. Some vitamins are known to play an important role in the prevention of atherosclerosis. It is known that vitamins have effects on numerous events such as decreasing inflammation in the vascular wall, prevention of endothelial injury, prevention of vessel wall stiffness and regulation of platelet functions that are important pathogenetic mechanisms in the development of atherosclerosis. In this chapter, the relationship between vitamins and the development, pathogenesis and prognosis of acute coronary syndromes will be explained.

4.2 Vitamin D and acute coronary syndromes

Vitamin D is a vitamin that can be produced endogenously in the body as well as it can be taken from outside through the food. 7-Dehydrocholesterol in skin exposed to ultraviolet B rays transforms into cholecalciferol (vitamin D3), which can also be taken by nutrients or vitamin supplements. The 25-hydroxylation of vitamin D occurs in the liver. 25-hydroxy vitamin D is then converted to 1,25-dihydroxy vitamin D in the kidneys with the enzyme 1-alfahydroxylase. This active form of vitamin D shows its biological effect by binding to the vitamin D receptors in the body. Most tissues and cells in the human body have vitamin D receptors. Vitamin D plays a role in cellular proliferation, differentiation, apoptosis and angiogenesis (Holick, 2007).

Normal serum vitamin D level is 100-150 nmol/l. If the serum level is below 50 nmol/l, it is expressed as severe vitamin D deficiency. vitamin D deficiency is seen in almost half of the healthy individuals in developed countries. Risk factors for vitamin D deficiency are older age, female sex, darker skin color, less exposure to sunlight, and undernutrition (Lugg *et al.*, 2015).

In recent years, most studies carried out on the relationship between acute coronary syndromes and vitamins have been related to vitamin D. Vitamin D directly affects endothelial dysfunction, vascular smooth muscle proliferation and migration (Kunadian *et al.*, 2014). It also has a regulatory influence indirectly on glucose and lipid metabolism (Kassi *et al.*, 2013). It leads to endothelial dysfunction, vasoconstriction, increased endothelial permeability, platelet aggregation, leukocyte adhesion, and atherosclerosis via cytokine production (Kunadian *et al.*, 2014). In a study conducted by Mahdavi *et al.* (2013), the level of 25-hydroxy vitamin D at admission of 216 patients admitted to the hospital with the diagnosis of acute coronary syndrome was compared with 25-hydroxy vitamin D level in 120 patients in the control group who were completely healthy without a history of cardiovascular disease. 25-hydroxy vitamin D levels in patients with coronary syndrome (under 20 ng/dl in 72% of patients) were found to be significantly lower than those of the control group (less than 20 ng/dl in only 27.4% of the patients in the control group). As a result of this study, it was reported that the group with the lowest level of 25-hydroxy vitamin D were the patients diagnosed with STEMI among the patients with acute coronary syndrome. However, no relationship was found between vitamin D levels and early prognosis in the study.

In a study of 1,801 patients with metabolic syndrome that underwent coronary angiography, 92% of patients had 25-hydroxy vitamin D levels below normal (75 nmol/l). In the study, 22.2% of the patients were found to have severe (25 nmol/l) vitamin D deficiency. During the mean follow-up period, 462 patients died and 267 (57.8%) of these patients were reported to have died of cardiovascular diseases. In addition, the optimal level of 25-hydroxy vitamin D was deduced to reduce cardiovascular disease related mortality rate (Thomas *et al.*, 2012).

Systemic inflammatory response develops during acute myocardial infarction. Along with being a reaction to repairing this damaged myocardium, it is also thought that the inflammatory response may damage the myocardium. In a study investigating the effects of vitamin D on the markers released during the inflammatory reaction occurred during acute myocardial infarction, levels of inflammatory markers in patients with myocardial infarction were found to have decreased, that is, inflammatory responses weakened when vitamin D was replaced in the acute phase (Arnson *et al.*, 2013). Patients who underwent acute myocardial infarction were divided into 2 groups and one group received 4,000 IU vitamin D orally for the first 5 days after acute myocardial infarction in the study. After 5 days, blood samples from both groups were taken and the levels of VCAM-1, CRP, IL-6, IL-8, vascular endothelial growth factor and tumor necrosis factor α levels were compared. As a result of the study, VCAM-1, CRP and IL-6 levels of the group given vitamin D supplementation were significantly lower than the group without vitamin D supplementation.

Vitamin D also functions in the function of pancreatic β cells through vitamin D receptors. Therefore, there is a close relationship between low 25-hydroxy vitamin D levels and the development of type 2 diabetes and metabolic syndrome. In patients with type 2 diabetes, the likelihood of developing acute ischemic coronary event is much higher. This is directly related to the adverse effect on endothelial activity and to preparing the ground for more unstable plaque formation. In a study with 166 acute coronary syndrome patients (66 with type 2 diabetes, 100 nondiabetic), 54% of patients with diabetic acute coronary syndrome and 33% of patients

with nondiabetic acute coronary syndrome had severe D vitamin deficiency (<20 ng/dl). The prevalence of vitamin D deficiency in patients with acute coronary syndrome with type 2 diabetes was significantly lower than the prevalence in nondiabetic patients (p = 0.006). In addition, in this study, while the rate of the serum 25-hydroxy vitamin D level of the cases with <20 ng/dl was 47.8% in patients diagnosed with STEMI, the rate of those with <30 ng/dl was found 13.4% (*P*=0.03). Coronary angiography showed a significantly higher incidence of multivessel lesions (69%) in diabetic patients with low serum 25-hydroxy vitamin D levels compared with nondiabetic patients (31.8%) with low serum 25-hydroxy vitamin D levels (*P*=0.007). At the end of the study, vitamin D deficiency was reported to be independently associated with the development of more severe acute coronary syndrome and to be more predictive of more common coronary lesions in patients with type 2 diabetes (Gondim *et al.*, 2016).

Vitamin D has a significant protective effect on the cardiovascular system with its antiangiogenic, antioxidant and antiproliferative properties. Cardiomyopathy is the best example of this in the cases with rickets caused by malnutrition. Histologically significant decrease in myofibrils and a significant increase in the extracellular space which disturbs myocardial contractility have been determined in these patients. In both experimental and clinical vitamin D deficiency, altered myocardial contractility returns to normal after vitamin D replacement therapy (Abdullah *et al.*, 1999).

Cardiovascular problems associated with vitamin D deficiency have been reported in all age groups. However, especially in postmenopausal women, vitamin D insufficiency has been dtermined to be associated with impaired pancreatic beta cell functions and glucose metabolism, lipoprotein exchange, overweight and obesity, hypertension and consequent increased cardiovascular risk (Perez-Lopez, 2009).

Verdoia *et al.* (2014) found vitamin D deficiency in 70.4% of 1,484 patients that underwent elective coronary angiography in their study. In this study, significant relationship between the severity of coronary artery disease and vitamin D deficiency was reported. Vitamin D levels in patients with at least 1% coronary artery stenosis less than 50% were found to be lower than those in patients without coronary artery stenosis on angiography. It was also found that there was a strong correlation between the severity of coronary artery disease including especially the left main coronary artery and/or 3 vascular disease and hypovitaminosis D level, and this relationship was found to be stronger if vitamin D level was below 10 ng / ml.

It has been supported by some studies that Vitamin D deficiency is an important factor not only in atherogenesis and pathogenesis of acute coronary syndromes but also in their prognosis. In a study involving 814 patients admitted to the hospital with the diagnosis of acute coronary syndrome, the morbidity and mortality of the patients during the hospital stay were examined. A relationship between low vitamin D levels and outcome during hospitalization was established in the study. In the group with the lowest level of vitamin D, the rate of in-hospital mortality and major complications such as hemorrhage required for transfusion, respiratory insufficiency and left ventricular failure were higher. In this study, it was found that there was a significant

correlation between D vitamin insufficiency and in-hospital and 1-year poor outcome (De Metrio *et al.*, 2015). This suggests that correcting vitamin D deficiency and achieving an optimal level of vitamin D may be promising in preventing acute coronary syndrome and its negative consequences.

As is seen, studies have supported that vitamin D insufficiency is an important risk factor for the development of both atherogenesis and acute coronary syndromes and an important factor in the development of poor prognosis, high mortality, more severe coronary lesions and serious complications.

4.3 The relationship between vitamin E and acute coronary syndromes

The protective effect of vitamin E on certain diseases due to its antioxidant effect is known. Oxidation is important in the pathogenesis of carcinogenesis and for this reason vitamin E is thought to be protective against cancer. Researchers have investigated not only the cancer-inhibiting effect but also the protective effect of vitamin E on cardiovascular disease.

Oxidation of unsaturated lipids on LDL particles and sequelae that occur in the flow of LDL due to this oxidation support the hypothesis that there is an association between vitamin E and cardiovascular diseases (Cathcart *et al.*, 1985; Morell *et al.*, 1984; Steinbrecher *et al.*, 1984). The most important effects of alpha-tocopherol are the reduction of lipid peroxidation, the proatherogenic activity of monocytes and platelet aggregation. In addition, by inhibiting the enzyme 5 lipoxygenase, it suppresses the inflammatory response. Endothelial cells inhibit monocyte adhesion. It has an important role on vascular homeostasis. NO release and NO response are required for normal vascular functions. Alpha tocopherol regulates NO release. Vitamin E support protects endothelium-dependent vasodilatation in male hypercholesterolemic and smokers. At physiological concentrations, vitamin E inhibits the proliferation of vascular smooth muscle cells via proteinkinase C inhibition. It inhibits COX-1, COX-2 and 5-lipoxygenase with its potent antiinflammatory effect (Mathur *et al.*, 2015).

Dietrich *et al.* (2009) classified 4,270 patients who participated in the Framingham Heart study according to their basal cardiovascular status, and compared groups receiving and not receiving vitamin E supplementation for 10 years in terms of cardiovascular disease and death incidence for all causes. In this study, researchers found no significant difference in term of the risk of cardiovascular disease and all mortality reasons between the group receiving supplementation and the group without vitamin E supplementation. As a result of the study, he pointed out that vitamin E supplementation did not reduce the risk for cardiovascular disease in those with and without pre-existing cardiovascular disease.

In a randomized, double-blind, placebo-controlled study on male patients with coronary artery disease, the effects of vitamin E and omega-3 fatty acid supplementation on the adiponectin level, the most important antiatherogenic adipocytokine, were investigated. In the study, vitamin E

supplementation was found to have no effect on adiponectin in patients with coronary artery disease (Ramezani *et al.*, 2015).

Contrary to the study above, Saboori *et al.* (2016) found in a placebo-controlled randomized study that vitamin E supplementation had positive effects on patients with coronary artery disease. In this study, 60 patients with coronary artery disease were divided into 3 groups. Group 1 received 4 g/day omega-3 and placebo-E vitamin, group 2 received 4 g/day omega-3 and 400 IU/day vitamin E and group 3 received placebo omega-3 and placebo vitamin E for 2 months. At the end of this period, total antioxidant capacity and antioxidant enzymes levels were found higher in the groups given omega-3 and vitamin E.

When 400 IU of vitamin E supplementation was administered to a patient with acute coronary syndrome and smoking habit daily for 6 months after discharge from hospital, the level of CRP, the most important indicator of vascular inflammation due to cigarette smoking, decreased significantly. High CRP levels measured during the first 6 months after acute coronary syndrome are associated with high myocardial infarction and high mortality rates. CRP is not only a marker for vascular inflammation, it also has prothrombotic potency. It activates the complement and stimulates monocyte release of the tissue factor. Thus it creates a strong stimulus for thrombosis. It is known that healthy cigarette addicts have higher levels of CRP compared to non-smokers. High CRP levels are associated with accelerated atherosclerosis (Murphy *et al.*, 2004). That is, if sufficient vitamin E level is provided, atherosclerosis can be slowed down, and reinfarction and mortality rates can be reduced.

Serum vitamin E and vitamin A levels were reported to be significantly lower in some studies with patients admitted with acute coronary syndrome diagnosis than in the healthy control group. In addition, serum E and vitamin A levels were found to be lowest in the ST elevated MI group and highest in the unstable angina pectoris group (Serdar *et al.*, 2007).

Söğüt *et al.* (2015), in a study with 189 patients underwent coronary angiography and with at least 1 major or near complete occlusion in epicardial coronary artery, classified as good collateral circulation and poor collateral circulation group according to the Rentop scoring system and compared plasma vitamin E and vitamin A of both groups. There was no significant difference in vitamin E and vitamin A levels between good collateral circulation and poor collateral circulation groups in the study. Relevy *et al.* (2015) investigated the effects of vitamin A deficiency on apolipoprotein and atherogenesis.

Whether vitamin E supplementation has a preventive, risk-reducing effect on coronary artery disease has been researched and discussed in a number of studies. Besides advocating that vitamin E supplementation is beneficial, some studies suggest that high dose vitamin E supplementation may be harmful or may increase mortality risk. Miller *et al.* (2005) reported in a meta-analysis that receiving daily supplementation of vitamin E more than 400 IU daily increased mortality caused by all reasons. A total of 19 clinical trials covering 135,967 patients were evaluated in this meta-analysis. In another meta-analysis of 232,606 participants and 68 randomized studies, it was

reported that either alone or combined vitamin E and vitamin A supplementation may increase mortality (Bjelakovic *et al.*, 2007).

In a study involving 8,415 patients with acute myocardial infarction who were initially without congestive heart failure, patients were divided into 2 groups as patients receiving vitamin E (4,202 patients) and those without vitamin E (4,213 patients) supplementation. Patients were followed in terms of the development of congestive heart failure for 3.5 years. During the follow-up period, a total of 220 patients developed congestive heart failure. In the group receiving vitamin E, the risk of developing congestive heart failure was high, 50% and the risk increase was found statistically significant (Marchioli *et al.*, 2006).

When the relationship between vitamin E and coronary artery diseases is investigated, it is seen that low vitamin E levels may be a potential health risk for coronary atherosclerosis and acute coronary syndromes while administrating vitamin E more than required may lead to some negative health problems. Concerning this issue, it can be said that the optimal level of vitamin E is an important factor for cardiovascular health.

4.4 Cardiovascular diseases, acute coronary syndromes and vitamin C

Intracellular ascorbic acid is the most important cofactor involved in collagen synthesis in arterial vessel wall and plaque capsule. In addition, endothelial dysfunction and inflammation, which are the most important factors for sensitive plaque rupture, can be reduced by the concentration of vitamin C in the cell. Vitamin C improves endothelial function by reducing the reactive oxygen species on the vessel wall with its anti-oxidant effect and by preventing the oxidative modification of low-density lipoproteins (Duvall, 2005). Dalgård *et al.* (2013) reported a high risk of acute coronary syndrome in women with sodium-dependent vitamin C transporter 2 gene variant responsible for the vitamin-C transport.

In a study with 102 patients diagnosed with acute coronary syndrome, the patients were divided into 2 as unstable angina pectoris and acute myocardial infarction, and then the acute myocardial infarction group was divided into 2 groups as with ST elevation and acute myocardial infarction without ST elevation. Vitamin C levels of the patient groups were compared with a healthy control group consisting of 45 individuals. In the study, the highest level of vitamin C was detected in control group (54.2 μmol/l). Vitamin C levels were found lower in all patient groups than in control group; 40.7 μmol/l in the group of unstable angina pectoris, 29.6 μmol/l in the group with acute myocardial infarction without ST elevation, and 31.5 μmol/l in patients with ST elevation myocardial infarction. In study, vitamin C levels of patient groups were significantly higher than control group. In addition, patients with ST elevation and non-ST elevation myocardial infarction had significantly lower levels of vitamin C than patients with unstable angina Pectoris (Serdar *et al.*, 2007).

A total of 85,118 healthy (30-55 years) women who did or did not receive vitamin C supplements were followed for 16 years for coronary heart disease in a study carried out by Osganian *et al.* (2003). At the end of follow-up period, 1,356 coronary heart diseases were detected. As a result, an inverse relationship was found between the intake of vitamin C supplementation and the development of coronary heart disease.

In another study investigating the relationship between plasma C vitamin level and the risk of lethal and non-fatal heart disease development, 11,112 female and 9,187 male participants aged 39-79 years were followed for 4 years. Studies have shown that every 20 μmol/l increase in vitamin C in the plasma reduces the risk of heart failure by 9% (Pfister *et al.*, 2011). In a similar study, Khaw *et al.* (2001) followed the plasma C vitamins of 19 496 patients for 4 years in terms of mortality due to ischemic cardiac diseases, cancer-related mortality and all-cause mortality. It was found that mortality rate fell to half at the highest vitamin C levels, that is, there was a reverse significant correlation between the mortality rate and vitamin C levels.

Randomized double blind placebo-controlled studies investigating the association of vitamin C supplementation with acute myocardial infarction, major cardiovascular events and stroke were also performed. In a placebo-controlled study involving a total of 14,641 male volunteers over 50 years of age, volunteers were followed for major cardiovascular events (death due to major cardiovascular events, acute myocardial infarction and stroke) for a mean of 8 years. In this study, 400 IU/day E vitamins and 500 mg/day C vitamins were administered to volunteers throughout the follow-up period. A total of 1,245 major cardiovascular events were detected at the end of follow-up. There were no significant differences in the major cardiovascular events between the groups receiving E and C vitamins and those receiving placebo. As a result, both E and C vitamin supplementation were found to have no effect on the prevention of major cardiovascular events in middle-aged and older men (Sesso *et al.*, 2008).

In another randomized controlled study, 8,171 female participants aged over 40 years with a history of cardiovascular disease or at least 3 risk factors for cardiovascular disease were followed for an average of 9.4 years administrating 500 mg/day C vitamins, and 600 IU E vitamins and 50 mg A vitamins every other day. At the end of the study period, a total of 1450 participants had one or more major cardiovascular events (acute myocardial infarction, coronary revascularization, death due to cardiovascular disease, stroke). It was reported that vitamin C, A and E supplementation in patients at high risk for cardiovascular disease had no protective effect on major cardiovascular events (Cook *et al.*, 2007).

On the other hand, among the postmenopausal women receiving hormone replacement therapy, the patient group receiving a high dose of vitamin C supplementation (1000 mg/day, 2 doses) was compared with the control group for non-fatal myocardial infarction, death and stroke (Waters *et al.*, 2002)

In this study, it was stated that high dose C vitamin supplementation may have no preventive effect on the development of non-fatal myocardial infarction, death and stroke, and even had harmful effects.

As can be seen, although studies have supported that vitamin C insufficiency may have adverse effects on ischemic coronary diseases and cardiovascular events, large-scale randomized controlled studies, which report that these diseases can be prevented with vitamin C supplement, are inadequate. Though few there are also some researchers who point out in some studies that high-dose vitamin C supplementation may be harmful to coronary vascular diseases. Vitamin C that is taken to meet physiological needs with daily meals may be protective in the development of cardiovascular problems or acute ischemic coronary diseases. It has been understood, however, that high-dose vitamin C or vitamin C taken as an external supplement in addition to nutrition, may not have a preventive effect and may even have adverse effects.

4.5 Acute coronary diseases, vitamin B6, B12 and folic acid

Hyperhomocysteinemia is a risk factor for cardiovascular diseases. Homocysteine is an amino acid containing sulfur that causes endothelial damage. It emerges as an intermediate product of methionine metabolism. Folic acid and vitamin B12 serve as cofactors in methionine metabolism. There is a negative correlation between homocysteine level and B12 and folic acid levels. Garcia *et al.* (2007) found that B12 and folic acid levels in patients with acute coronary syndrome were significantly lower than those in the healthy control group. They also determined a significant negative correlation between folic acid level and acute coronary syndromes. In a study of patients with acute coronary syndrome, the prevalence of hyperhomocysteinemia was 79.1%. In the same study, the prevalence of hyperhomocysteinemia was 5% in the group without acute coronary syndrome. In addition, the prevalence of hyperhomocysteinemia increased from the control group to the group of acute myocardial infarction, and the prevalence of the highest hyperhomocysteinemia was found in the group of acute myocardial infarction (93.15%). It was found that there was a negative correlation between homocysteine level and folic acid level and vitamin B12 level, and low folic acid level was detected in 51.1% and low vitamin B12 level in 42% of the patients with hyperhomocysteinemia and acute coronary syndrome, (Liu *et al.*, 2015).

Hodis *et al.* (2009) followed 506 patients aged 40-89 years administrating 5 mg folic acid, 0.4 mg vitamin B12 and 50 mg vitamin B6 for 3.1 years in a double-blind, placebo-controlled study. The effect of folic acid, B12 and B6 supplementation on intima and media thickness in 3 vascular beds was measured in the study. In the study, it was reported that high dose vitamin B supplementation reduced the progression of early atherosclerosis (carotid artery intima-media thickness).

Bleys *et al.* (2006) investigated the effects of vitamins A, C, E and B on atherosclerosis in a meta-analysis in a randomized controlled study. These studies have separately evaluated the studies using antioxidants (C, E, A vitamins and selenium) and studies using group B vitamins (folate, B6 and B12) support. Progression of atherosclerosis was calculated by B-mode ultrasound,

intravascular ultrasonography and angiography. Participants in the study were divided into two groups as support groups and non-support groups. As a result of this meta-analysis, antioxidant and vitamin B group vitamin supplementation were reported to have no reducing effect on atherosclerosis progression.

In a randomized placebo-controlled study by Imasa *et al.* (2009), 240 patients who were diagnosed with non ST elevation MI and unstable angina pectoris within the last 2 weeks were divided into two groups: folate group (116 patients) and placebo group (124 patients). Patients in folate group were followed-up for 6 months in terms of death, non-fatal acute coronary syndromes and serious health problems requiring re-admission to the hospital by giving daily 5 mg folic acid, 400 mg vitamin B12 and 10 mg vitamin B6 daily.

In this study, mortality, non-fatal acute coronary syndromes and re-hospitalization rates in the folate group were found higher in the placebo group during the follow-up period. As a result, it has been reported that folic acid-based supplementation may not be beneficial in preventing cardiovascular events, and may even be harmful.

As can be seen, the results of studies on reduction of mortality prevention and reduction of acute coronary syndromes and prevention of morbidity with B6, B12 vitamins and folic acid supplementation are still controversial. Although there is some data in some of the randomized controlled studies indicating that the risk is reduced with folic acid and group B vitamin supplements, other studies have reported that supportive therapy has no effect and may even be harmful. Insufficient folic acid and vitamin B12 and B6 levels are the causes of hyperhomocysteinemia, an important risk factor for cardiovascular diseases.

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5. Genomic and nongenomic controls of vitamin D on cardiovascular health and disease

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Abstract

Ectopic or vascular calcification caused primarily by dysregulation of vitamin D and phosphate homeostasis is a major contributor to development of atherosclerosis and cardiovascular disease (CVD). Both deficiencies and excesses of vitamin D affect cardiovascular factors that mediate development of CVD. Atherosclerosis results from accumulation of plaque which is comprised of oxidized lipids, cellular debris, fibrin material and marked calcification. Vitamin D deficiency affects macrophage activation, adhesion and migration, which underlies the 'response to injury' hypothesis that initiates atherosclerotic progression. Vitamin D coupled with other epigenetic modifications can subdue cellular inflammation, reduce age-related systolic hypertension and vascular rigidity, and improve overall endothelial functions. Thus, understanding molecular changes and pathways that preserve function and integrity of vascular endothelia and smooth muscle cells will enable investigators to development effective dietary and/or therapeutic measures to remediate CVD. This chapter will explore the major contributions of vitamin D in controlling vascular endothelial and smooth muscle cell integrity, activation and inflammation. Emphasis is placed on: (1) evidence that links vitamin D deficiency to cardiovascular disease; (2) redox sensitivity and control of the vitamin D receptor; (3) role of vitamin D in augmenting the antioxidant network associated with the transcription factor, nuclear factor (erythroid-derived 2)-like 2; and (4) the association of vitamin D with fibroblast growth factor 23 and Klotho, the endocrine axis which regulates phosphate homeostasis among bone, parathyroid gland, and kidney.

Keywords: vitamin D, endothelium, cytokines, atherosclerosis, ROS/RNS

Key facts

- Cardiovascular disease (CVD) is a major cause of death worldwide and low circulating levels of 25-hydroxyvitamin D co-associates with CVD and atherosclerosis.
- Vitamin D benefits CVD by increasing endothelial nitric oxide and influencing vascular tone, decreasing endothelial oxidative stress, inhibiting release of pro-inflammatory cytokines, modulating immune response, and inhibiting proliferation and migration of vascular smooth muscle cells.
- Vitamin D controls responses of two critical anti-oxidative pathways, namely, nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and Klotho/fibroblast growth factor 23 (FGF23) transcription factors.

Summary points

- Vitamin D sufficiency along with properly functioning receptors and transporters protect against cardiovascular disease by inhibiting hypertension, endothelial dysfunction, atherosclerosis, vascular calcification, cardiac hypertrophy and progressive renal dysfunction.
- Vitamin D and its receptors prevent formation of oxidized lipids, mitigate endothelial and smooth muscle cell dysfunction, block formation of inflammatory cytokines, and quench ROS/RNS; collectively, these contribute to maladaptive cardiac remodeling and dysfunction.
- Vitamin D supplementation interacts with the Nrf2 detoxification/antioxidant network.
- Vitamin D in co-association with FGF23 and Klotho, decrease hyperphosphatemia, control renal production of 1a,25$(OH)_2$D3, and block synthesis of parathyroid hormone.

Abbreviations

CAD	Coronary artery disease
CYP	Cytochrome p450
CVD	Cardiovascular disease
DBP	Vitamin D binding protein
FGF23	Fibroblast growth factor 23
FGFR1	Fibroblast growth factor receptor 1
eNOS	Endothelial nitric oxide synthase
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidized)
HDL-C	High-density lipoprotein cholesterol
Keap1	Kelch-like ECH-associating protein 1
LDL-C	Low-density lipoprotein cholesterol
Maf	Musculoaponeurotic fibrosarcoma protein
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NO	Nitric oxide
PSH	Protein sulfhydryl
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RXR	Retinoid X receptor
SOD	Superoxide dismutase
VDR	Vitamin D receptor
VSMC	Vascular smooth muscle cells

5.1 Introduction

Vitamin D and its receptors are intricately involved in maintaining heart health and preventing CVDs. This chapter will explore the impact of this essential nutrient in controlling vascular endothelial cell integrity and in regulating inflammatory responses within the cardiovascular system via genomic and non-genomic pathways.

5.2 Vitamin D activation and VDR interaction

Vitamin D3 is a lipophilic nutrient that was discovered as a dietary constituent (primarily in cod liver oil) that helps remediate childhood rickets, a disease of defective mineralization that occurs prior to epiphyseal closure. In addition to its acquisition through dietary intake, vitamin D3 can be produced by direct exposure of the provitamin D, 7-dehydrocholesterol, in skin to sunlight (UV range of 260-360 nm). The photolysis of 7-dehydrocholesterol involves bond cleavage of its B ring between carbons 9 and 10 to form an opened-ring steroid (secosteroid) or cholecalciferol (vitamin D3). Following its production by keratinocytes within the stratum basale and stratum

spinosum layers of the skin, cholecalciferol is transported to the liver bound in plasma to a DBP. Cholecalciferol is hydroxylated to 25(OH)D3 primarily in liver, and marginally in other tissues, by five microsomal CYP hydroxylases (CYP2R1, CYP2J2/3, CYP3A4, CYP2D25, and CYP2C11) and one mitochondrial hydroxylase (CYP27A1) (Pinto and Cooper, 2014; Prosser and Jones, 2004; Zhu and DeLuca, 2012). Each of the CYP proteins exhibits different catalytic properties that are not exclusive in their formation of 25(OH)D3 from cholecalciferol because they also hydroxylate other vitamin D analogs as well as other sterol and bile acid metabolites. Among the 25-hydroxylases, CYP2R1 plays a key role in the vitamin D 25-hydroxylation in humans as kinetic studies show that 25-hydroxylation can occur using physiological concentrations of the zoosterol, cholecalciferol, and plant-derived, ergocalciferol (vitamin D2). In addition, animal knock-out models and mutations of the CYP2R1 exhibit symptoms of vitamin D-dependent rickets and marked decreases in 25(OH)D3 concentrations, which is the major plasma form of vitamin D bound to DBP. Uptake of the circulating 25(OH)D3-DBP complex occurs via a megalin/cubilin-mediated endocytosis. 25(OH)D3 is physiologically inactive until hydroxylated to 1α,25-$(OH)_2$ vitamin D3 (1α,25$(OH)_2$D3) in the kidney. Final conversion of circulating 25(OH)D3 to the active hormone, 1α,25$(OH)_2$D3 or calcitriol, is under regulatory control by a renal mitochondrial CYP27B1 (Urushino *et al.*, 2006). In addition to renal production of 1α,25$(OH)_2$D3, mitochondria in placenta, macrophage, astrocytes, skin, intestine, as well as breast and prostate can also synthesize 1α,25$(OH)_2$D3 (Jones, 2013). The activity of CYP27B1 in kidney is highly regulated by the calciotropic hormones, parathyroid hormone and calcitonin and by fibroblast growth factor-23 (FGF-23), and is the major producer of systemic 1α,25$(OH)_2$D3. By contrast, 1α-hydroxylase activity in the aforementioned extrarenal tissues functions in a paracrine/autocrine manner and is influenced by cytokines such as interleukins and interferon-γ (Prietl *et al.*, 2013).

Once inside target cells, 1α,25$(OH)_2$D3 binds to the VDR in the cytoplasm and translocates to the nucleus where it heterodimerizes with a RXR. This complex then binds to vitamin D response elements on DNA to increase transcription of vitamin D-regulated genes. These genes enhance innate immune responses and suppress adaptive immune responses, thereby shifting immune status from a proinflammatory to a more tolerogenic state (Prietl *et al.*, 2013). Other important biological functions of vitamin D include maintenance of muscle strength and size, stimulation of insulin production in β-cells, suppression of renin secretion from kidneys and reduced cellular proliferation of both normal and cancer cells. The more sustained function of vitamin D is reserved for the regulation of calcium, phosphate and bone homeostasis (Thomas and Demay, 2000).

5.3 Pleiotropy of vitamin D: evidence linking vitamin D deficiency to CVD

The importance of vitamin D extends beyond control of bone calcium homeostasis. Vitamin D metabolites impact not only on the health of cardiovascular cells but on the pathogenesis of CVD. This is evidenced by findings that VDRs are expressed in target tissues that include major cardiovascular cell types, namely, endothelial cells (Bozic *et al.*, 2015), VSMC (Mary *et*

al., 2015), myocardiocytes (Chen *et al.*, 2011), and platelets (Silvagno *et al.*, 2010). Exerting its activity through the VDR, vitamin D regulates vascular inflammation, calcification of soft tissue, and hypertension via the renin-angiotensin aldosterone system. Thus, 1α,25$(OH)_2$D3 plays a significant role in influencing blood pressure, atherosclerosis and other factors that lead to the development of coronary artery and other vascular diseases. Based on gene expression profiles, VDR activation regulates expression, either directly or indirectly, of approximately 0.8-5% of the total genes in the human genome (Bouillon *et al.*, 2008). This includes pathways that control cell proliferation, differentiation, apoptosis, oxidative stress, membrane transport, matrix homeostasis, tissue mineralization, and cell adhesion (Bossé *et al.*, 2007; Burgess *et al.*, 1990; Li *et al.*, 2004; Lind *et al.*, 1995; Shalhoub *et al.*, 2006; Simpson *et al.*, 2007; Wu-Wong *et al.*, 2007; Xiang *et al.*, 2005).

Epidemiological studies as well as observational analyses including randomized control trials, case-controlled and prospective cohort studies have demonstrated that higher CVD morbidity and mortality correlate with seasonal variations in vitamin D levels and that plasma 25(OH)D3 inversely associates with risk factors for CVD namely hypertension, hyperlipidemia, and type 2 diabetes (Adams and Hewison, 2010; Judd and Tangpricha, 2009; Lee *et al.*, 2008; Pilz *et al.*, 2016; Temmerman, 2011). Dietary intake information derived originally from the NHANES and the second NHANES 2003-2006 indicated associations between vitamin D status and CVD (Curtin *et al.*, 2012). Earlier NHANES studies as well as cohort and case-control studies revealed that vitamin D deficiency is associated with increased prevalence of self-reported coronary heart disease, heart failure, and peripheral vascular disease. Cardiovascular risk factors and vitamin D status were investigated in a population with a high prevalence of type 2 diabetes. Studies showed that vitamin D deficiency occurred in normoglycemic but especially in hyperglycemic patients in combination with cardiometabolic risk factors, such as increased blood pressure (Braun *et al.*, 2012). In a similar fashion, cardiovascular risk factors and vitamin D status have been widely investigated in patients with chronic renal disease. Advancements of these investigations were made evident by understanding strong relationships between renal disease and cardiovascular events (Kiuchi and Mion, 2016; Luft, 2000). In brief, hypovitaminosis D is associated with peripheral arterial disease and other subclinical CVD markers not only in patients with chronic renal failure but in patients with non-dialysis kidney disease (Căpuşa *et al.*, 2016).

5.4 Genomic and non-genomic actions of vitamin D3

Epidemiological evidence notably correlates deficiencies of vitamin D with the development of CVD. In particular, low levels of vitamin D, as indicated by serum total 25(OH)D3 levels, are positively associated with increased risk of CVD-related outcomes. The Endocrine Society Clinical Practice Guidelines define vitamin D deficiency as serum 25(OH)D3 levels <50 nmol/l, insufficiency as 52.5-72.5 nmol/l, and sufficiency as >75 nmol/l. The data are presented in nmol/l and can be divided by 2.496 to convert nmol/l to ng/ml (Heber *et al.*, 2010).

By contrast to other steroid hormones, the conversion of 7-dehydrocholesterol to the secosteroid, vitamin D, provides three structural features that contribute to the conformational flexibility of 1α,25$(OH)_2$D3; these include the presence of (1) an eight-carbon side chain, (2) the opened B ring which 'unlocks' the A ring, and thus (3) provides the 'unlocked' A ring with the steric flexibility to undergo an extremely fast *cis-t*rans chair-chair interconversion (Bouillon *et al.*, 1995). This rapid interchange promotes tight binding between 1α,25$(OH)_2$D3 and its cognate VDR which then enables heterodimer formation with a subset of other type II steroid receptors, particularly the 9-cis-retinoic acid receptor (RXR) and also the T3 receptor (Cooney *et al.*, 1993). The interaction of the heterodimeric complex with a specific vitamin D-responsive element within the DNA causes recruitment of co-activators or co-repressors, which lead to positive or negative transcriptional regulation of gene expression (Norman, 2006).

Given that 1α,25$(OH)_2$D3 functions in part through binding to VDR, dysfunctionality of the VDR also contributes to the composite of CVD-associated events by regulating the expression of genes that control smooth muscle cell proliferation, immunomodulation, angiogenesis, inhibition of the renin–angiotensin–aldosterone system, and expression of neurotrophic factors. Integrated within its genomic responsibilities, 1α,25$(OH)_2$D3 exerts non-genomic activity through direct signal transduction pathways that involve phospholipases, phosphatidylinositol-3 kinase, p21ras, and direct generation of second messengers that enable activation of a variety of protein kinases and opening of calcium and chloride channels (Hii and Ferrante, 2016). Although the cell-type specific distribution of the VDR is not fully resolved, the existence of a nuclear VDR for genomic action and a membrane-associated VDR responsible for non-genomic signaling is recognized (Dormanen *et al.*, 1994). Membrane VDRs were identified to explain observations that vitamin D analogues that failed to complex with nuclear VDRs were able to elicit rapid cellular responses. Efforts to understand the complexities of binding vitamin D and its analogues to both nuclear and membrane VDRs and their therapeutic implications have been reviewed elsewhere (Belorusova and Rochel, 2014; Carlberg and Molnár, 2015; Hii and Ferrante, 2016; Zhang *et al.*, 2013).

5.5 Composite evidence that deficiency results in an increase in risk for CHD

5.5.1 Vitamin D3 deficiency is linked to hyperlipidemia

In a large cross-sectional study involving the USA population (Lupton *et al.*, 2016), investigators examined the effects of serum 25(OH)D levels on atherogenic lipid and lipoprotein profiles. Subjects were divided into groups based on serum 25(OH)D levels. The deficient group was categorized by having serum 25(OH)D <20 ng/ml, intermediate ≥20-30 ng/ml, and optimal ≥30 ng/ml. The deficient and optimal 25(OH)D groups from a sample size of 20,360 subjects were compared. Compared to the optimal vitamin D group, patients who were deficient in serum 25(OH)D showed lower serum HDL-C coupled with higher total cholesterol and non-HDL-C. In addition, deficient serum 25(OH)D was associated with significantly higher LDL-C, intermediate-density lipoprotein cholesterol, very LDL-C, remnant lipoprotein cholesterol, and triglycerides. Chylomicrons, very low-density lipoprotein, intermediate-density lipoprotein, and

LDL components are contributors to CVD because the particles are not eliminated by the liver but instead are taken up in arterial walls to eventually be engorged in lipid laden macrophages or foam cells (Colin *et al.*, 2014). These cells represent the hallmark of initiation and progression of atherosclerosis and co-associate with infiltration of oxidized lipids, cytokines and in situ vascular inflammation. It is important to consider here that the phenotypic activation of macrophages acquiesce to the availability of 1α,25$(OH)_2$D3. Vitamin D inhibits T-cell proliferation and decreases expression of pro-inflammatory cytokines which includes inhibition of nuclear factor kappa-B signaling (Menezes *et al.*, 2014). In addition, 1α,25$(OH)_2$D3 shifts proliferation of T-helper 1 to T-helper 2 phenotypes which dampen the atherogenic response characteristic of T-helper 1 cells. Thus, vitamin D is important in modulating immunoplasticity by altering the polarization and activation of proatherogenic macrophages.

5.5.2 VDR knockout mice show endothelial dysfunction

Among the numerous benefits of vitamin D, the ability to control vascular smooth muscle and endothelial cell proliferation is vital to the proper functioning of endothelial tissue. Endothelial dysfunction is among the most significant risk factors for the development of CVD. To determine whether endothelial dysfunction is associated with impaired signaling of VDR, several investigators (Ni *et al.*, 2014) assayed endothelium-dependent vasorelaxation using mice with a deletion in their VDR gene (VDR ECKO). Aortae isolated from VDR ECKO mice were compared to those from control mice, in terms of phenylephrine-induced contraction and acetylcholine induced endothelium-dependent relaxation. VDR ECKO mouse aortae demonstrated impaired vasorelaxation compared to the control mouse aortae, suggesting that the deletion of the VDR gene can cause endothelial dysfunction. Moreover, VDR ECKO and control mice administered angiotensin II revealed aortic intimal wall thickening and vascular fibrosis. These results show that VDR plays an integral protective role in maintaining the vascular endothelium.

In a cross-sectional study that evaluated the metabolic association between osteoporosis and vascular calcification, results indicated an inverse correlation between bone mineral density and carotid intimal thickness (Hmamouchi *et al.*, 2009). These studies suggested that vascular disease exhibits a close associated with vitamin D status and proper calcium balance. In other studies that compared patients undergoing elective coronary angiography with those requiring coronary angiography because of significant CAD (defined as single vessel stenosis >50%) and severe CAD (defined as left main and/or trivessel disease), vitamin D deficiency was prevalent in the majority of patients with CAD (Verdoia *et al.*, 2014). These studies implicate a strong relationship between vitamin D and CVD. Although not completely substantiated by randomized controlled trials, epidemiologic data and observational studies demonstrate a clear association between vitamin D deficiency and CVD.

The genomic effects of vitamin D which involve binding of 1α,25$(OH)_2$D3 to the VDR have been directly implicated in endothelium-mediated vasodilation, anti-coagulant activity and inhibition of inflammatory responses. In addition, coupled with its genomic manifestations, non-genomic effects of vitamin D occur rapidly through membrane-associated receptors that

influence signaling cascades such as calcium channels, cyclic guanosine monophosphate and mitogen-activated protein kinases. These pathways, in turn, contribute to maintenance and control of blood pressure and reductions in myocardial hypertrophy and incidents of ventricular arrhythmias (Temmerman, 2011).

It is important to consider that genetic variations in the VDR can modify the efficacy of vitamin D on the aforementioned pathways that can influence outcomes of CVD. For example, VDR polymorphisms such as ApaI (VDR 7975232 C > T), BsmI (VDR 1544410 A > G), FokI (VDR 2228570 C > T), and TaqI (VDR 731236 T > C) have been associated with marked variations in pathophysiological conditions that affect CVD (Valdivielso and Fernandez, 2006). These genetic variances have been shown to exacerbate the prevalence of CAD in type 2 diabetics, modify insulin secretory capacity, and diminish transcriptional activity by causing variation in the length of the VDR (Jurutka *et al.*, 2000; Ogunkolade *et al.*, 2002; Ortlepp *et al.*, 2001). Thus, individual risks and responses to vitamin D among patients can vary according to common genetic differences in the VDR gene. A long-term prospective study identified an association between a VDR polymorphism (VDR7968585 C > T) and a composite of risk for hip fracture, cancer, myocardial infarction, and mortality. In this genetic variation, association with disease and VDR was only apparent in individuals with compromised vitamin D levels, highlighting the importance of taking into account genetic considerations when identifying disease relationships with vitamin D. Thus, in order to better understand the role of vitamin D and CVD, investigations need to consider the relationship among circulating 25(OH)D, VDR genetic variants, and biomarkers of CVD (Reis *et al.*, 2005). Given the complexity of the clinical environment, such studies will help explain some of the inconsistent results across studies that correlate vitamin D status and cardiovascular health and disease. In depth understanding of genetic variants in the VDR and clinical outcomes of CVD can be found in several recent reviews (García-Bailo *et al.*, 2013; Levin *et al.*, 2012; Schuch *et al.*, 2013).

5.5.3 Redox sensitivity of transcription factor VDR

A number of transcription factors, such as, NF-κB, tumor suppressor encoded p53, steroid hormone receptors, e.g. glucocorticoid receptor, estrogen receptor as well as the VDR, contain conserved cysteine residues in their structure. These conserved cysteinyl moieties ensure binding of the VDR with DNA specific elements present in the promoter region of genes under their control. These interactions occur through hydrogen bonding between sulfhydryl (thiol) groups of transcription factors and nitrogenous bases on DNA. In addition, intra- or intermolecular disulfide (-S-S-) and mixed disulfide (-S-S-R) bonds occur within transcription factors that permit proper tertiary orientations. Formation and/or disruption of disulfide bridges are crucial for binding DNA and complexing cations as exemplified by the orchestration of DNA-interactive zinc-finger motifs within transcription factors. Because cysteines contain free thiol moieties that are prone to oxidative and nitrosative events and can engage in mixed sulfide-disulfide exchange reactions within the intracellular milieu, many transcription factors have been categorized as redox sensitive (Carter and Ragsdale, 2014).

In the case of VDR, early studies of steroid hormone and VDR interactions demonstrated that thiol-attacking and modifying compounds such as mercurials and iodoacetamide adversely affect ligand and DNA binding (Pike, 1981). Importantly, the binding activity can be reactivated using reductants such as dithiothreitol, implying that maintenance of thiol integrity or free sulfhydryl groups was required for ligand and/or DNA binding. Most importantly, it became evident that changes in the intracellular redox environment may be exploited to regulate the ambient intracellular redox potential and influence homeostasis. Several studies have shown that VDR cysteinyl thiol moieties are critical for ligand and DNA binding (Pike, 1981; Wecksler *et al.*, 1979). In studies examining the VDR ligand binding domain, site-directed mutagenesis that replaced conserved cysteine residues with glycine clearly implicated thiols as being important for high affinity ligand binding and VDR-driven transcriptional activation (Nakajima *et al.*, 1996). These investigators found that mutagenesis of cysteine to glycine at positions 288 and 337 in the 1α,25$(OH)_2$D3 binding domain of the VDR markedly compromised transcriptional activation. By contrast, heterodimer formation with retinoid X receptors in binding to the vitamin D-responsive element and nuclear localization were not appreciably affected by cysteinyl alterations at these sites.

As mentioned earlier, heterodimerization of the VDR and RXR is critical for transcriptional activation of target genes. Numerous *in vitro* and *in vivo* studies have demonstrated that the zinc finger binding motif of the VDR:RXR heterodimer and the extent of DNA binding are sensitive to exposures of intra- and extracellular oxidants that include H_2O_2, superoxide, singlet oxygen, peroxyl radicals, NO and peroxynitrite. Studies performed *in vitro* show that these reactive oxygen and nitrogen species inhibit VDR/RXR-DNA complex formation in a dose-dependent manner (Kröncke *et al.*, 2002). Accordingly, singlet oxygen, peroxyradical, and peroxynitrite species irreversibly inhibit receptor function. In studies performed *in vivo* using a VDR/RXR reporter system, these investigators showed that cells can repair zinc finger domains after nitrosative stress but cannot after oxidative stress. Further studies indicate that of the ROS affecting VDR receptor function, interaction with the vasodilator gas, NO, is less detrimental and easier to reverse. In this context and relative to interactive events with antioxidant cuproenzymes that degrade superoxide, nitrosative events in signaling cascades can have activating as well as inhibitory effects. Thus, cells are able to repair binding of VDR:RXR heterodimers that have been altered by exposure to certain ROS/RNS but not others. This indicates that the extent of oxidative stress occurring within cells may influence the ability of reducing agents to reverse oxidation of VDR thiols and thus influence transcription (Bogdan, 2001).

The varying degrees of thiol oxidation by ROS have been observed using cysteinyl containing peptides and proteins (PSH). Thiol moieties may be sequentially oxidized to sulfenic acid (PSOH), then to sulfinic acid (PSO_2H) and finally to their highest oxidation level of sulfonic acid (PSO_3H). The oxidative states of PSOH and PSO_2H (Jönsson *et al.*, 2008; Lei *et al.*, 2008), but not PSO_3H, are potentially reversible. The former two oxidation states occurring in redox sensitive domains of proteins can be easily reduced by antioxidant enzymes such as glutaredoxins, thioredoxin reductase, sulfaredoxin and by protein disulfide isomerase (Cooper *et al.*, 2011). In some cases, the deliberate oxidation of PSH to PSOH may be beneficial within unfolded proteins as this assists

in the correct formation of intramolecular disulfide bonds (Rehder and Borges, 2010). In other cases, accumulation of unfolded proteins through excessive oxidation of cysteinyl moieties may cause increased expression of genes involved in growth arrest and apoptosis (Boot-Handford and Briggs, 2010). Thus, a wide range of redox modifications regarding sulfur moieties are involved in redox signaling with H_2O_2/superoxide-mediated oxidations of cysteine residues in proteins being ones most widely investigated. Protein cysteinyl moieties with low pKa are susceptible to oxidation to form usually reversible intra- or inter-molecular disulfides.

In terms of the importance of redox signaling in the heart, ROS and redox signaling are critical factors that enable the cardiovascular system to adapt to both physiological and pathological stresses. Compared to endothelial cells within the vasculature, cardiomyocytes exhibit the highest oxygen consumption and consequently must endure ROS generation during mitochondrial reduction of molecular oxygen to water within the electron transport chain. In view of the continuous generation of ROS during regular metabolic processes, cardiac tissue is able to acutely adapt to meet contractile performance and chronically remodel to meet demands of prolonged workloads. Cardiac hypertrophy is a major component of chronic cardiac remodeling which involves enlargement of cardiomyocytes and increased thickness of ventricular walls (Santos *et al.*, 2011). During sustained cardiac stress irreversible structural and contractile abnormalities will lead to complete cardiac dysfunction. During these adaptive phases, redox signaling pathways play critical roles both in acute cardiac adaptations and in chronic cardiac remodeling that can lead to heart failure. Detailed reviews of the importance of ROS and redox signaling in cardiomyocytes have been published elsewhere (Santos *et al.*, 2016; Silva-Palacios *et al.*, 2016; Wang and Hai, 2016).

5.6 VDR signaling and CVD

Vitamin D and the VDR regulate expression of numerous signaling pathways that associate vitamin D deficiency and receptor defects with CVD and its contributing illnesses, hypertension, diabetes, and obesity. Although there is consensus in the literature that oxidative stress contributes to the development of CVD, debate continues over the level and duration of exposure that may precipitate or even exacerbate the disease (Tada and Suzuki, 2016). The theory underlying oxidative stress and CVD implies that increased production of ROS and RNS species induces a variety of macromolecular oxidative modifications that promote mitochondrial and cytosolic damage gradually leading to endothelial and smooth muscle cell dysfunction. Intracellular oxidative stress within the vascular endothelium is one of the most fundamental triggers in the complex chain of events that lead to atherosclerosis. Vascular oxidative stress and inflammation are thought to promote the development of myocardial infarction and stroke, thus increasing incidence of cardiovascular mortality (Barančík *et al.*, 2016).

Vitamin D is the custodial regulator of two important redox signaling, antioxidant pathways, namely Klotho and Nrf2. Both pathways regulate a cadre of antioxidant responses, including induction of numerous genes for proteins that detoxify ROS and regulate synthesis of GSH, the

cell's master antioxidant (Berridge, 2015). Proatherogenic conditions such as obesity, diabetes, and hypertension which co-associate with vitamin D deficiency invoke adaptive mechanisms that involve induction of both Klotho and Nrf2 (Ding and Ma, 2015; Espinosa-Diez *et al.*, 2015; Kalaitzidis *et al.*, 2016; Ungvari *et al.*, 2011). Vitamin D controls formation of Klotho and Nrf2 which counter oxidative challenges. The former protein is recognized as an anti-aging protein whose decline in expression contributes to age-related conditions such as CVD and ectopic calcification of soft tissue and the latter protein is a transcription factor that up-regulates numerous ROS detoxifying and antioxidant genes.

5.7 Klotho

The Klotho gene is classified as an aging-suppressor gene which is abundantly expressed in kidney and brain choroid plexus (Xu and Sun, 2015). It encodes a transmembrane protein that functions as a co-receptor for FGFR1. Studies on the sequence analysis of Klotho complementary DNA revealed two transcripts presumably due to alternative RNA splicing (Matsumura *et al.*, 1998). The identified transcripts encode for a membrane-bound form and a secreted form of Klotho. Investigators have shown that the secreted protein is the major Klotho isoform which has undergone subsequent cleavage by several membrane-bound proteases before being released into the systemic circulation (reviewed in (Pavlatou *et al.*, 2016)). Once secreted, Klotho functions as an obligate co-receptor for FGF23 which, in the absence of Klotho, exhibits low affinity binding to the FGFR1. Thus, the resultant high affinity, ternary complex exhibits protection against ROS/RNS, increases production of NO (Saito *et al.*, 1998), and regulates multiple ion channels including calcium and phosphate (Chang *et al.*, 2005).

The cooperative binding of Klotho and FGFR1 to the ligand FGF23 increases phosphaturia (Hu *et al.*, 2010, 2013), a physiological corrective response designed to counteract hyperphosphatemia (Razzaque, 2009) that can arise from prolonged or excessive 1α,25$(OH)_2$D3-stimulation of intestinal calcium and phosphate absorption. Elimination of excess phosphate is especially critical in renal disease patients since hyperphosphatemia is an independent risk factor that contributes to increased incidence of aortic and mitral stenosis and CVD (Qunibi, 2004). Hyperphosphatemia may cause arterial calcification by altering the phenotype of VSMC into osteoblast-progenitor cells that are capable of calcifying its extracellular matrix (Giachelli *et al.*, 2001). Accordingly, in terms of mechanistic control of negative and positive pathways, 1α,25$(OH)_2$D3 and Klotho may be considered 'yin and yang' regulators that function in reciprocation to ensure phosphate and calcium homeostasis in humans. Both FGF23 and Klotho expression are subject to control by exposure to vitamin D3 (Haussler *et al.*, 2012). Upregulation of FGF23 accelerates urinary phosphate excretion and suppresses 1α,25$(OH)_2$D3 synthesis by decreasing renal CYP27B1 (1α-hydroxylase) and inducing CYP24A1 (24-hydroxylase) in the presence of FGFR1 and its co-receptor Klotho. Thus, Klotho ameliorates vascular endothelial dysfunction and delays vascular calcification factors associated with various cardiovascular events (Ding and Ma, 2015). Studies of genetic polymorphisms (e.g. G395A) within the promoter region of the Klotho gene are associated with the development of essential hypertension (Kalaitzidis *et al.*, 2016). Moreover,

rodent studies have shown that administration of VDR agonists elevates serum and urine Klotho as well as increases renal synthesis of Klotho (Lau *et al.*, 2012). These studies provide support for the thesis that vitamin D3 plus its cognate receptor, VDR, together with Klotho and FGF23 exist as a tightly regulated, interactive endocrine network that serves to mitigate calcification of soft tissue, fibrosis within the vasculature, and other age-associated cardiovascular dysfunctions.

What is the mechanism that underlies the yin-yang relationship between 1α,25$(OH)_2$D3 and Klotho? As summarized in the previous section, the cardioprotective effects of vitamin D, manifested as collective activities that include detoxification of xenobiotics, attenuation of oxidative stress, and suppression of inflammation, are regulated in part through the transcription of gene sets under control by cytosol-nucleus shuttling of Nrf2. Conceivably, the same mechanistic underpinning might govern the reciprocal interplay between vitamin D3 and Klotho expression.

Klotho is required for FGF23 to activate FGFRs. Together, FGF23 and Klotho suppress the expression of CYP27B1 and induce CYP24A1, thereby concomitantly inhibiting the synthesis and promoting the catabolism of 1,25$(OH)_2$D3 (Hardcastle and Dittmer, 2015). It is important to consider here the circuitous control of this pathway in that 1,25$(OH)_2$D3 increases Klotho expression independently of FGF23 (Takenaka *et al.*, 2013). Deficiencies of FGF23 and/or Klotho manifest with similar characteristic phenotypes that include hyperphosphatemia, enhanced synthesis of 1,25$(OH)_2$D3, ectopic calcification, and pathophysiological conditions associated with aging, namely osteoporotic lesions and atherosclerosis (Komaba and Fukagawa, 2012; Kuro-o, 2012).

In the subsequent section, we provide an overview of the Nrf2 pathway and examine its role in cardiac hypertrophy and heart failure. This will be followed by a potential perspective regarding how the Nrf2 actively participates in the control of Klotho by vitamin D.

5.8 Evidence supporting a role of Nrf2 in preventing cardiac hypertrophy, antioxidant detoxification and control of heart failure

Vitamin D activates Nrf2 which is sequestered in the cytosol and negatively regulated by binding to Keap1, an actin-binding repressor protein (Hsieh *et al.*, 2006). The binding of Keap1 with Nrf2 mediates its ubiquitination and subsequent proteasomal degradation thus preventing its nuclear translocation (Kobayashi *et al.*, 2004). Keap1 is a redox sensor protein with multiple sulfhydryl moieties that respond to a diverse array of electrophiles (Bryan *et al.*, 2013). Oxidation of critical sulfhydryl moieties on Keap1 enables Nrf2 to evade capture and localize in the nucleus where it heterodimerizes with a small Maf protein, which has been characterized as a leucine zipper-type transcription factor that can bind to DNA (Li *et al.*, 2008). The Nrf2-sMAF heterodimer binds to a cis-acting regulatory gene termed the antioxidant/electrophile response element and induces several cytoprotective enzymes and factors which protect cells against oxidative and electrophilic challenges. Nuclear accumulation of Nrf2 evolves from two sources, translocation from the pre-existing cytosolic pool when released from Keap1 but mainly from *de novo* protein biosynthesis

(Shay *et al.*, 2012). It is interesting to speculate that the rapid accumulation of nuclear Nrf2 following stress stimuli is mediated by 1α,25$(OH)_2$D3_VDR interaction.

Since oxidative stress plays a critical role in the pathogenesis of atherosclerosis, induction by Nrf2 of a network of anti-oxidant and phase II detoxifying enzymes is critical to prevent endothelial dysfunction and activation of monocyte/macrophage lineage. This process leads to foam cell formation within the vascular endothelium. A number of reviews have identified that Nrf2 controls the basal and inducible expression of over 200 detoxification genes (Nerland, 2007) that encode for cytoprotective phase II detoxifying enzymes such as SOD 1 and 2, glutathione reductase, glutathione S-transferases, catalase, peroxiredoxins, NAD(P)H quinone oxidoreductase 1 and heme oxygenase-1 (Bobilev *et al.*, 2011; Chen and Kunsch, 2004; Cho *et al.*, 2006; Kensler *et al.*, 2007; Kobayashi *et al.*, 2004; Kobayashi and Yamamoto, 2006; Lee *et al.*, 2005; Wang *et al.*, 2015). In view of the aforementioned sulfhydryl oxidation states of redox sensitive proteins, Nrf2 also increases the expression of sulfiredoxins, thioredoxin reductase, and glutaredoxins to reduce the higher oxidation states of sulfhydryl moieties (Abbas *et al.*, 2013).

As mentioned earlier, GSH, ROS and NO are physiological modulators that interact with cysteine domains, in particular on redox sensitive proteins. Signal proteins especially within the intermembrane and matrix compartments of mitochondria exhibit an efficient SH/S-S redox system that controls the state of sulfhydryl oxidation of mitochondrial proteins (Hu *et al.*, 2008). Nrf2 controls synthesis of GSH which is the most important intracellular redox buffer (Mishra *et al.*, 2014). Moreover the intracellular redox balance of signal proteins is influenced by GSH/GSSG ratio (Thomas *et al.*, 2016). Among the various mechanisms of regulation via sulfhydryl groups is protein S-glutathionylation which represents a prototypical endogenous mechanism that modulates protein function (Cooper *et al.*, 2011). Nrf2 inducible glutaredoxins catalyze formation of glutathionylation of redox signal proteins, which is an important regulatory mechanism that is particularly important to control oxidative stress (Kalinina *et al.*, 2008; Park *et al.*, 2009).

Of particular note in mitochondria, studies show that oxidative stress causes reversible *S*-glutathionylation of mitochondrial Complex I. Using isolated Complex I, investigators showed that S-glutathionylation (P-SSG) of Complex I involves an initial exposure of the protein to superoxide forming a protein thiyl radical (P-S▪) which then cross reacts with endogenous GSH to form P-S-SG (Kang *et al.*, 2015). Exposure of murine heart to SOD markedly diminishes detection of P-S▪ supporting the notion that oxidative stress regulates *in vivo* mitochondrial function beginning at least at complex I in the electron transport chain. Both P-S▪ complex I and P-S-SG complex I are elevated in NO synthase (eNOS) (-/-) knock-out mice. Overexpression of SOD in these animals diminishes formation of P-S▪ complex I which suggests that complex I may be a redox switch for regulating mitochondrial function (Kang *et al.*, 2015). The relevance of these studies to CVD is that eNOS(-/-) mice develop hypertension and exhibit pathological phenotypes of progressive cardiac hypertrophy.

Thus, diminishing oxidative stress can have profound influences on attenuating development of cardiac hypertrophy and myocardial dysfunction (Espinosa-Diez *et al.*, 2015). By adopting sulfhydryl-containing antioxidants along with use of 1α,25$(OH)_2$D3 may have multiple roles in mitigating CVD by upregulating Nrf2 and Klotho synthesis, preventing Keap1 capture of cytosolic Nrf2, and diminishing oxidation of reactive sulfhydryl domains on redox sensitive signaling proteins, such as VDR (Date *et al.*, 2002; Lu *et al.*, 2016; Silva-Palacios *et al.*, 2016; Su *et al.*, 2016).

Taken together, these results suggest that Nrf2 plays an important role in maintenance of mitochondria functional integrity in the heart via multiple mechanisms that involve membrane stability and expression of an array of anti-oxidative enzymes that counteract oxidative stress-induced cardiac disorders. Importantly, vitamin D deficiency has been linked to increased CVD risk (Artaza *et al.*, 2009; Meems *et al.*, 2011) while its supplementation modulates cardiac alterations following different modes of injury caused by inflammatory cytokines and direct interaction with the VDR (Bodyak *et al.*, 2007; Kong *et al.*, 2010; Mancuso *et al.*, 2008). Furthermore, vitamin D activates the Nrf-2-Keap1 antioxidant pathway (Li and Kong, 2009; Nakai *et al.*, 2014) and, lastly with its direct association with the endocrine axis mediated by FGF23 and Klotho (Ellidag *et al.*, 2016), vitamin D may provide the key to understanding the physiology and pathophysiology of multiple metabolic processes that include CVD.

5.9 Summary

Vitamin D is an essential nutrient that plays several key roles in maintaining cardiovascular health. Vitamin D protects NO and its vasodilating action on endothelial cells to ensure healthy vascular tone. Through proper transportation and regulation of vitamin D and control of ROS via various anti-oxidative enzymes, cardiac health is maintained and hypertrophy prevented. Early intervention of vitamin D through diet or controlled exposure to sunlight may provide an ideal approach towards preventive therapy against vascular conditions. Numerous studies have proven that vitamin D and the VDR have protective effects beyond metabolism of calcium balance. Vitamin D sufficiency along with properly functioning receptors and transporters protect against CVD by inhibiting hypertension, endothelial dysfunction, atherosclerosis, vascular calcification, cardiac hypertrophy and progressive renal dysfunction. The primary mechanisms that underlie these pathophysiological factors are the release of reactive oxygen and nitrogen species-induced cytotoxicity and cardiac signaling which contribute to maladaptive cardiac remodeling and dysfunction. Thus, novel findings show that vitamin D and its receptor can prevent formation of oxidized lipids, mitigate endothelial and smooth muscle cell dysfunction, block formation of inflammatory cytokines, and quench ROS/RNS many of the factors involved in progression of CVD and related disorders. Therefore, sufficient levels of vitamin D and proper functioning VDR are crucial to maintenance of cardiovascular health. Further studies will provide better understanding of the beneficial effects of timing of vitamin D supplementation, its interaction with the Nrf2 detoxification/antioxidant network, and its co-association with FGF23 and Klotho to decrease hyperphosphatemia, control renal production of 1α,25$(OH)_2$D3, and block synthesis

of parathyroid hormone. Thus, vitamin D exhibits potential for preventive treatment and even as a potential reversing agent for progression of CVD.

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6. Vitamin D and cardiovascular disease and heart failure prevention

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Abstract

Vitamin D deficiency is highly prevalent worldwide. Animal and clinical studies suggest that low vitamin D has a role in the pathogenesis of cardiovascular disease (CVD). A growing body of research indicates that vitamin D deficiency may contribute to high blood pressure, poor insulin sensitivity, inflammation, and other processes that underlie heart disease. Large scale cross sectional and prospective population studies have shown favourable associations of circulating vitamin D with CVD risk factors in particular hypertension, impaired glucose tolerance or diabetes and inflammation. There is clear evidence from observational studies that vitamin D is associated with reduced risk of incident CVD. The association between vitamin D and risk of heart failure in contrast to coronary heart disease is less consistent. Despite the strong evidence from observational studies, randomised controlled trials (RCTs) so far have been inconclusive and individual intervention trials and meta-analysis of RCTs have failed to show a significant reduction in cardiovascular risk with vitamin D supplementation. The current trials and evidence to date are not sufficient to establish causative links between vitamin D deficiency and CVD and highlights the need of large RCTs with long term follow-up. Several large RCTs designed to evaluate the effect of vitamin D supplementation on CVD are in progress which will provide further evidence of whether vitamin D supplementation has any beneficial effect on CVD outcomes.

Keywords: vitamin D deficiency, randomised controlled trials, observational studies, cardiovascular risk

Key facts

- Vitamin D deficiency increases with distance from the equator and is a widespread problem particularly in the elderly
- Vitamin D deficiency is associated with hypertension, diabetes, inflammation and significantly increased risk of cardiovascular disease (CVD) events.
- Animal studies suggest a pathophysiologic role of vitamin D in heart failure but observational studies have reported conflicting results on the association between low vitamin D and incident heart failure (HF).
- Randomised controlled trials have failed to find a beneficial effect of vitamin D supplementation on cardiovascular risk.
- Vitamin D supplementation is currently not recommended for the prevention of cardiovascular disease.

Summary points

- Vitamin D deficiency is common in the elderly. The circulating 25-hydroxyvitamin D (25(OH)D) is a measure of a person's vitamin D status.
- There is no consensus on optimal vitamin D levels but deficiency may be defined as 25(OH)D level of <20 ng/ml.
- Considerable evidence from observational studies and meta-analyses has shown low vitamin D to be associated with many cardiovascular risk factors including hypertension, diabetes and inflammation.
- Observational studies and meta-analyses have consistently shown that low vitamin D is associated with increased risk of CVD events (CVD deaths, myocardial infarction or stroke).
- However, randomised controlled trials have failed to find a beneficial effect of vitamin D supplementation on reducing hypertension, incident type-2 diabetes and cardiovascular events.
- Animal studies suggest a pathophysiologic role of vitamin D in heart failure but observational studies have reported conflicting results on the association between low vitamin D and incident HF.
- There is also evidence that too much vitamin D may be harmful.
- Several large randomised controlled trials designed to evaluate the effect of vitamin D supplementation on CVD are in progress to determine the role of vitamin D in reducing CVD risk.

Abbreviations

25(OH)D	25-hydroxyvitamin D
CHD	Coronary heart disease
CRP	C-reactive protein
CVD	Cardiovascular disease
HF	Heart failure
IL-6	Interleukin-6
MI	Myocardial infarction
RCTs	Randomised controlled trials
RR	Relative risk
UVB	Ultraviolet B

6.1 Introduction

CVD is one of the major causes of morbidity and mortality throughout the world. In the last few decades there has been considerable interest among both the clinical professionals and the public regarding the cardiovascular health benefits of vitamin D. Traditionally vitamin D has been associated with bone and mineral metabolism and is known to influence skeletal health. In the 2000s a growing number of studies support the idea that low levels of vitamin D are linked to an increased risk of heart disease, and that adding vitamin D supplements can help reduce this risk. Accumulating evidence from both clinical and observational studies has shown vitamin D deficiency to be associated with increased risk of hypertension and CVD events. Numerous reviews and meta-analyses of prospective studies conducted in recent years have shown a robust association between vitamin D and CVD (Brøndum-Jacobsen *et al.*, 2012; Chowdhury *et al.*, 2014; Grandi *et al.*, 2010; Pilz *et al.*, 2016; Schöttker *et al.*, 2014; Wang *et al.*, 2012). However, randomised trials that have examined the effects of vitamin D supplementation on CVD have not been convincing (Ford *et al.*, 2014; Pilz *et al.*, 2016). Moreover, studies that have related vitamin D to CVD have largely focused on CHD. HF is a major epidemic and significant public health burden in the ageing society; the incidence of HF increases steeply with age. With the growing elderly population and the high prevalence of vitamin deficiency in the elderly, attention has also turned to vitamin D, specifically in the prevention of HF. In contrast to CHD observational studies on the relationship between vitamin D and HF have yielded inconsistent findings (Bansal *et al.*, 2014; Kestenbaum *et al.*, 2011; Lutsey *et al.*, 2015; Thomas *et al.*, 2012; Wannamethee *et al.*, 2014). This chapter discusses the epidemiological evidence on the effects of vitamin D on CVD and HF.

6.2 Sources of vitamin D

Over 90% of vitamin D in humans is produced in the skin through UVB irradiation of 7-dehydrocholesterol, which subsequently transforms this to vitamin D3 (Boonen *et al.*, 2006;

De Luca, 2004). Few foods naturally contain or are fortified with vitamin D. Vitamin D is found in a few foods – including fish, fish liver oils, and egg yolks as well as some dairy and grain products. Vitamin D is a collective term for D2 or D3 (total vitamin D referring to D2 plus D3). Both vitamin D3 and dietary sources are metabolized in the liver to 25(OH)D which is then converted to the active form of vitamin D, 1,25-dihydroxy vitamin D by 1-alpha-hydroxylase in the kidney. 25(OH)D is the storage form of vitamin D and is the metabolite used to assess an individual's vitamin D status; it reflects vitamin D concentrations over the preceding one month (De Luca *et al.*, 2004; Holick, 2007). Sunlight exposure is essential for maintaining adequate levels. Thus time spent outdoors, skin pigmentation, body mass and amount of skin exposed influence vitamin D levels.

6.3 Vitamin D deficiency

There is no consensus on optimal vitamin D levels but deficiency may be defined as 25(OH) D levels of <20 ng/ml (50 nmol/l) (Holick, 2007). Vitamin D deficiency is extremely common affecting more than half of adults (Lee *et al.*, 2008) and the majority of the elderly who often have less sun exposure due to limited outdoor activity and a limited capacity of the skin to produce vitamin D metabolites (Mosekilde, 2005). The prevalence of vitamin D deficiency also increases with greater distance from the equator because of increased atmospheric filtering of the UVB radiation. High proportions of the Northern Hemisphere populations, such as the UK, and increasingly of countries where covering up skin has reduced exposure, have sub-optimal levels of vitamin D. Correction of vitamin D deficiency is easily accomplished with oral supplementation in most cases.

6.4 Vitamin D and CVD

There is much evidence from experimental and clinical studies that high levels of vitamin D contribute to CVD (Norman and Powell, 2014). Expression of the vitamin D receptor (VDR) and vitamin D metabolizing enzymes in the heart and blood vessels suggests a role of vitamin D in the cardiovascular system (Pilz *et al.*, 2011). A growing body of research indicates that vitamin D deficiency may contribute to a number of conditions such as high blood pressure, poor insulin sensitivity, inflammation, and other fundamental processes that underlie heart disease. Vitamin D deficiency has been associated with cardiovascular events such as myocardial infarction and stroke as well as congestive HF (Pilz *et al.*, 2016). It could be suggested that vitamin D deficiency contributes to the development of CVD through its association with risk factors, such as diabetes and hypertension. Hypothesised mechanisms linking vitamin D deficiency to CVD may be direct or indirect. These include increased insulin resistance and pancreatic β cell dysfunction predisposing to diabetes; over activation of the renin angiotensin system increasing blood pressure leading to left ventricular hypertrophy; effects on vascular smooth cells, the endothelium and cardiomyocytes and stimulation of systemic and vascular inflammation (Ford *et al.*, 2014; Norman and Powell, 2014).

6.5 Clinical and epidemiological studies of vitamin D and CVD

6.5.1 Vitamin D and CVD risk factors

Large scale cross sectional and prospective population studies have shown favourable associations of circulating vitamin D with CVD risk factors in particular hypertension, impaired glucose tolerance or diabetes and inflammation with less convincing data for dyslipidemia (Anderson *et al.*, 2010; Pittas *et al.*, 2011).

Hypertension

Large observational studies and meta-analyses have shown that low vitamin D is associated with arterial hypertension (Burgaz *et al.*, 2011; Kunutsor *et al.*, 2013). A large meta-analysis of 11 prospective studies which comprised over 283,000 participants found those in the highest tertile of 25(OH)D to have a significant reduction in risk of hypertension compared to those in the lowest tertile of baseline 25(OH)D [RR=0.73 (0.57-0.86)] (Kunutsor *et al.*, 2013). Possible mechanisms for the inverse association between vitamin D levels and blood pressure include its regulatory effects on the renin-angiotensine-aldosterone system. Although large meta-analyses of observational studies provide strong evidence for a relationship of vitamin D and blood pressure, data from RCTs have yielded inconsistent results. While some reviews and meta-analyses conclude a significant effect of vitamin D supplements on blood pressure others found either no change or only reduction in systolic blood pressure in specific subgroups only such as those with vitamin D deficiency at baseline (Al Mheid, 2013; Kunutsor *et al.*, 2014). It is suggested that many of the trials were not adequately designed to answer the question whether correction of vitamin D deficiency is effective for the treatment of hypertension as many did not include participants with both hypertension and vitamin D deficiency. However, The Styrian Vitamin D Hypertension Trial, a double blind placebo controlled study showed no significant effect on blood pressure of vitamin D supplementation in hypertensive patients with low 25(OH)D concentrations (Pilz *et al.*, 2015).

Glycaemia and type 2 diabetes

Vitamin D also affects mechanisms related to type 2 diabetes pathophysiology including impaired β-cell function and insulin resistance (George *et al.*, 2012; Pittas *et al.*, 2007). In observational and prospective studies low vitamin D has been associated with glucose metabolism as well as higher risk of developing diabetes (Forouhi *et al.*, 2012; George *et al.*, 2012; Pittas *et al.*, 2010; Song *et al.*, 2013). In a meta-analysis of 21 cohort studies with a total of 76,000 participants high 25(OH)D levels (top tertile) was associated with a 38% reduction in risk of developing diabetes compared to those with low 25(OH)D (bottom tertile) (Song *et al.*, 2013). RCTs on the other hand have largely failed to show clear beneficial effects of vitamin D supplementation on improving glycaemia and insulin sensitivity or reducing incident type-2 diabetes (George *et al.*, 2012; Pittas *et al.*, 2007; Seida, 2014). In a systematic review and meta-analysis of 31 RCTs which included 35 trials (43,407 participants) the authors concluded that vitamin D supplementation had no significant

effect on glucose homeostasis or diabetes prevention (Seida *et al.*, 2014). However, there is lack of data on large long term trials on vitamin D supplementation and diabetes risk. The Endocrine Society statement emphasised the lack of solid evidence supporting benefits of vitamin D therapy in diabetes mellitus (Rosen, 2012). It has been suggested that the inconsistencies in both the hypertension and type 2 diabetes trials may be due to the various vitamin D disease, achieved 25(OH)D levels, small sample sizes and study designs which included primary secondary and tertiary trials (Lavie *et al.*, 2011)

Inflammation

It is well established that inflammation is involved in the process of plaque rupture. Experimental studies have shown vitamin D to increase the expression of anti-inflammatory cytokines and to decrease expression of pro-inflammatory molecules including TNF-alpha and IL-6 (Wang, 2016). Blood markers of inflammation such as CRP and IL-6 are known to predict CVD (Wannamethee *et al.*, 2009, Emerging Risk factor Collaboration, 2010). Vitamin D deficiency has been associated with elevated CRP. However clinical trials of vitamin D supplementation on CRP have shown mixed results with most trials reporting no effects on CRP concentration whereas some have shown reduction in CRP concentration (Fry and Sanders, 2015).

6.5.2 Vitamin D and CVD events

Since Robert Scragg first hypothesised that increasing ultra-violet-related vitamin D status may confer protection against CVD over 30 years ago (Scragg, 1981) there have been numerous epidemiological observational studies and RCTs which have examined the effects of vitamin D on CVD risk particularly in the last 10 years. Several comprehensive reviews regarding the cardiovascular health benefits of vitamin D have been conducted in the last decade (Al Mheid *et al.*, 2013; Carvalho and Sposito 2015; Elamin *et al.*, 2011; Ford *et al.*, 2014; Fry and Sanders 2015; Grandi *et al.*, 2010; Kienreich *et al.*, 2013; Norman and Powell, 2014; Pilz *et al.*, 2013, 2016; Pittas *et al.*, 2010) and there is clear evidence from observational studies that vitamin D is associated with reduced risk of CHD generating vast interest among both medical professionals and the public for vitamin D supplementation for the prevention of CVD. However results from vitamin D supplementation trials have been inconclusive. The evidence from observational and RCT studies are discussed below.

Observational studies

Several meta-analyses of prospective studies have consistently shown that low 25(OH)D concentrations is associated with increased risk of overall CVD incidence and CVD mortality (Brøndum-Jacobsen *et al.*, 2012; Chowdhury *et al.*, 2014; Grandi *et al.*, 2010; Pilz *et al.*, 2016; Schottker *et al.*, 2014; Wang *et al.*, 2012). In a meta-analysis of 26,018 participants those in the bottom quintile showed over a 50% increase in risk of CVD mortality compared to those in the top quintile (Schottker *et al.*, 2014). Another large meta-analysis of 19 studies in 65,994 participants with over 6,000 CVD cases demonstrated a generally inverse linear relationship

between circulating 25(OH)-vitamin D ranging from 20-60 nmol/l (8-24 ng/ml) and risk of CVD (Wang *et al.*, 2012) with no further decrease above 60 nmol/ (24 ng/ml). The pooled RR for total CVD was 1.52 (1.30-1.77) in a comparison of the lowest with the highest category, 1.42 (95% CI: 1.19-1.71) for CVD mortality, 1.38 (95% CI: 1.21-1.57) for CHD and 1.64 (95% CI: 1.27-2.10) for stroke. A few studies have reported a U shaped association with both low and high levels of vitamin D showing increased risk cardiovascular risk (Durup *et al.*, 2015; Pilz *et al.*, 2016). In a large cohort study of 247,574 subjects from the Copenhagen general practice sector a J shaped relationship was seen between 25(OH)D levels and CVD mortality. Low levels of 25(OH)D was associated with the highest risk of CVD mortality but high levels of 25(OH)D (~125 nmol/l; 50 ng/ml) was also associated with a 30% increase in risk of CVD mortality compared to those with levels of 70 nmol/l (28 ng/ml) (Durup *et al.*, 2015). The risks of CVD in those with excessive high levels of vitamin D have not been well studied and the nature of this association is unclear. There is a need for RCT to also include information on the effects of 25(OH)D levels above 125 nmol/l (50 ng/ml).

Randomised control trials

Despite the strong evidence from observational studies, RCTs so far have been inconclusive and individual intervention trials and meta-analysis of RCTs has failed to show a significant reduction in risk CVD with vitamin D supplementation. In the largest trial to date the Women's Health Initiative, with 36,282 postmenopausal women, supplementation with vitamin D and calcium revealed no significant effect on the incidence of CVD events after 7 years follow-up (Hsiah *et al.*, 2007). Several critical reviews and meta-analyses have addressed the role of vitamin D supplementation in reducing the risk of CVD (Bolland *et al.*, 2014; Elamin *et al.*, 2011; Ford *et al.*, 2014; Fortmann *et al.*, 2013). In an analysis of 8 randomised trials there was a slight but statistically non-significant 10% reduction in CV risk with vitamin D supplementation at moderate to high doses (~1000 IU daily) (Wang *et al.*, 2010). A meta-analysis of 51 eligible randomised trials that included over 31,000 patients failed to demonstrate benefit with vitamin D supplements on CVD risk or risk factors including stroke, MI, lipid fractions, blood pressure, and blood glucose value even in planned subgroup analyses of trials involving vitamin D-deficient patients (Elamin *et al.*, 2011). In a comprehensive trial sequential meta-analysis of over 80,000 participants it was concluded that vitamin D supplementation does not reduce CVD outcomes (Bolland *et al.*, 2014). A recent review with a meta-analysis of 21 RCTs involving 13,033 people concluded that vitamin D supplementation did not reduce risk of MI or stroke (Ford *et al.*, 2014). The authors concluded that the findings suggest that the link between vitamin D deficiency and increased risk of CVD is not causal. However several concerns have been raised regarding these trials to evaluate the effects of vitamin D on cardiovascular risk. Most large previous RCTs were designed to study vitamin D effects on bone health and were not primarily designed to study vitamin D effects on CVD (Wang, 2016). Some reviews included trials that compared calcium and vitamin D compared with a placebo or control as well as trials of vitamin D alone, which is problematic because calcium supplements have been shown to increase risk of cardiovascular events (Bolland *et al.*, 2010) and may attenuate a potential effect of vitamin D alone. In addition most trials included study participants regardless of their vitamin D status.

Future randomised controlled trials

Several large RCTs designed to evaluate the effect of vitamin D supplementation on CVD endpoints in chronically ill as well as in the general population are now in progress. The VITamin D and OmegA-3 triaL VITAL trial is one of the ongoing double blind placebo controlled RCT to evaluate the impact of 2,000 IU vitamin D on CVD and cancer mortality in over 20,000 older subjects in the USA without cancer or CVD at baseline (Manson *et al.*, 2012). Other ongoing studies include the Vitamin D assessment Study (ViDA) in New Zealand among over 5,000 older individuals (Scragg *et al.*, 2015) as well as the D-Health trial in Australia of over 25,000 older adults (Neale *et al.*, 2016). Results from these trials are expected between 2017 and 2020.

6.5.3 Vitamin D and incident heart failure

HF is a major epidemic and significant public problem in the ageing society and much attention has turned to the role of mineral and bone metabolism in recent years specifically vitamin deficiency. Vitamin D deficiency is commonly seen in patients with HF and animal studies suggest a pathophysiologic role of vitamin D in HF (Agarwal *et al.*, 2011; Meredith and McManus, 2013). Prospective studies that have related vitamin D to CVD endpoints have largely focused on CHD. Fewer prospective studies have examined the association between vitamin D and incident HF but in contrast to CVD events (MI or stroke), the association between vitamin D and HF have been inconsistent with some prospective studies showing positive associations while others find no association. Table 6.1 summarises the prospective studies that have examined the association between vitamin D and HF. In the Intermountain Heart Collaborative a USA study of over 40,000 adults (average age 50 years) low 25(OH)D levels was associated with increased risk of HF events (Anderson *et al.*, 2010). In the Ludwigshafen Risk and Cardiovascular Health Study (LURIC) (aged 56-70 years) and the Third National Health and Nutrition Examination Survey (>35 years) 25OHD deficiency was associated with increased HF deaths (Liu *et al.*, 2012; Thomas *et al.*, 2012). Neither of these two studies included non-fatal HF events and did not take incident MI into account. In the Atherosclerosis Risk in Communities Study (ARIC) low levels of 25(OH) D was associated with incident HF in whites only and only in those genetically predisposed to high diastolic blood pressure (Lutsey *et al.*, 2015). In contrast, reports from the British Regional Heart Study (mean age 69 years) (Wannamethee *et al.*, 2014), the Physicians' Health Study (mean age 58.6 years) (Robbins *et al.*, 2016), the EPIC Potsdam study (Di Giuseppe *et al.*, 2014), the Cardiovascular Health study (>65 years) (Kestenbaum *et al.*, 2011) and the Multi-Ethnic Study of Atherosclerosis (mean age 62 years) (Bansal *et al.*, 2014) showed no association between vitamin D with risk of HF. The differences in results could also be explained by the age differences between study populations; studies showing positive associations were generally conducted in younger people. In older adults, HF with preserved ejection fraction is a common type of HF and is often not associated with MI (Kaila *et al.*, 2012). The finding that 25(OH)D deficiency is not associated with HF in older adults is consistent with the findings from several cross sectional population studies that 25OHD is not associated with any biochemical conduction or echocardiographic outcomes (Anderson *et al.*, 2013; Van Ballegooijen *et al.*, 2013a,b). Moreover the association

Table 6.1. Prospective studies of vitamin D on heart failure.[1]

Studies	Location	Study sample	Vitamin D measurement	Age-range (years)	HF outcome	Main findings
Studies finding no association						
BRHS (Wannamethee *et al.*, 2014)						
	UK	3,731 men	25(OH)D	60-79	incident HF (F or NF)	no association
Cardiovascular Health Study (Kestenbaum *et al.*, 2011)						
	USA	2,312 men and women	25(OH)D	≥65	incident HF (F or NF)	no association
EPIC (di Giuseppe *et al.*, 2014)						
	Europe	1,228 men and women	25(OH)D3	35-65	incident HF (F or NF)	no association
MENSA (Bansal *et al.*, 2014)						
	USA	6,459 men and women	25(OH)D	45-84	incident HF (F or NF)	no association
Physician's Health Study (Robbins *et al.*, 2016)						
	USA	19,635 men	Dietary vitamin D	50-97	incident HF (F or NF)	no association
Studies finding an association						
ARIC (Lutsey *et al.*, 2015)						
	USA	12,215 men and women	25(OH)D	45-64	incident HF (F or NF)	low vitamin D associated with increased risk of HF in whites or in those predisposed to high diastolic blood pressure
Intermountain Heart Collaborative (IHC) Study Group (Anderson *et al.*, 2010)						
	USA	41,504 men and women	25(OH)D	mean age 55 years	incident HF (F or NF)	low (16-30 ng/ml) and very low (≤15 ng/ml) vitamin D levels associated with significant increased HF risk compared to normal vitamin D status; HR=1.31 and 2.01. respectively
LURIC (Thomas *et al.*, 2012)						
	Germany	1,801 men and women	25(OH)D	56-70	HF deaths	high vitamin D associated with reduced risk of HF; optimal level (>75 nmol/l) compared with severe vitamin D deficiency (<25 nmol/l), HR=0.24 (0.06-1.04)
NHANES III (Liu *et al.*, 2012)						
	USA	13,131 men and women	25(OH)D	≥35	HF deaths	low vitamin D showed increased risk of HF; <20 ng/ml vs >30 ng/ml), HR=2.06 (1.01-4.25)

[1] F = fatal; HF = heart failure; HR = hazard ratio; NF = non-fatal.

between vitamin D deficiency and left ventricular systolic and diastolic function has not been consistently observed (Agarwal *et al.*, 2011).

However, contradictory to these findings, a recent study which analysed the data of a clinical trial involving 5,292 individuals aged 60 and over given a daily vitamin D supplement or placebo every day for 5 years found that vitamin D supplements will not prevent heart attack or stroke, although it could protect against HF in older individuals (Ford *et al.*, 2014). They found a lower risk of dying from cardiac failure among seniors who took a daily supplement although nonfatal HF events were not included in the trial. In a secondary analysis of the randomized trial of vitamin D plus calcium of the Women's Health Initiative calcium plus vitamin D supplements did not reduce HF incidence (Donneyong *et al.*, 2015). There are no reported studies directly investigating increasing vitamin D levels to reduce the risk of congestive heart failure incidence. More trials on vitamin D in HF needs to be conducted before any conclusions can be drawn.

6.6 Guidelines for vitamin D

Vitamin D deficiency is particularly widespread in elderly adults and those who spend little time in the sun. Many health experts now advise adults to supplement with 1,000 IU or more each day (Holick *et al.*, 2011). There is, however, significant controversy in the literature regarding the acceptable lower limit of 25(OH)D concentration in adults. Although The Endocrine Society recommendations advise adults to achieve a minimum blood level of 30 ng/mL of 25(OH)D (Holick *et al.*, 2011) an analysis by the Institute of Medicine suggests that 25(OH)D levels of 20 ng/ml or greater are adequate (Ross *et al.*, 2011). Similarly, guidelines related to the acceptable upper limit of 25(OH)D levels in adults are unclear. Serum 25(OH)D levels greater than 150 ng/mL are associated with toxicity (Holick *et al.*, 2011), but levels up to 60 ng/ml had been reported as optimal (Lugg *et al.*, 2015). On the other hand, the 2011 Institute of Medicine guidelines suggest 50 ng/ml as the upper threshold for desirable 25(OH)D levels (Ross *et al.*, 2011).

6.7 Conclusions

While observational studies have shown strong associations between vitamin D and CVD risk factors and incident CVD events the majority of RCTs to date have been null. The 2011 Institute of Medicine report on dietary intakes for calcium and vitamin D conclude that the evidence for a role of vitamin D in preventing CVD is inconclusive and not yet sufficient to inform nutritional recommendations. This is subsequently supported by more recent meta-analyses of RCTs showing inconclusive results. Results from ongoing clinical trials designed to assess the effects of vitamin D supplementation on CVD will provide further evidence on whether vitamin D supplementation has any beneficial effect on CVD outcomes.

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Nutrition and nutrition counseling in heart function and growth

7. The role of diet in systemic and neural inflammation in obesity and metabolic syndrome

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Abstract

Obesity is defined as the excessive accumulation of body fat that represents health consequences. The excessive adipose tissue is associated with chronic inflammation, which is related to metabolic complications. The prevalence of obese patients has increased in recent years, and this is observed even in children and adolescents. The combination of cardiometabolic alterations including elevated triglycerides, reduced high-density lipoprotein, high blood pressure, impaired fasting glucose, and increased abdominal circumference characterize metabolic syndrome (MetS). In addition, the prevalence of MetS has been associated with increased occurrences of coronary artery disease, stroke, and cardiovascular risks in adults. Recently, there is rising evidence regarding nutrients and bioactive compounds and nutrigenetic/nutrigenomic mechanisms that can trigger obesity and MetS control, in order to reduce cardiovascular risk factors. Thus, the present chapter aims to review the role of diet in systemic and neural inflammation in obesity and MetS. In summary, the importance of nutrition in disease prevention and treatment has gained much attention recently. The involvement of nuclear factor κB (NF-κB) as the link between nutrients and inflammation suggests the close integration of nutrient and metabolic disease. Taken together, omega-3 polyunsaturated fatty acids and bioactive compounds have an important role in the inhibition of NF-κB signaling pathway, which may be a clinical strategy for the treatment of inflammation and metabolic disorders in obese individuals. On the other hand, excessive caloric intake, trans fatty, and saturated fatty acids can be involved in activation of NF-κB, promoting inflammation. This chapter will highlight mechanisms and clinical studies examining the effects of bioactive compounds and polyunsaturated fatty acids in order to contribute to obesity and MetS approach.

Keywords: bioactive compounds, omega-3 polyunsaturated fatty acid, saturated fatty acid, inflammation

Key facts

- Obesity is defined as a chronic disease related to several comorbidities, including cardiovascular disease. At least 2.8 million people die each year as a result of being overweight or obese.
- Excessive adipose tissue is related to a low-grade chronic inflammation. In obesity, many cytokines are secreted by inflammatory leukocytes and adipocytes, and act as intercellular mediators.
- Inflammation is related to several metabolic disturbances, including insulin resistance, dyslipidemia, hypertension, and consequently, metabolic syndrome (MetS).
- Nutrigenomic includes investigation of interactions between nutrients and gene expression. Therefore, nutrients and food components can promote changes in inflammatory profile by affecting transcriptional and posttranscriptional mechanisms.
- Some food components able to modulate inflammatory process include n-3 polyunsaturated fatty acid, saturated fatty acid (SFA) and bioactive compounds (curcumin, quercetin, gingerol, resveratrol, anthocyanin, capsaicin, genistein).

Summary points

- Adipose tissue is much more than a single tissue responsible for fat energy reserves, thermoregulation and for protecting the organs. Nowadays, adipose tissue is considered an endocrine organ.
- Several adipokines are secreted in high degree from adipose tissue in obesity condition (leptin, tumor-a necrosis factor, interleukin 6 and 1). This low-grade inflammatory state is related to MetS development, systemic and hypothalamic inflammation, and insulin resistance.
- Studies have shown that the saturated fats present in large amounts in western diets can activate an inflammatory response in the hypothalamus, affecting the capacity of such neurons to respond appropriately to satiety and adipostatic signals.
- Omega-3 polyunsaturated fatty acids (n-3 PUFA) improves inflammation in obesity by modulating many metabolic pathways in its adipose tissue. Therefore, diets including food rich in n-3 PUFA and fish oil supplement can contribute to down-regulation of inflammation.
- Excessive consumption of SFA contributes to weight gain and inflammation thought several mechanisms, including oxidative or endoplasmatic reticulum stress, generation of ceramide and reactive oxygen species, IKK and protein kinase C signaling.
- Bioactive compounds, including curcumin, resveratrol, flavonoids, catechins, quercetin and isoflavones, are known to suppress nuclear factor κB, and consequently systemic and adipose tissue inflammation.

Abbreviations

AgRP	Agouti-related protein
AMPK	Activated protein kinase
BAT	Brown adipose tissue
CART	Cocaine and amphetamine-regulated transcript peptide
CNS	Central nervous system
CRP	C reactive protein
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
ERS	Endoplasmic reticulum stress
FFA	Free fatty acid
GPR-120	G-protein coupled receptor-120
hs-CRP	High sensitivity C-reactive protein
IKK	I kappa B kinase
IL	Interleukin
IRS	Insulin receptor substrates
JNK	C-Jun N-terminal kinase
LPS	Lipopolysaccharide
MC3R	Melanocortin 3 receptor
MC4R	Melanocortin 4 receptor
MetS	Metabolic syndrome
NF-κB	Nuclear factor kappa B
NPY	Neuropeptide Y
n-3 PUFA	Omega-3 polyunsaturated fatty acid
PKC-θ	Protein kinase C-θ
POMC	Pro-opiomelanocorticotin
PTP1B	Protein-tyrosine phosphatase 1B
ROS	Reactive oxygen species
SFA	Saturated fatty acid
SNS	Sympathetic nerve system
SOCS3	Suppressor of cytokine signaling 3
TFA	Trans fatty acid
TNF-α	Tumor necrosis factor alpha
TLR	Toll-like receptor
α-MSH	α-melanocytes-stimulating hormone

7.1 Introduction

Obesity is considered a chronic disease defined as an excessive accumulation of fat in adipose tissue as a consequence of a positive energy balance. The World Health Organization indicates that obesity has more than doubled since 1980. The prevalence of obesity is increasing not only in adults, but among children and adolescents. In 2014, more than 1.9 billion adults were overweight. Of these over 600 million were obese (WHO, 2016).

Obesity is a significant risk factor contributing to increased morbidity and mortality, most importantly from cardiovascular disease. In the USA, human obesity is responsible for up to 20% of all deaths (Antonopoulos *et al.*, 2016; Pi-Sunyer, 2009).

Adipose tissue is currently considered an endocrine organ, capable of producing and secreting pro-inflammatory cytokines and adipokines responsible for a low-grade systemic inflammatory state, a key feature of the metabolic disturbances linked to obesity. In this way, obesity, mostly considering central and visceral fat and insulin resistance, has been strongly associated with the development of MetS, type 2 diabetes, dyslipidemia, non-alcoholic fatty liver disease, hypertension, cancer and chronic disability (Pi-Sunyer, 2009).

The cause of obesity is very complex and is the result of multifactorial elements, being affected by some genetic, behavioral and environmental factors, such as dense foods and limited physical activity. Nutritional therapy research is not focused solely on reduced energy intake and managed macronutrient intake, but is investigating the role of micronutrients and bioactive components of food. Recently, the role of nutrition in chronic disease treatment and prevention has gained much attention. Many researchers have sought to identify nutrients and bioactive components in food capable of minimizing obesity and inflammation (Rosa, 2012). Thereby, this chapter will highlight studies examining the effects of bioactive compounds and nutrients on obesity and inflammatory markers.

7.2 Adipose tissue, obesity and inflammation

Over the last decade, increasingly evidence has been found that adipose tissue is much more than a single tissue responsible primarily for three basic functions: (1) storage and supply of energy reserves (storage and release of fatty acids); (2) thermal insulation (thermoregulation); and (3) responsible for protecting the organs of the chest cavity from mechanical shocks. The metabolic functions extend far beyond these classic actions.

Actually, adipose tissue is considered an important secretory organ responsible for maintaining energy homeostasis through the production and release of numerous proteins, conceptualized as adipokines. The adipokines interact in central and peripheral ways and in different organs including the brain, liver, pancreas, heart and other tissues such as muscle and skeletal tissue. Adipocytes are cells specialized in storing stemmed lipid through the degradation of fatty acids

into triglycerides and stored in lipid droplets format. When necessary to obtain energy by body tissues, triacylglycerols stored in adipose tissue are hydrolyzed and both glycerol and fatty acids are released into the bloodstream (Berg and Scherer, 2005; Ouchi *et al.*, 2003; Oyama *et al.*, 2009; Harwood, 2012).

Among the adipose tissue disposal sites, there are two more abundant, which are the subcutaneous and visceral regions, responsible for increased production and release of adipokines. In obesity, there may be ectopic fat accumulation in different body regions in association with multiple organs, including the heart and kidneys. The adipocytes are also found in bone marrow, lung and tunica adventitia of the main blood vessel as shown in the following Figure 7.1 (Ouchi *et al.*, 2011; Samaras *et al.*, 2010).

White adipose tissue is composed in its entirety by adipocytes; however, there is the presence of other cells in that tissue performing essential functions for growth and maintenance. Among them, we highlight the preadipocytes, fibroblasts, lymphocytes, vascular cells and macrophages. The latter, macrophages, play essential role in sustaining inflammatory status and the development of insulin resistance which is present in individuals with obesity. It was found in the literature that its amount is relatively more abundant in visceral adipose tissue and proportional to the increased release of pro-inflammatory markers. This is one of the main reasons why the accumulation of body fat in the abdominal region has a higher metabolic risk for individuals (Ouchi *et al.*, 2011; Xu *et al.*, 2003).

Ouchi *et al.* (2011) suggested that adipose tissue could be described as structures and functions in three different conditions: (1) eutrophic individual with metabolic functions considered within the normal range; (2) individual with obesity and the presence of mild metabolic disorders; and finally (3) individual with obesity and serious metabolic disorders. In these three possibilities, the

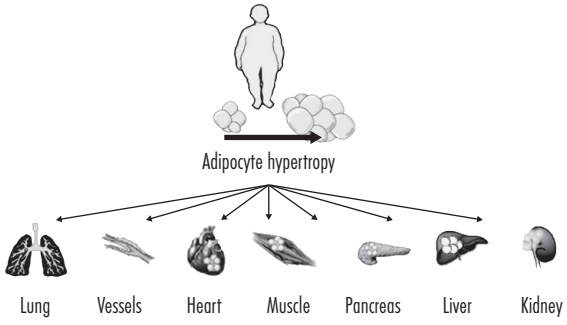

Figure 7.1. Local deposits of body fat (adapted from Evans *et al.*, 2004). Fat deposits are mainly found in the visceral and subcutaneous regions. In obesity condition may occur accumulation of this tissue in other body areas that commonly include heart region, kidney and tunica adventitia of blood vessels. The ectopic fat accumulation in different body areas causes changes in the functions of organs and consequently in metabolic activity.

adipose tissue shows striking phenotypic characteristics that are easily identified and associated with the degree of metabolic dysfunction.

In condition 1, it is possible to verify the presence of adipocytes with appropriate volumes, type M2 macrophages, which produce anti-inflammatory markers such as IL-4, IL-10, IL-13 and CD4+T cell type, which inhibit the presence and activity of the type M1 macrophages, and consequently pro-inflammatory markers and the subsequent insulin resistance frame (Feuerer *et al.*, 2009).

However, for condition 2, there is the hypertrophy accompanied adipocyte the presence of infiltrates of type M1 macrophages by increased release of pro-inflammatory markers, particularly TNF-α, IL-6 and ROS, and the presence of CD8+T cell type responsible for the recruitment and activation of macrophages M1 type. In this condition, vascular function is retained, but with reduced metabolic control and increased inflammation.

Lastly, condition 3 is strongly influenced by the presence of the structure referred to as 'crown-like' (similar to the crown) in which lies the presence of adipocytes in the necrotic layer (death adipocytes) surrounded by macrophages of the type M1. Furthermore, the increase in the inflammatory state degree as well as in the metabolic and vascular dysfunctions are characteristic of this pattern of change of adipose tissue (Ouchi *et al.*, 2011).

In literature three pathways are suggested as the mechanisms involved in the development of inflammation in obesity (Geloneze *et al.*, 2010):

1. Increased oxidative stress resulting from stemmed nutrients from caloric diet that stimulates the cell to begin the process of apoptosis and has increased release of pro-inflammatory markers from stimulating molecules such as JNK and IKK;
2. Reduced blood flow leading to hypoxia in adipose tissue, and thus favoring the release of pro-inflammatory adipokines;
3. Direct toxicity exerted by the lipidic overload, since lipids are able to activate TLR-2 and TLR-4 and the production of pro-inflammatory adipokines from macrophage into the tissue adipose.

7.3 Obesity and metabolic syndrome

Once obesity is diagnosed, the presence of metabolic alterations that predispose the development of many comorbidities, such as MetS and cardiovascular disease, is very common. The criteria of MetS diagnosis is based in a cluster of risk factors, including visceral adiposity, insulin resistance, hypertension, high triglyceride and low high-density lipoprotein-cholesterol, all of which increase the risk for the development of type 2 diabetes and cardiovascular disease (Alberti *et al.*, 2009). MetS confers a 5-fold increase in the risk of type 2 diabetes mellitus and 2-fold the risk of developing cardiovascular disease over the next 5 to 10 years (Kaur, 2014).

Studies from our research team showed that those young and obese present approximately 27.5% of MetS. However, long-term multicomponent therapy, including nutritional approaches, may reduce it to 13%, corroborating early prevention of cardiovascular disease (Masquio *et al.*, 2015a).

The mechanisms of how adipose tissue dysfunction may contribute to inflammation and metabolic alterations include adipocyte hypertrophy, which leads to insulin resistant adipocytes with a high lipolytic capacity and secretion of proinflammatory adipokines (Bashan *et al.*, 2007; Rudich *et al.*, 2007). As previously described, not only an energy font is the function of white adipocyte, more than 50 different molecules, known as adipokines, are secreted by this tissue and is associated with the development of MetS (Spite *et al.*, 2014).

The inflammatory state related to obesity is characterized by secretion of pro-inflammatory adipokines including IL-6, TNF-α, plasminogen activator inhibitor type 1, leptin, CRP, resistin, and reduced secretion of anti-inflammatory adiponectin (Piya *et al.*, 2013). Figure 7.2 illustrates the main mechanisms related to obesity and MetS development.

The MetS etiology origin from increased abdominal adipose tissue, promotes an elevation in influx of FFA to portal circulation. This increase in FFA on liver promotes consequences, stimulating lipoprotein synthesis and liver steatosis. In muscle, increased influx of FFA promotes insulin resistance, leading to hyperinsulinemia and β cells dysfunctions in pancreas. Beyond that, increased secretion of pro-inflammatory adipokines presents many roles on physiopathology

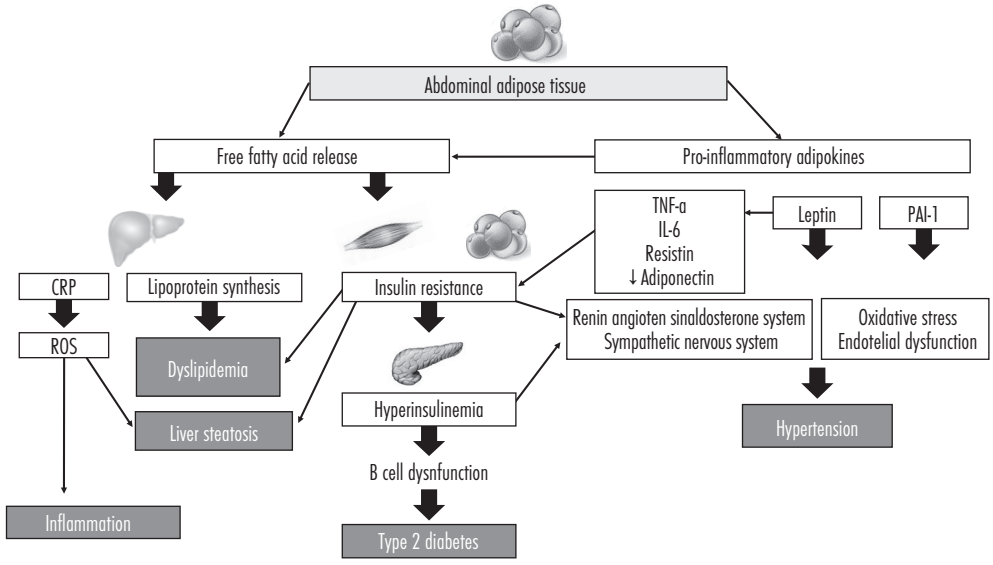

Figure 7.2. Physiopathology of metabolic syndrome in obesity (CRP = c reactive protein; IL-6 = interleukin 6; PAI-1 = plasminogen activator inhibitor type 1; ROS = reactive oxygen species; TNF-a = tumor-a necrosis factor).

of MetS, including insulin resistance, oxidative stress, endothelial dysfunction and increase in CRP production by liver. Altogether, inflammation and FFA release lead to inflammation, dyslipidemia, type 2 diabetes and hypertension.

The prolonged inflammatory state appears to be the most important mechanism that links the pathophysiology of insulin resistance and MetS. In particular, adipose tissue dysfunction, insulin resistance, sympathetic hyperactivity, and endothelial dysfunction have emerged as pivotal players (Kahn and Flier, 2000).

Moreover, the pathophysiology of MetS is associated with a diet containing excess calories and/or high saturated fat or glucose content and physical inactivity. In consequence of a continuous positive energy balance, both fat accumulation and body weight increases contribute to development of MetS. Moreover, it is necessary to elucidate the possible influence of genetic and environmental factors and their interactions that can contribute to the positive energy balance by adipocyte hypertrophy, and frequently is associated with pathogenic factors causing impaired adipose tissue function (Figure 7.3) (Klöting and Blüher, 2014).

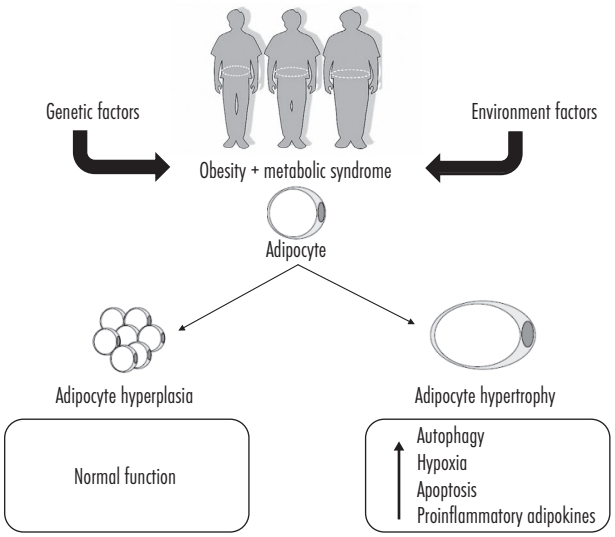

Figure 7.3. Effects of interaction between among genetic and environment factors in development of obesity + metabolic syndrome (adapted from Klöting and Blüher, 2014). Alterations in adipocyte hyperplasia and hypertrophy.

7.4 Neuroendocrine control of energy balance and obesity

Despite the fact that adipocytes have a close relationship with inflammation, they also play an important role in energy balance. Far beyond storing triglycerides, adipose tissue is responsible for neural and hormonal signals, and the secretion of adipokines that are involved in thermogenesis, feeding regulation and neuroendocrine activities (Ahima, 2006).

This is characterized as an energy balance and aims to stabilize the body fat stores through hunger control and satiety neuroendocrine mechanisms. The hypothalamus is the main anatomical structure of CNS involved in this process to control orexigenic and anorexigenic pathways stimulating food intake and appetite inhibition. In this system, the neuropeptides responsible for stimulating orexigenic pathways are NPY, AgRP and melanin-concentrating hormone. On the other hand, anorexigenic pathways are modulated by expression by CART and POMC, the main precursor of α-MSH, which is an important regulator of energy balance (Coll *et al.*, 2008; Hillebrand *et al.*, 2002; Vendrell *et al.*, 2004).

Several circulating factors, mostly produced by adipose tissue, gastrointestinal tract and pancreas, in addition to own nutrients may influence energy balance by acting on hypothalamus and other CNS areas, such as solitary tract nucleus (Figure 7.4).

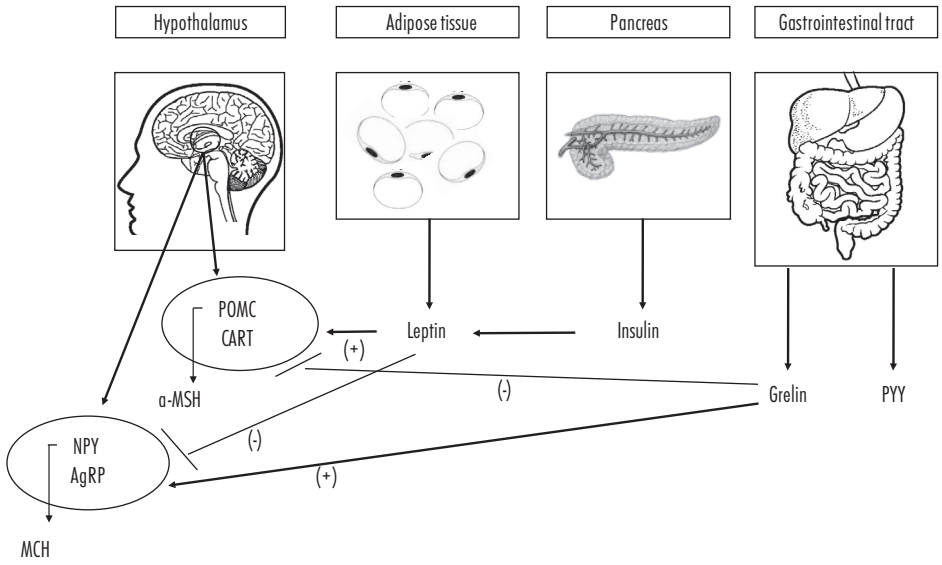

Figure 7.4. Neuroendocrine control of energy balance and obesity (AgRP = agouti-related protein; CART = cocaine and amphetamine-regulated transcript peptide; MCH = melanin-concentrating hormone; NPY = neuropeptide Y; POMC = pro-opiomelanocorticotin; PYY = peptide YY; α-MSH = α-melanocytes-stimulating hormone).

Adipose tissue produces and secretes leptin, one peptide hormone mainly expressed in white adipose cells in response to food intake. It crosses blood-brain barrier and binds to its receptors (ObR or LEPR), exerting two different hypothalamic signals. First stimulating CART and POMC neurons, which is cleaved giving rise to α-MSH binding to MC3R and MC4R. This subgroup of neurons present anorexigenic properties (Bischoff *et al.*, 2012; Coll *et al.*, 2008; Seufert *et al.*, 1999; Vendrell *et al.*, 2004). Simultaneously, leptin inhibits expression of both orexigenic neuropeptides, NPY and AgRP. The AgRP is an antagonist of MC3R and MC4R, and NPY acts in Y receptors stimulating food intake.

The gastrointestinal tract is also involved in the control of energy balance. Stomach releases ghrelin, which increases NPY gene expression and inhibits signaling satiety action of leptin. These signals always increase in hunger and pre feeding times. On the other hand, intestine produces peptides signals that influence satiety process by increasing glucagon like peptide-1 and peptide YY which decreases intestinal motility processes and increases satiety sensation (Van der Lely, 2004; Woods and D'Alessio, 2008).

Insulin is a hormone secreted by pancreatic beta cells in post-prandial status and is also responsible for neuroendocrine control of energy balance. Similar to leptin, it is transported across the blood-brain barrier and acts on receptors predominantly expressed in hypothalamus arcuate nucleus neurons. In contrast to its anabolic effect and classic role in glucose metabolism regulation, insulin has a central role in energy balance control by exerting anorexigenic and catabolic properties. Leptin expression is controlled by increased insulin release in the fed state, inhibiting food intake and increasing energy expenditure and controlling glucose and fat metabolism. Together with leptin, insulin inhibits appetite (Benoit *et al.*, 2004; Seufert *et al.*, 1999; Trayhurn and Wood, 2004).

Positive energy balance usually maintained by obese individuals acts as one important factor that disrupts neuroendocrine control of energy balance, leading to increase in number and size of fat cells and modification in adipokines function, mainly leptin (Kaur, 2014; Sanches *et al.*, 2014; Trayhurn and Wood, 2004). Most obese patients present hyperleptinemia, which suggests leptin resistance (Ramachandrappa and Farooqi, 2011). Interesting, this condition corroborates to impairments in weight loss process by inhibition of α-MSH and downregulation of adiponectin. These mechanisms may suggest alterations in energy balance and systemic inflammatory process (Dâmaso *et al.*, 2011; Moraes *et al.*, 2013; Sanches *et al.*, 2014).

Peripheral insulin resistance, induced by chronic pro-inflammatory state, was already well described in the literature. In the last 10 years, studies have pointed out a state of insulin and leptin resistance in the hypothalamus, which is commonly associated to defects on its receptors (De Souza *et al.*, 2005; Thaler and Schwartz, 2010).

Lifestyle changes remain the cornerstone of management of food intake and energy expenditure. Many thermogenic foods can be added to conventional diet as a strategy to modify energy balance and current dietary guidelines. Furthermore, supplements are formulated with multiple

ingredients of these foods purported to increase energy expenditure and fat oxidation, or suppress appetite. Among others, use of natural herbal ingredients such as teas (catechins), caffeine, and chili peppers (capsaicin) has attracted interest, especially because these ingredients do not contain any energy themselves, but can stimulate energy expenditure (Hursel and Westerterp-Plantenga, 2010; Ludy *et al.*, 2012; Vogel *et al.*, 2015; Whiting *et al.*, 2014). Table 7.1 summarizes the mechanisms by which catechins, caffeine and capsaicin can contribute to energy balance.

These functional ingredients have the potential to produce significant effects on metabolic targets such as thermogenesis and fat oxidation. Factors such as ethnicity, genetic effect, age, dependent doses, tolerance and habitual intake may act as confounders; this remains to be revealed. Furthermore, long-term randomized trials are now needed to investigate influence of these effects.

Nowadays, the new perspective in thermogenesis studies is the existence of metabolically BAT in adult humans. BAT is a promising target for combating obesity and related metabolic disorders in humans and anti-obesity possible effects of this thermogenic foods is also attributable to the activation of the sympathetic nerve and BAT system (Saito, 2015).

7.5 Hypotalamic inflammation

Leptin and the integrity of signaling pathways are crucial for energy homeostasis, since null mutations in genes of leptin, the leptin receptor, or POMC causes hyperphagia and severe obesity, which can be reversed by treatment with recombinant human leptin, in the case of disability leptin.

Recently, studies have shown that hypothalamic inflammation is induced during diet-induced obesity both in rodents and in humans. Excessive amounts of fatty acid in the diet cause hypothalamic inflammation and lead to obesity, even though intracellular fatty-acid sensing within the hypothalamus is important for the regulation of energy balance. An intake of a diet rich in SFAs promotes inflammation, gliosis, and neuronal stresses in the mediobasal hypothalamus, as well the inflammatory activation of microglia. SFAs can trigger toll-like receptor 2 and 4 dependent signaling, favoring an induction of the pro-inflammatory flagging mediated by JNK and NF-κB (Cai, 2012).

Excess amounts of FFAs, glucose, and amino acids due to over-nutrition induce ERS and oxidative stress, which also promote the activation of pro-inflammatory signaling and defective autophagy. These metabolically induced pro-inflammatory alterations in the hypothalamus cause defective intracellular leptin and insulin signaling, leading to central leptin/insulin resistance (Posey *et al.*, 2009).

The mechanism by which ERS is induced in the presence of fat diet is not well understood. It was suggested that lipids could directly affect homeostasis of the endoplasmic reticulum, by

Table 7.1. Effects of thermogenic compounds in energy balance.[1]

Thermogenic	Mechanism	Safety	References
Catechins	• Inhibit the enzyme COMT that is present in almost every tissue and degrades catecholic compounds such as norepinephrine. • COMT decreases the hydrophilicity by methylation, followed by sulfation and glucuronidation to make the excretion in urine and bile possible. • Norepinephrine cannot be degraded through the inhibition of COMT, and consequently the SNS will be stimulated, which attaches to b-adrenoceptors and causes an increase in energy expenditure and fat oxidation.	A possible side effect of green tea consumption is a minor increase in blood pressure.	Saito (2015); Hursel and Westerterp-Plantenga (2010); Hursel *et al.* (2009); Belza *et al.* (2007)
Caffeine	• Inhibit the enzyme phosphodiesterase which degrades intracellular cyclic amino mono phosphate. • After consumption of caffeine, cyclic adenosine monophosphate concentration rises and SNS activity will be increased and inactive hormone-sensitive lipase will be activated, which promotes lipolysis. • Affects the thermogenesis through the stimulation of substrate cycles such as the Cori-cycle and the FFA-triglyceride cycle. • Caffeine antagonizes the inhibitory effects of adenosine on lipolysis by adenylyl cyclase.	Use of caffeine is relatively safe, although acute caffeine consumption may alter some cardiovascular variables, chronic ingestion of caffeine has little or no health consequences. Optimal dose for caffeine is estimated at 5 to 10 g/day. It's necessary to consider individual tolerance and dose-dependence.	Hursel *et al.* (2011); Hursel and Westerterp-Plantenga (2010); Westerterp-Plantenga (2010); Belza *et al.* (2007); Acheson *et al.* (2004)
Capsaicin	• Stimulates catecholamine production by the TRPV1 receptor. • Increased energy expenditure by stimulation of the SNS and the upregulation of UCPs.	The long-term use and several doses day of capsaicin may be limited by its strong pungency and sensorial effect. Studies doses varied from 0.4 mg to 33 mg/day.	Saito (2015); Whiting *et al.* (2014); Ludy *et al.* (2012); Hursel and Westerterp-Plantenga (2010)

[1] COMT = catechol O-methyl-transferase; FFA = free fat acids; SNS = sympathetic nerve system; TRPV1 = transient receptor potential vanilloid; UCPs = uncoupling protein.

changing membrane composition of this organelle and in calcium depletion (Belgardt *et al.*, 2010). However, a study performed with animals showed that inhibition of TLR-4 is sufficient to improve the ERS, independently if they were fed with rich fat diet or received injection of SFAs, suggesting that this is a secondary event to the activation of TLR-4 (Milanski *et al.*, 2009).

Souza *et al.* (2015) showed that high fat diet causes an increase in gene expression of inflammatory proteins in the hypothalamus, such as TNF-α and IL-6. This phenomenon accompanies the activation of protein kinase sensitive inflammation such as JNK and inhibitor of NF-κB (IKK) (Unger *et al.*, 2010).

The JNK activation catalyzes the phosphorylation of IRS serine, reducing activation of the phosphoinositide 3-kinase/protein kinase B pathway. Furthermore, the pharmacological or genetic inhibition of JNK in the rodents' hypothalamus restores insulin signaling. The IKK protein is expressed in hypothalamic neurons mediobasal, but typically it is inactive. When activated, it phosphorylates IκB, which is a protein that sequesters NF-κB in the cytoplasm, keeping it inactive. The phosphorylated IκB is degraded, releasing NF-κB to perform their actions. Thus, NF-κB translocates to the nucleus and leads to the transcription of inflammatory genes (De Souza *et al.*, 2005; Unger *et al.*, 2010; Zhang *et al.*, 2008). The activation of this pathway, in mice, leads to reduced leptin and insulin signaling in hypothalamus, resulting in weight gain and hyperphagia, while its suppression protects against the development of obesity (Zhang *et al.*, 2008).

Another mechanism by which hypothalamic inflammation causes the local resistance to leptin and insulin is the activation of proteins like SOCS3 and PTP1B, which run as physiological inhibitors of these signaling hormones. The SOCS3 can connect directly to receptors or signaling proteins, directly inhibiting or facilitating their degradation. Münzberg and colleagues have showed that SOCS3 expression is increased in the arcuate nucleus of the hypothalamus of mice fed to a high fat diet (Münzberg *et al.*, 2004). It was also demonstrated that the deletion of SOCS3, specifically in neurons, results in protection against diet-induced obesity, while overexpression of SOCS3 in neurons POMC induces leptin resistance, obesity and intolerance glucose (Mori *et al.*, 2004; Reed *et al.*, 2010).

The PTP1B inhibits signaling by leptin and insulin by the dephosphorylate of the insulin receptor, JAK2 and other signaling molecules in both pathways. Its expression in the hypothalamus increases in response to high-fat diet and systemic administration of TNF-α. Moreover, the inhibition of PTP1B in the CNS results in resistance to diet-induced obesity by improving the hypothalamic leptin sensitivity and insulin (Picardi *et al.*, 2008; Zabolotny *et al.*, 2008).

There is another protein, PKC-θ which can mediate deleterious effects of a diet high in fat on the core adiposity signs. It has been shown that exposure to palmitic acid (SFA) induces the activation of PKC-θ, reducing insulin signaling in the hypothalamus. In addition, the knockdown of this protein, in the arcuate nucleus, improves signaling site of insulin and glucose homeostasis in the periphery, alleviating induced weight gain by diet (Benoit *et al.*, 2009).

According to the studies presented above, it is observed that the manipulation of the many mechanisms involved in resistance to insulin and leptin in the hypothalamus is capable of modifying the adiposity, suggesting that the hypothalamic changes are not just an extension of the periphery inflammation, but can also be involved in the genesis of obesity in model animals (Velloso and Schwartz, 2011).

Another level of regulation which may be important in the control of energy homeostasis is the synaptic plasticity. It was reported that diet and peripherals metabolic hormones can influence in the organization of synaptic connections between hypothalamic neurons. Changes in these connections, along with neuronal apoptosis may be related to the difficulty on completely reversing the increased adiposity, even with the cessation of stimulus given by hypothalamic inflammation (Horvath *et al.*, 2010; Velloso and Schwartz, 2011).

In summary, some evidence suggests that high fat diet promotes central leptin and insulin resistance by the excessive amounts of fatty acid seen in the hypothalamus promoting inflammation leading to a vicious cycle between high fat diet, inflammation and obesity (Figure 7.5).

7.6 The role of nutrients and bioactive compounds in systemic inflammation

As described above, obesity is often associated with a low-grade inflammatory state. It has been demonstrated that immune cells infiltrated in adipose tissue contribute to and perpetuate the inflammatory state of fat, systemic insulin resistance, and the promotion of systemic inflammation. The ability to harness control of an immune response could break this pro-inflammatory cycle, related to several chronic diseases (Johnson and Makowski, 2015).

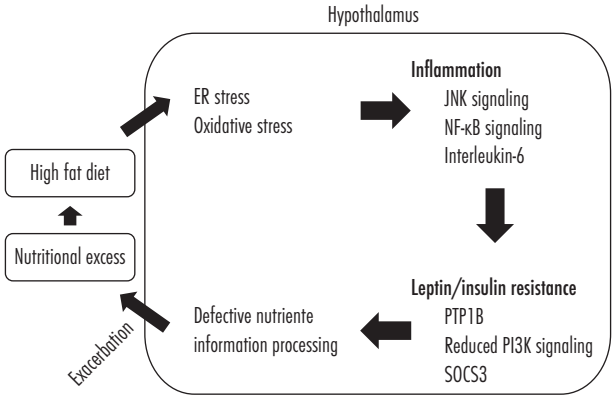

Figure 7.5. Mechanisms related to hypothalamic inflammation (ER = endoplasmatic reticulum; JNK = C-Jun N-terminal kinase; NF-κB = nuclear factor κB; PI3k = phosphoinositide 3-kinase; PTP1B = protein-tyrosine phosphatase 1B; SOCS 3 = suppressor of cytokine signaling 3).

The role of diet and nutrients in maintaining health and reducing the risk of chronic diseases is undoubted. Eating habits are considered to be one of the main factors that affect health or disease development, once certain genes can be regulated by nutrients/food compounds. The comprehensive mechanisms by which metabolism, nutrients, and bioactive compounds regulate macrophage and inflammation are considered potential therapeutic targets (Johnson and Makowski, 2015; Sales *et al.*, 2014).

One concept is that the nutrients themselves are inflammatory and physiological immune response is activated while they are metabolized. It has also been suggested that an overfeeding or high fat intake can trigger an inflammatory response. Furthermore, nutrients and bioactive compounds can influence the synthesis of mRNA (transcriptomics), protein synthesis (proteomics) and metabolite production (metabolomics). Thus, diet can influence cellular response elicited by nutritional stimulus (Isaak and Siow, 2013; Neeha and Kinth, 2013; Rosa *et al.*, 2012; Sales *et al.*, 2014).

NF-κB is the main transcription factor involved in upregulation of inflammatory cytokines, being considered a key mediator of inflammation in adipose tissue (Zoico *et al.*, 2009). NF-κB is a nuclear transcription factor expressed in the cytoplasm of all cell types, where its activity is controlled by a family of regulatory proteins IkB, called inhibitors of NF-κB. The involvement of NF-κB as the link between nutrients and inflammation suggests the close integration of nutrient and metabolic diseases (Tornatore *et al.*, 2012). Taken together, nutrients and bioactive compounds have an important role in the modulation of NF-κB signaling pathways, which may be a clinical strategy for the treatment of inflammation and metabolic disorders in obese individuals.

Activation of NF-κB signaling is initiated by extracellular stimuli. These stimuli are recognized by receptors and transmitted into the cell, where adaptor signaling proteins initiate a signaling cascade. These signaling cascades culminate in the activation of IKK, JNK, mitogen AMPK and activator protein 1 (Napetschnig and Wu, 2013). The Figure 7.6 shows the resume of the pathways between nutrients and inflammation, including the TLR-4 and NF-κB pathways in adipocytes and macrophages. The NF-κB is also related to insulin resistance. IKK B activation is key to development of insulin resistance, since it disrupts insulin receptor signaling through direct serine phosphorylation of IRS, which mediates many of the metabolic effects of insulin.

7.6.1 Omega-3 polyunsaturated fatty acid

n-3 PUFA are a family of PUFA characterized by having the last double bond between carbon numbers 3 and 4. Longer chain n-3 fatty acids include (EPA (C20:5n-3), DPA (C 22:5n-3) and DHA (C22:6n-3)). Omega-3 represents the fundamental component of phospholipids in cellular membranes and surface layer of intracellular lipid droplets in human (Calder, 2015).

EPA, DPA and DHA are found in significant quantities in fish and other seafood, and so they may be collectively referred to as marine n-3 PUFA. This fatty acid is known to have anti-inflammatory properties. Since the original report of Bang *et al.* (1976) on the diet of Greenland Eskimos

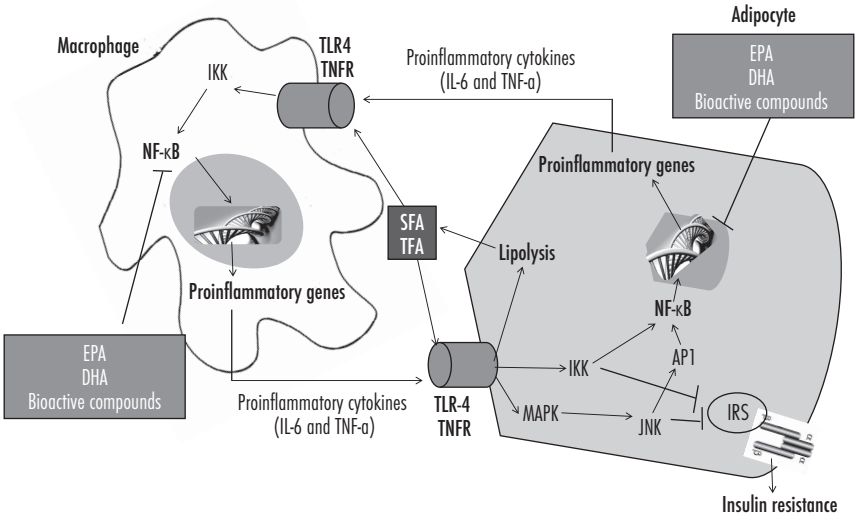

Figure 7.6. Relation between nutrients/bioactive compounds, inflammation and insulin resistance (adapted from Masquio *et al.*, 2014) (AP1 = activator protein 1; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; IKK = I kappa B kinase; IL-6 = interleukin 6; IRS = insulin receptor substrates; JNK = C-Jun N-terminal kinase; MAPK = mitogen activated protein kinase; NF-κB = nuclear factor κB; SFA = saturated fatty acid; TFA = trans fatty acid; TLR-4 = toll-like receptor 4; TNF-a = tumor-a necrosis factor; TNFR = tumor-a necrosis factor receptor).

and the decreased incidence of cardiovascular disease, there has been considerable interest in the use of n-3 fatty acids as dietary supplements. Several studies have shown promising results against inflammatory disorders including cardiovascular disease, MetS, non-alcoholic fatty liver disease and inflammatory parameters in humans (Flachs *et al.*, 2011; Yamaoka and Tango, 2012) (Table 7.2).

The mechanism of which n-3 fatty acid mediate inhibition of inflammation and cardiovascular disease is not fully understood, but includes several hypotheses. A great deal of supportive evidence indicates that n-3 PUFAs are able to improve inflammation in obesity by modulating main metabolic pathways in adipose tissue, as demonstrated in Figure 7.7 (Martínez-Fernández *et al.*, 2015).

n-3 PUFA can reduce adiposity by activating AMPK, which in turn promotes fatty acid β-oxidation in adipose tissue, leading to adipocyte hipotrophy. EPA and DHA are also known to promote mitochondrial biogenesis, which potentially contributes to increased energy metabolism. EPA and DHA also improve adipose tissue function and inflammation by increasing anti-inflammatory adipokines, such as, adiponectin and decreasing pro-inflammatory cytokines, such as, TNF-α and IL-6. The increase in adiponectin secretion seems to be peroxisome proliferator-activated receptor-dependent. The reduction of inflammation is also promoted by activation of GPR-

Table 7.2. Trials investigating the effects of omega-3 polyunsaturated fatty acids (PUFA) supplementation on inflammation markers in human.[1]

References	N sample	Study design	Main results
Ortega *et al.* (2016)	36 patients with MetS	placebo group: ingested 500 ml/day of semi-skim milk (8 g of fat; placebo milk) × supplementation group: 500 ml/day of skim milk enriched with 275 mg of n-3 PUFA and 7.5 g of oleate (Ω-3 + OLE); both groups were submitted to 24 weeks of high-intensity interval training	both groups: improvement of blood pressure, waist circumference, body fat mass and trunk fat mass; supplementation group: improvement of insulin sensitivity, CRP and HDL-C
Polus *et al.* (2016)	Obese women	control group × intervention group: moderate dose of n-3 PUFAs (1.8 g EPA+DHA per day) for 3 months	↓ inflammatory markers (MCP-1, sVCAM-1, sPECAM-1, and hs-CRP), TG and insulin plasma and ↑ concentrations of pro-resolving DHA derivatives in plasma
Barden *et al.* (2014)	21 healthy volunteers	supplementation with n-3 fatty acids (2.4 g/day) for 7 days with random assignment to take aspirin (300 mg/day) or placebo from day 5 to day 7	supplementation with n-3 fatty acids significantly increased proresolving mediators: RvE1, 18R/S-HEPE, 17R/S-HDHA, and 14R/S-HDHA
Flock *et al.* (2014)	116 adults	young adults with low fish intake received one of five doses (0, 300, 600, 900, or 1,800 mg/d EPA+DHA) for 5 months	no significant effects of supplemental EPA+DHA on IL-6 or CRP; however, there was a marginal treatment effect for TNF-a ($P<0.08$)
Kiecolt-Glaser *et al.* (2012)	138 healthy middle-aged and older adults	three-arm randomized, placebo-controlled, double-blind 4-month trial compared responses to (1) 2.5 g/d n-3 PUFAs; or (2) 1.25 g/d n-3 PUFAs; or (3) placebo capsules	serum IL-6 decreased by 10% and 12% in low and high dose n-3 groups; similarly, low and high dose n-3 groups showed modest 0.2% and -2.3% changes in serum TNF-a compared to a 12% increase in the control group
Nobili *et al.* (2011)	60 children with biopsy-proven NAFLD	DHA supplementation (250 mg/day) × DHA supplementation (500 mg/day) × placebo group	DHA supplementation improves liver steatosis and insulin sensitivity in children with NAFLD
Dangardt *et al.* (2010)	25 obese adolescents	volunteers were randomized to receive capsules containing either 1.2 g/day n-3 or placebo for 3 months	the serum n-3 PUFA concentration increased with n-3 treatment; N-3 supplementation also decreased the lymphocyte, monocyte, TNF-a, IL-6 and IL-1β levels
Ebrahimi *et al.* (2009)	120 subjects with MetS	control group × intervention group: 1 gram of fish oil as a single capsule, containing 180 mg EPA and 120 mg DHA daily for 6 months	supplementation with omega-3 was associated with significant fall in body weight, systolic blood pressures, serum low-density lipoprotein cholesterol, and total cholesterol, TG and hs-CRP
Kelley *et al.* (2009)	17 hypertriglyceridemic men	double-blind, randomized, placebo-controlled parallel study; volunteers received no supplements for the first 8 d and then received either 7.5 g/d DHA oil (3 g DHA/d) or olive oil (placebo) for the last 90 days	supplementation reduced concentrations of CRP by 15%, interleukin-6 by 23%, and granulocyte monocyte-colony stimulating factor by 21% and DHA increased the concentration of anti-inflammatory matrix metalloproteinase-2 by 7%

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Table 7.2. Continued.

References	N sample	Study design	Main results
Lee *et al.* (2014)	59 subjects with early-stage type 2 diabetes or MetS	randomized, single-blind, parallel intervention study. Individuals received either corn oil (CO), a botanical oil (BO) combination (borage [*Borago officinalis* L.]/echium oil [*Echium plantagineum* L.]) or fish oil (FO) for eight weeks	supplementation with BO significantly lowered total and LDL cholesterol levels and FO reduced serum triglycerides, HbA1C and increased HDL-C
Spadaro *et al.* (2008)	40 NAFLD patients	group 1 (n=20) received an AHA recommended diet and PUFA 2 g/day; group 2 (n=20) received only the AHA regular diet	ALT, TG, TNF-a and fatty liver improved after PUFA supplementation
Capanni *et al.* (2006)	56 NAFLD patients	42 patients received n-3 PUFA 1-g capsule daily for 12 months, whereas 14 refused the treatment and were analysed as controls	PUFA supplementation group: ↓ liver enzymes, fasting glucose and improvement of ultrasonographic and haemodynamic features of liver steatosis
Warensjo *et al.* (2006)	576 men	cross-sectional and prospective (20 y) analyses	n-3 PUFA predicted MetS development over 20 y, independent of smoking habits, physical activity, and BMI
Meydani *et al.* (1991)	young (23-33 y) and older (51-68 y) women	subjects supplemented their diets with 2.4 g of (n-3) fatty acid/d for 3 months	n3 supplementation reduced total IL-1 beta synthesis by 48% in young women but by 90% in older women; tumor necrosis factor was reduced by 58% in young and 70% in older women; interleukin-6 was reduced in young women by 30% but by 60% in older women; the (n-3) supplementation reduced IL-2 production in both groups; however, this reduction was significant only in older women
Endres *et al.* (1989)	9 healthy volunteers	addition of 18 g of fish-oil concentrate per day to diet for six weeks	n-3 PUFA inhibit the production of IL-1 and TNF-a

[1] AHA = American Heart Association; ALT = alanine aminotransferase; BMI = body mass index; BO = botanical oil; CO = corn oil; CRP = c reactive protein; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; FO = fish oil; HbA1C = hemoglobin A1c; HDL-C = high-density lipoprotein-cholesterol; hs-CRP = high sensitivity c reactive protein; IL-1 β = interleukin 1 β; IL-2 = interleukin 2; IL-6 = interleukin 6; MCP-1 = monocyte chemoattractant protein-1; MetS = metabolic syndrome; n-3 PUFA = omega-3 polyunsaturated fatty acids; NAFLD = non-alcoholic fatty liver disease; RvE1 = resolvinE1; sPECAM-1 = soluble platelet endothelial cell adhesion molecule-1; sVCAM = soluble vascular cell adhesion molecule 1; TNF-a = tumor-a necrosis factor; TG = triglycerides; 14-HDHA = 14-hydroxydocosahexaenoic acid; 17R/S-HDHA = 17R/S-hydroxydocosahexaenoic acid; 18R/S-HEPE = 18R/S-hydroxye icosapentaenoic acid.

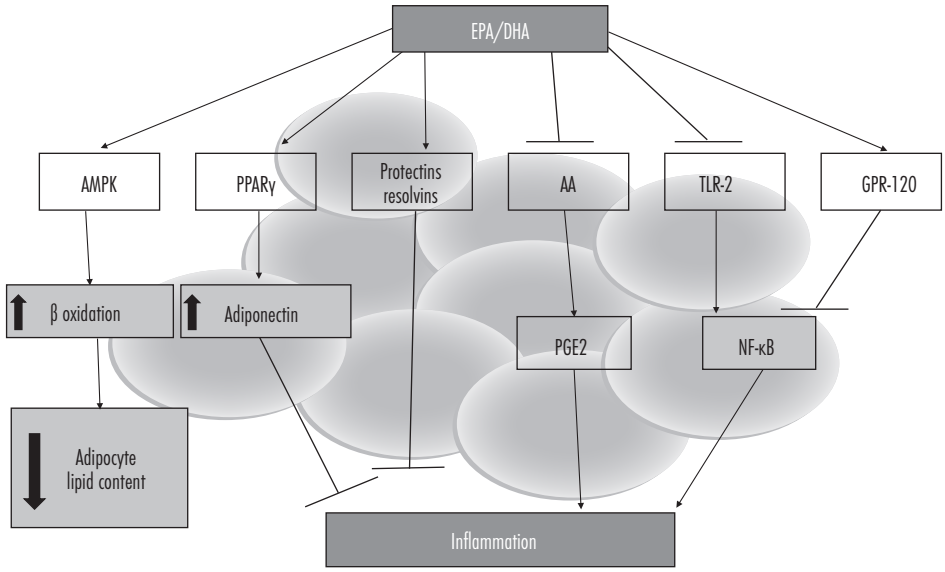

Figure 7.7. Actions of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in adipose tissue (adapted from Siriwardhana *et al.*, 2013) (AA = arachidonic acid; AMPK = activated protein kinase; GPR-120 = G-protein coupled receptor-120; NF-κB = nuclear factor κB; PGE2 = prostaglandin E2; PPARγ = peroxisome proliferator-activated receptor; TLR-2 = toll-like receptor 2).

120 and secretion of resolvins and protectins. In macrophages, activation of GPR-120 leads to inhibition of the NF-κB pathway (Siriwardhana *et al.*, 2013).

Furthermore, it is considered that the influence of fatty acids on inflammatory cell responses, and inflammatory processes, involves their incorporation into cell membrane phospholipids. Since increased intake of marine n-3 fatty acids decreases the amount of arachidonic acid in the membrane phospholipids of cells involved in inflammation, it might be expected that production of its derived mediators, such as prostaglandin 2, would be decreased simply because of a reduced amount of substrate available (Calder, 2015).

Adipose tissue TLRs (TLR-2 and 4) are cell surface receptors that are activated by several dietary stimulants including LPS, SFA and TFA. The TLRs activate NF-κB, a transcription factor and potent inducer of gene transcription of several proinflammatory cytokines such as IL-6 and TNF-α (Siriwardhana *et al.*, 2013). In summary, it is important to note diverse biological effects of omega-3. Clearly, its holistic anti-inflammatory action should be considered. This is important in respect of the low-grade inflammation associated with obesity and MetS (Flachs *et al.*, 2014).

7.6.2 Saturated fatty acids

Fat is an important component of the normal human diet. It is a source of energy and provides essential fatty acids and fat-soluble vitamins. However, several fatty acids, especially SFAs and TFA may have adverse effects on human health (Estadella *et al.*, 2013).

In the human diet, SFAs are derived from animal sources, including high fatty dairy products, red meats and pork, and fast and processed foods. The consumption of SFA for American population exceeds in 16 g from recommended amount. High SFA intake, the typical dietary pattern of western populations, favors a proinflammatory status that contributes to the development of insulin resistance (Estadella *et al.*, 2013; Kennedy *et al.*, 2009)

Excessive consumption of SFA contributes to weight gain and inflammation thought several mechanisms, including oxidative or endoplasmatic reticulum (ER) stress, generation of ceramide and ROS, IKK and PKC signaling (Figure 7.8). It is important to note that hypertrophied and lipid filled adipocytes containing SFA are involved in the incerased secretion of pro-inflammatory mediators, contributing to promote systemic inflammation (Kennedy *et al.*, 2009).

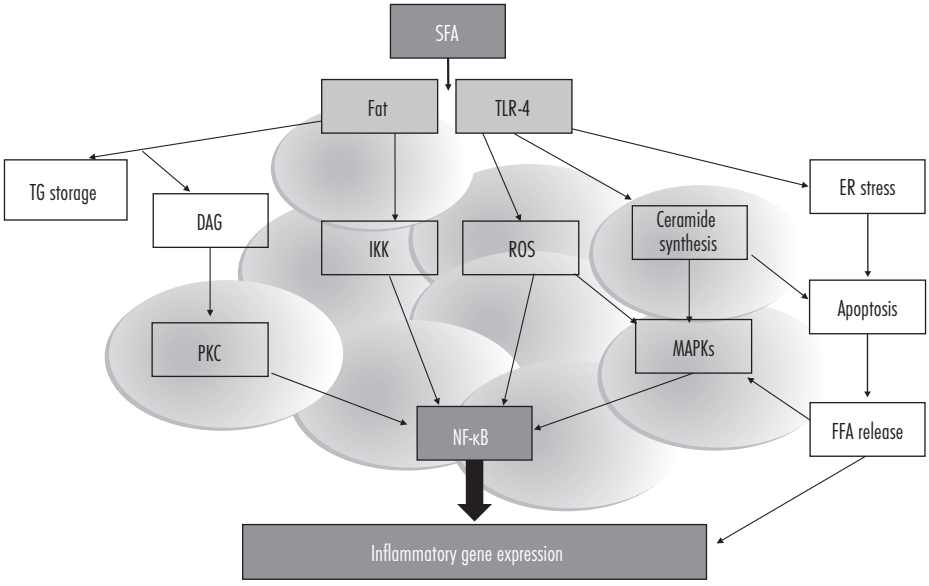

Figure 7.8. Proposed mechanism by which saturated fatty acid mediates inflammation (adapted from Kennedy *et al.*, 2009) (DAG = diacylglycerol; ER = endoplasmatic reticulum; FFA = free fatty acid; IKK = I kappa B kinase; MAPKs = mitogen activated protein kinases; NF-κB = nuclear factor κB; PKC = protein kinase C; ROS = reactive oxygen species; SFA = saturated fatty acid; TG = tryglicerides; TLR-4 = toll-like receptor 4).

The SFA is able to bind to TLR-4 receptors present on membranes from peripheral tissues, including white adipose tissue and macrophages, and trigger a cascade reaction which promotes the activation of NF-κB, and consequently gene expression of pro-inflammatory cytokines and insulin resistance. Interestingly, SFA promotes cross-talk among adipocytes, macrophages and myotubes from muscle tissue. SFA can directly and indirectly cause inflammation and insulin resistance in muscle, through diacylglycerol, PKC, IKK, and impaired in insulin signaling in IRS-1 (Figure 7.9) (Estadella *et al.*, 2013; Kennedy *et al.*, 2009).

Recently, our research group demonstrated that serum SFA was positively correlated with leptin/adiponectin ratio, the number of MetS parameters, and negatively correlated with adiponectin in obese adolescents (Masquio *et al.*, 2016). Furthermore, we verified that reduction greater than 3.67 g of SFA plus physical exercise during 1 year promoted decrease in insulin, leptin/adiponectin ratio, carotid intima media thickness, and increase in adiponectin and adiponectin/leptin ratio (Masquio *et al.*, 2015b). Altogether, these data suggested the pro-inflammatory role of SFA in adolescents, which can be reverted reducing the intake of this type of fatty acid.

In a representative sample of United States residents, saturated fat consumption was modestly associated with elevated CRP (King *et al.*, 2003). In a systematic review, Santos *et al.* (2013)

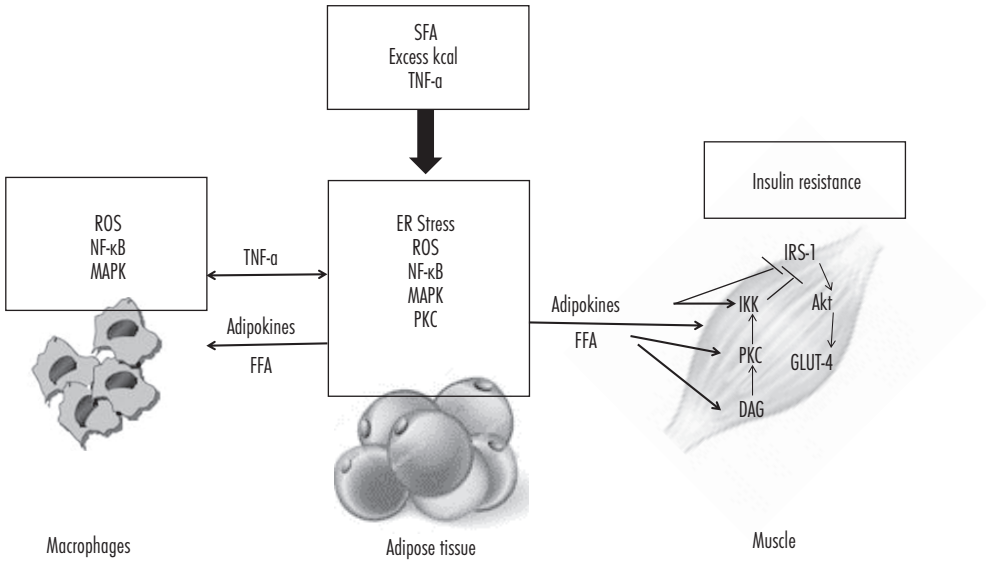

Figure 7.9. Cross-talk between inflamed adipose tissue, macrophages and muscle (adapted from Kennedy *et al.*, 2009) (Akt = protein kinase B; DAG = diacylglycerol; ER = endoplasmatic reticulum; FFA = free fatty acid; GLUT-4 = glucose transporter 4; IKK = I kappa B kinase; IRS = insulin receptor substrates; MAPK = mitogen activated protein kinase; NF-κB = nuclear factor κB; PKC = protein kinase C; ROS = reactive oxygen species; SFA = saturated fatty acid; TNF-a = tumor-a necrosis factor).

suggested a potential positive association of SFA with hs-CRP, however it did not support an association with adipokines. Further studies are needed to confirm the relationship between SFA and adipokines in human studies.

7.6.3 Bioactive compounds

The finding that diet rich in vegetables reduce the risk of chronic disease has encouraged research that identified nutrient and non-nutrient substances acting on specific targets and thus interfering in physiological and pathogenic process. Beneficial effects of some diets, such as Mediterranean and Dietary Approaches to Stop Hypertension, are attributed for the significant amounts of bioactive compounds present especially in vegetables and fruits (Bastos *et al.*, 2009; Wang *et al.*, 2014).

The consumption of foods rich in bioactive compounds have been documented to decrease inflammation, since they present anti-inflammatory and anti-oxidant properties. A multiplicity of plant extracts with anti-inflammatory properties has been shown to have a significant effect on adipose tissue, to act as anti-diabetic agents, or to be effective for the treatment of chronic inflammatory conditions (Leiherer *et al.*, 2013).

As described above, the inflammatory trigger could be a variety of stimuli, including TNF-α, IL-1, ROS, LPS, TFA and SFA, which promote the activation of NF-κB, considered the central regulator of inflammation (Rosa *et al.*, 2012). However, bioactive compounds, including curcumin, resveratrol, flavonoids, catechins, quercetin and isoflavones, are known to suppress NF-κB, and consequently systemic and adipose tissue inflammation (Siriwardhana *et al.*, 2013). The anti-inflammatory mechanisms of bioactive compounds and food sources are described in Table 7.3.

Table 7.4 demonstrates the results of clinical studies about the beneficial effects of bioactive compounds in inflammatory markers. According to the review conducted by Rosa *et al.* (2012), some points must be taken in account:

- Quercetin: although quercetin supplementation has shown anti-inflammatory effects on animals and *in vitro* experiments, the results have not yet been reproduced in humans (Rosa *et al.*, 2012).
- Isoflavones: are similar to estradiol molecules and thereafter have been classified as phytoestrogens. Three forms of this polyphenol are genistein, daidzein and glycitein, and they have affinity to estrogen receptors, exerting potential hormone properties. The lack of positive results on inflammation markers is also considered to be due to the healthy and relatively normal body weight of women profile (Charles *et al.*, 2009).
- Anthocyanin: seems to be a promising substance to be applied even to prevent or to counteract inflammation process, since results were found in healthy people and also in patients with MetS and cardiovascular disease.
- Resveratrol: no articles in humans were found while evaluating the anti-inflammatory effects of an extract containing, exclusively, resveratrol. It is justified that once water insoluble structure of resveratrol might render its applicability as a supplement difficult.

Table 7.3. Bioactive compounds in foods involved in modulating the inflammatory response (adapted from Bastos *et al.*, 2009; Rosa *et al.*, 2012; Leiherer *et al.*, 2013; Licznerska *et al.*, 2016).

Bioactive compounds	Food source	Anti-inflammatory mechanisms[1]
anthocyanin	berry fruits such as cranberries, chokeberries and blueberries as a red and blue pigment	↓ NF-κB, ↑ Nrf2
capsaicin	chilli peppers	↓ NF-κB
catequins	green tea (*Camellia sinensis*)	↓ NF-κB, ↓ AP-1, ↓ JNK, ↓ IL-6, ↓ COX2
curcumin	*Curcuma longa*	↓ NF-κB, ↓ AP-1, ↓ JNK, ↓ PKC, ↓ TNF-a, ↓ IL-6, ↓ LOX, ↓ COX2, ↓ iNOS, ↑ PPARγ, ↑ Nrf2
elagic acid	pomegranate	↓ NF-κB, ↓ COX2, ↓ MMP-9
gingerol	ginger (*Zingiber officinalle*)	↓ NF-κB, ↓ AP-1, ↓ COX2, ↓ TNF-a, ↓ iNOS, ↓ p38MAPK
genistein	soy (*Glycine max*)and soy derivated products	↓ NF-κB, ↑ GSH-px
indole-3-carbinol	*Brassica* (cabbage, broccoli, cauliflower, Brussels sprouts, kale, bok cho)	↓ NF-κB, ↓ JNK, ↓ COX2
quercetin	citrus fruits, onion, apple, broccoli and lettuce	↓ NF-κB, ↓ MAPK, ↓ AP-1, ↓ iNOS
resveratrol	grape (*Vitis vinifera*), red wine, peanuts, cranberries, blueberries	↓ NF-κB, ↓COX2, ↓ iNOS, ↓ JNK, ↓ AP-1, ↓ PKC, ↓ LOX, ↓ IL-6, ↓ IL-8, ↓ IL-1, ↑ Nrf2

[1] AP-1 = activator protein 1; COX2 = cyclooxygenase 2; GSH-px = glutathione peroxidase; IL = Interleukin; iNOS = inducible nitric oxide synthase; JNK = C-Jun N-terminal kinase; LOX = lipoxygenase; MMP-9 = matrix metalloproteinase-9; NF-κB = nuclear factor κB; Nrf2 = nuclear factor erythroid 2; PKC = protein kinase C; PPARγ = peroxisome proliferator-activated receptor; p38MAPK = P38-mitogen-activated protein kinase; TNF-a = tumor-a necrosis factor.

7.7 Considerations and conclusions

Obesity is defined as a chronic and inflammatory disease with increasing prevalence around the world. Systemic low-grade inflammation related to expansion in adipose tissue, especially visceral adipose tissue, has been linked with metabolic alterations, such as, insulin resistance, dyslipidemia, hypertension, and the development of MetS.

Dietary components such as bioactive compounds and PUFA can activate molecules related to gene expression and increased pathways which are able to promote health benefits and metabolic homeostasis, and also inhibit proinflammatory cytokine gene expression. This integrative omics approach, combined with mechanistic studies and appropriate experimental models, will help further understanding of the effective action of nutrients, bioactive compounds on the control of obesity and obesity-related diseases in humans.

Table 7.4. Trial investigating the effects of bioactive compounds on inflammation markers in human (adapted from Rosa *et al.*, 2012).[1]

Bioactive compounds	References	N sample	Study design	Main results
anthocyanin	Kaspar *et al.* (2011)	36 healthy men	groups: supplemented with 150 g/d of cooked white, yellow (58 mg of carotenoids and 0.3 g of /kg), and purple (1.3 mg of carotenoids and 6.2 g of anthocyanin/kg) potato cultivars for 6 weeks	significant decrease in CRP in purple potato group; no changes in plasma IL-1, IL-1b, IL-2, IL-4, IL-8, IL-10, IFN-γ or TNF-a
anthocyanin	Karlsen *et al.* (2010)	62 men and women with high risk for CVD	groups: bilberry juice (330 ml/d diluted to 1 l water) and water for 4 weeks	decrease in concentrations of CRP, IL-6, IL-15, and decreased and TNF-a increased in the bilberry group
anthocyanin	Basu *et al.* (2010)	27 men and women with MetS	groups: supplemented with two cups of strawberry beverage (25 g of freeze-dried strawberry powder – 154-mg anthocyanin) and two cups of water/d or control four cups of water/d for 8 weeks	decreased plasma levels of VCAM but no effects on ICAM
anthocyanin	Naruszewicz *et al.* (2007)	44 adults after myocardial infarction	groups: supplemented 3 £ 85 mg/d chokeberry flavonoid extract (anthocyans 25%, monomeric and olygomeric procyanidins 50% and phenolic acids 9%) or placebo for 6 weeks	lowering effect on ICAM, VCAM, MCP-1, hsIL-6 and hs-CRP
anthocyanin	Karlsen *et al.* (2007)	118 healthy men and women BMI 17-35 kg/m^2	groups: supplemented with four capsules of 75-mg/d corresponding to 300-mg anthocyanins/d or placebo for 3 weeks	reductions in IL-8, RANTES, IL-4 and IL-13 compared to placebo group; CRP did not differ between the groups
curcumin	Yang *et al.* (2015)	14 type II diabetes mellitus patients	supplementation with 500 mg/day of curcumin for a period of 15-30 days	curcumin reduced plasma MDA level with enhanced the Nrf2 system specifically regulated protein; decrease in plasma LPS content and increase in IκB (inhibitory protein on NFkB) were observed
curcumin	Ganjali *et al.* (2014)	30 obese individuals	groups: supplemented with curcumin (1 g/d) or placebo for 4 weeks	IL-1β, IL-4, and VEGF were reduced by curcumin therapy; no significant difference was observed in the concentrations of IL-2, IL-6, IL-8, IL-10, IFNγ, EGF, and MCP-1

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Table 7.4. Continued.

Bioactive compounds	References	N sample	Study design	Main results
curcumin	Ramirez-Bosca *et al.* (2000)	16 men and 14 women	10 mg of curcumin 2×/day per 15 days	↓ levels of plasma fibrinogen in both genders
gingerol	Arablou *et al.* (2014)	70 type 2 diabetic patients	double-blinded, placebo-controlled clinical trial. groups: ginger (1,600 mg ginger daily) or control group (1,600 mg wheat flour placebo daily) for 12 weeks	ginger reduced fasting plasma glucose, HbA1C, insulin, HOMA, triglyceride, total cholesterol, CRP and PGE2 compared with placebo group; there were no significant differences in HDL, LDL and TNF-a
green tea	Bogdanski *et al.* (2012)	56 obese, hypertensive subjects	double-blind, placebo-controlled trial; groups: GTE (1 capsule that contained 379 mg of green tea extract) or placebo for 3 months	decrease in systolic and diastolic blood pressure, fasting serum glucose, insulin levels, insulin resistance, total and low-density lipoprotein cholesterol, triglycerides, tumor necrosis factor a and C-reactive protein in the GTE; increase in high-density lipoprotein cholesterol and total antioxidant status
isoflavone	Charles *et al.* (2009)	75 healthy postmenopausal women	groups: 20 g of soy protein with 160 mg of total isoflavones (64 mg genistein, 63 mg daidzein, and 34 mg glycitein) or 20 g of soy protein placebo for 12 weeks	increase in adiponectin, but no effects on leptin, resistin, IL-6 and TNF-a
isoflavone (genistein)	Atteritano *et al.* (2007)	389 osteopenic post-menopausal women	groups: supplemented with 54 mg of genistein/d in tablets or placebo for 24 months	significant reductions in VCAM and ICAM compared to placebo

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Table 7.4. Continued.

Bioactive compounds	References	N sample	Study design	Main results
isoflavone	Huang *et al.* (2005)	12 post-menopausal women	intake of 36-oz portion of soymilk containing isoflavones daily for 16 week	serum levels of TNF-a decreased by 25.1% (as early as 2 week after soy consumption) and by 66.7% 10 week after soy consumption and recovered to the prediet levels 4 week after the termination of soy consumption; similar decrease of up to 56.6 and 14.4% was found for serum IL-1a and the mean percentage of blood monocytes during soy consumption, respectively, but not for IL-6
pomegranate	Asghari *et al.* (2012)	51 dyslipidemic patients	groups: pomegranate seed oil (PSO) (2×400 mg/d) or placebo for 4 weeks	serum concentration of TNF-a decreased in the PSO group
quercetin	Egert *et al.* (2010)	93 overweight and obese adults	groups: 150-mg quercetin/d or placebo; crossover trial with 5-wk wash-out period; individuals were classified into the following three genotypes: (1) apo E2 group; (2) apo E3 group; and (3) apo E4	decrease in serum TNF-a, in apo E3 and E4 subgroups, but no effect on serum CRP
quercetin	Egert *et al.* (2009)	93 overweight and obese adults	groups: 150-mg quercetin/d or placebo; crossover trial with 5-wk	no significant decrease in hs-CRP and hs-TNF-a compared to placebo
resveratrol	Macedo *et al.* (2015)	60 military firefighters	placebo-controlled double-blinded study; groups: 100 mg/day of RES or control for 90 days	plasma oxidative stress biomarkers (thiol content, 8-isoprostane and 8OHdG) showed no modifications, while IL-6 and TNF-a were decreased in the RES group
resveratrol	Faghihzadeh *et al.* (2014)	50 NAFLD patients	randomized, double-blinded, controlled clinical trial; groups: 500-mg resveratrol capsule or placebo for 12 weeks	resveratrol supplementation was associated with a significant reduction in liver enzymes, inflammatory cytokines, NF-κB, serum cytokeratin-18, and hepatic steatosis grade, as compared with placebo

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Table 7.4. Continued.

Bioactive compounds	References	N sample	Study design	Main results
resveratrol	Ghanim *et al.* (2010)	20 healthy subjects	groups: supplemented with 200 mg of *Polygonum cuspidatum* extract (equivalent to 40-mg/d trans-resveratrol) or placebo for 6 weeks	reduced intra-nuclear NF-κB DNA binding in MNCs; TNF-a and IL-6 mRNA expression in MNCs; and plasma TNF-a and CRP, JNK, IKkB mRNA expression compared to placebo
resveratrol	Brasnyó *et al.* (2011)	19 patients with type 2 DM	the patients were enrolled in the 4-week-long double-blind study: two groups: a resveratrol group receiving oral 2×5 mg resveratrol and a control group receiving placebo	resveratrol significantly decreased insulin resistance (HOMA-IR) and urinary ortho-tyrosine excretion and the ↑ pAkt:Akt ratio in platelets
resveratrol	Blanco-Colio *et al.* (2007)	16 healthy men and women	groups: red wine or vodka or brandy or rum or control without alcohol, all groups accompanied by a fat-enriched diet (44%); crossover trial with 5 days of duration and 2-week wash-out period	red wine, brandy and rum decreased NF-κB activation; red wine decreased plasma MCP-1

[1] AKt = protein kinase B; CRP = c reactive protein; CVD = cardiovascular disease; DM = diabetes mellitus; DNA = deoxyribonucleic acid; EGF = epidermal growth factor; GTE = green tea extract; HbA1C = hemoglobin A1c; HDL-C = high-density lipoprotein-cholesterol; HOMA = homeostasis model assessment; hs-CRP = high sensitivity c reactive protein; hsIL-6 = high sensitivity interleukin 6; ICAM = intercellular adhesion molecule; IκK = I kappa kinase; IKkB = I kappa B kinase; IL = interleukin; IFN-γ = interferon γ; JNK = Jun N-terminal kinase; LDL-C = low-density lipoprotein-cholesterol; LPS = lipopolysaccharide; MCP-1 = monocyte chemoattractant protein-1; MDA = malondialdehyde; MetS = metabolic syndrome; MNC = mononuclear cell; mRNA = messenger RNA; NAFLD = non-alcoholic fatty liver disease; NFκB = nuclear factor kappa b; Nrf2 = nuclear factor erythroid 2; PGE2 = prostaglandin E 2; PSO = pomegranate seed oil; RANTES = regulated on activation normal T cell expressed and secreted; RES = resveratrol; TNF-a = tumor-a necrosis factor; VCAM = vascular cell adhesion molecule 1; VEGF = vascular endothelial growth factor; 8OHdG = 8-hydroxy-2′-deoxyguanosine.

In summary, food intake is considered the main factor capable of promoting health and disease. In this way, the precise determination of molecular mechanisms, underlying human health and disease, offer a great potential for promoting health and lowering mortality and morbidity related to obesity and MetS. Indeed, changes in lifestyle and diet are mandatory to control the incidence of obesity and MetS.

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8. Role of food groups and dietary patterns in heart health

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Abstract

Cardiovascular disease (CVD) is a global burden and considered to be a multifactorial chronic disease. Foods are modifiable risk factors which may play a pivotal role in the development of CVD. Recent evidence emphasizing systematic reviews and meta-analyses of epidemiological and clinical trials from research on foods and dietary patterns on CVD risk are briefly reviewed in this chapter. The consistency of findings in all studies strongly suggests the cardio-protective properties of functional foods such as whole grains, legumes, fish, nuts, dairy products, fruit, vegetable, tea, olive oil and dark chocolate. Dietary pattern analysis as a complementary approach in nutritional epidemiology has evolved to explore associations between diet and disease. There are two different ways to define dietary patterns: 'a priori', focusing on the construction of patterns based on published dietary recommendations. The second is 'a posteriori', which builds on exploratory statistical methods. Adoption of a Mediterranean-style diet is important for the primary and secondary prevention of CVD and its risk factors. Evidence for the protective role of the dietary approach to stop hypertension pattern in prevention of CVD and its risk factors is strong. Dietary pattern indices found to be associated with a reduced risk of CVD and healthy dietary patterns were associated with reduced risk of CVD and its risk factors, whereas unhealthy/Western-type patterns are associated with an increased risk of CVD. These findings could be a useful tool to better understand the effectiveness of dietary patterns in managing CVD risk. Some aspects of healthy diets are already included in scientific and clinical guidelines available for the prevention of CVD.

Keywords: healthy patterns, Mediterranean dietary pattern, cardiovascular disease, functional foods

Key facts

- Dietary factors may play a vital role in the development of cardiovascular disease (CVD) and its risk factors, which can be modified in order to prevent and manage this disease.
- The consumption of whole grains, legumes, fish, nuts, dairy products, fruit and vegetables, as important parts of a healthy well-balanced diet has been recommended.
- Consumption of fast foods, high in sodium, fat, trans-fatty acids and cholesterol content and poor in essential nutrients and dietary fibers is a main dietary risk factor for chronic disease.
- Mediterranean diet (MD) including unrefined cereals, vegetable, fruit, olives, dairy, red or white wine, fish, pulses, nuts, eggs and low red meat intake is recommended for the prevention of CVD.
- Participants with high adherence to dietary guidelines and healthy dietary patterns have a lower risk of cardiovascular risk factors and its mortality.

Summary points

- Foods and dietary patterns are the fundamental unit in nutrition which can be modified; this is the main target for interventions aimed at primary prevention and management of CVD.
- Consistency of findings in all prospective and clinical trial studies strongly suggests the cardio-protective properties of functional foods such as whole grains, legumes, fish, nuts, dairy products, fruit and vegetables.
- Regarding the low essential nutrients and high fat and sodium content of fast foods, food policies providing nutritional information of fast-foods at restaurants help consumers to order more healthful foods.
- Current evidence demonstrates the beneficial effects of the MD on intermediate markers of cardiovascular risk, reducing CVD incidence, reoccurrence, and mortality.
- Unhealthy dietary patterns characterized by high consumption of processed meat, refined grains, sweets, high-fat dairy products, butter, and low fruit and vegetable intakes increase the risk of CVD.

Abbreviations:

AHEI	Alternate HEI
ALA	Alpha-linolenic acid
BP	Blood pressure
CAD	Coronary artery disease
CHD	Coronary heart disease
CIMT	Carotid intima media thickness
CLA	Conjugated linoleic acid
CRP	C-reactive protein
CVD	Cardiovascular disease
DASH	Dietary Approaches to Stop Hypertension
DHA	Docosahexaenoic acid (C22:6n3)
EGCG	Epigallocatechin-3-gallate
EPA	Eicosapentaenoic acid (C20:5n3)
FBG	Fasting blood glucose
HDL-C	High-density lipoprotein cholesterol
HEI	Healthy eating index
HR	Hazard ratio
IHD	Ischemic heart disease
LCn3PUFA	Long-chain n-3 polyunsaturated fatty acid
LDL-C	Low-density lipoprotein cholesterol
MD	Mediterranean diet
MetS	Metabolic syndrome
MI	Myocardial infarction
MUFA	Monounsaturated fatty acid
OR	Odds ratio
PCA	Principal component analysis
PREDIMED	The Prevención con Diet a Mediterránea
PUFA	Polyunsaturated fatty acid
RCT	Randomized clinical trials
RR	Relative risk
SFA	Saturated fatty acid
TG	Triglyceride
TLGS	Tehran Lipid and Glucose Study

8.1 Introduction

CVD is a global burden and varies between regions (Hartley *et al.*, 2013). It is well-known that CVD is considered a multifactorial chronic disease (Zhang *et al.*, 2015). Dietary factors may play a vital role in the development of CVD and its risk factors which may contribute to the geographic variability in CVD morbidity and mortality. Such factors are important because not only they

have been linked to CVD development, but they can also be modified, making them one of the main targets for interventions aimed at primary prevention and management of CVD (Hartley *et al.*, 2013).

Compelling scientific evidence has shown the role of certain specific foods such as functional foods in the prevention of CVD and certain diseases beyond basic nutrition, through the synergy and interaction of their main nutrients and bioactive phytochemical components. These specific foods include whole grains, fruit, vegetable, legumes, dairy, fish, green tea, olive oil, dark chocolate, garlic, cinnamon, turmeric, fenugreek, and red wine (Sikand *et al.*, 2015).

Foods, rather than nutrients in isolation, are the fundamental unit in nutrition; in this sense, consideration needs to be given to whether effects are due to single nutrients within a food, the food itself, or the whole dietary pattern in which the food is a significant part. Recent reviews have argued strongly for a greater focus on whole food research, because it provides the evidence in a form directly related to dietary guidelines (Warensjo *et al.*, 2010).

Considering all foods and their nutrient components with possible synergies and antagonisms between them, a technique known as dietary pattern analysis has evolved in nutritional epidemiology as a complementary approach to the study of individual foods which is best suited to explore associations between diet and disease (Rodriguez-Monforte *et al.*, 2015; Sala-Vila *et al.*, 2015). Diverse classifications have been used to group different dietary patterns, primarily categorizing them as healthy or prudent vs unhealthy or western.

In this chapter, we briefly review recent systematic reviews and meta-analyses of epidemiological and clinical trials evidence documented from 2010 by now about research on foods and dietary patterns on CVD risk.

8.2 Foods

8.2.1 Whole grains

Whole grains including rye, oats, barley, and whole wheat have protective effects against CVD risk factors, containing non-digestible complex polysaccharides, e.g. soluble and insoluble fibers, inulin, beta-glucan, and resistant starches. Additional bioactive components in whole grains include carotenoids, phytates, phytoestrogens, phenolic acids and tocopherols (Sikand *et al.*, 2015). A food providing at least 8 g of whole grains/30-g serving is defined as a whole-grain food (Sala-Vila *et al.*, 2015).

A meta-analysis of 11 prospective studies showed that individuals consuming 48 to 80-g/day of whole grains had about 20% lower risk of CVD, CAD, stroke, and fatal CVD, compared to individuals who rarely or never consumed whole grains. Short-term RCTs reported a modest lowering effect of whole grain interventions on LDL-C. A meta-analysis of RCTs using beta-

glucan-rich oats and barley showed that doses >3 g/day reduced LDL-C by 0.30 mmol/l (CI=0.24-0.35) (Sala-Vila *et al.*, 2015). Another meta-analysis of 67 controlled intervention trials, showed that daily consumption of 2-10 g/day of soluble fiber, mainly beta-glucan, psyllium, and pectin, lowered LDL-C by 2.2 mg/dl (95% CI: 1.7-2.7) with no significant changes in HDL-C or TG (Eilat-Adar *et al.*, 2013).

8.2.2 Legumes

Legumes including peas, beans, lentils, peanuts, and soybeans, are sources of plant protein, non-digestible carbohydrates (dietary fiber, resistance starches, oligosaccharides), bioactive compounds (linoleic acid, alpha-linolenic acid, isoflavones, phenolic compounds, saponins, and phytic acid) and some polyphenols (Sikand *et al.*, 2015). In a meta-analysis of five cohort studies, legume consumption was inversely associated with incident CAD, with a RR of 0.86 (CI=0.78-0.94) per four weekly 100-g servings. A meta-analysis of 10 RCTs evaluating the lipid effects of non-soy legume consumption indicated that significant mean decreases in total cholesterol of 0.31 mmol/l and LDL-cholesterol of 0.21 mmol/l, indicating that legumes, as a component of healthy diet, have a protective role against CVD (Sala-Vila *et al.*, 2015).

8.2.3 Nuts

Nuts including almonds, hazelnuts, walnuts, pistachios and peanuts are an easily available source of unsaturated fat intake. Although almost 80% of energy from nuts comes from fat but its fat is low in saturated (SFA) (4-16%) and high in both MUFA and PUFA, which have beneficial effects on inflammation, lipid markers and BP. The dietary guidelines for Americans recommend consumption of 4 ounces of nuts and seeds or soya products per week for a 2,000-kcal diet, emphasizing the benefits of nut consumption (Mayhew *et al.*, 2016). Moreover nuts also are a good source of plant proteins, bioactive peptides and many micronutrients (including folate, fiber, antioxidant vitamins and compounds, plant sterols, Ca, Mg and K) that are individually associated with decrease in CVD risk. Unsalted varieties of nuts have low sodium content. Nuts contain few carbohydrates and thus contribute little to postprandial glycemia. The antioxidant effect of nuts is attributed to the presence of alpha and gama tocopherol, phenolic acid, melatonin, oleic acid, and selenium (Sikand *et al.*, 2015).

In a systematic review of prospective cohort studies, compared to low nut consumers, higher nut consumption was associated with lower risk of all-cause mortality, total CVD, death from any type of CVD and CHD. Furthermore, a 4-weekly serving increment in nut intake, an amount consistent with the DASH diet, was associated with lower risk of all-cause mortality and lower risk of total CVD and CHD, findings compatible with those of previous systematic reviews (Mayhew *et al.*, 2016).

Inflammation has been linked to risk of CVD and diabetes, and evidence available suggests that frequent nut consumption has an inverse association with circulating inflammatory cytokines and a positive relation with plasma adiponectin (Luo *et al.*, 2014). Dietary patterns high in nuts

are associated with lower inflammatory cardiovascular risk markers, e.g. intercellular adhesion molecule 1 and the vascular cell adhesion molecule. Nuts exhibit a beneficial effect on the endothelium due to a high content of L-arginine, a main precursor of nitric oxide along with antioxidants and polyphenols that potentiate this effect (Sikand *et al.*, 2015). Six studies describe the protective effect of nut consumption on fatal and nonfatal CAD, resulting in an inverse association with both fatal CAD (RR=0.76; CI=0.69-0.84) and nonfatal CAD (RR=0.78; CI=0.67-0.92) per four servings of nuts/week (one serving equals 28.4 g). The result for each serving/day includes a pooled RR for CAD (fatal and nonfatal) 0.72 (CI=0.64-0.81). A dose-response relationship between nut consumption and reduced CAD outcomes was observed in all studies. In a meta-analysis, nut consumption was associated with a decreased risk of incident IHD and diabetes. Consumption of nuts was inversely associated with fatal IHD (six studies; 6,749 events; RR per four servings weekly, 0.76; CI=0.69-0.84) and inversely associated with nonfatal IHD (four studies; 2,101 events; RR per four servings weekly, 0.78; CI=0.67-0.92) (Sikand *et al.*, 2015).

A pooled analysis of 25 RCTs, comparing the effects of nut enriched versus nut-free diets on the lipid profile indicated a consistent cholesterol-lowering effect, with a mean 7.4% LDL-C reduction for an average consumption of 67 g (2.4 oz) of nuts, independent of the types of nut intakes. In the PREDIMED trial, individuals consuming the MD enriched with one serving of nuts per day resulted in a 30% reduction in the incidence of CVD and 49% reduction in stroke (Sala-Vila *et al.*, 2015); although no other evidence of an association between nut intakes and total stroke was found, but the quality of evidence for this outcome was very low (Mayhew *et al.*, 2016).

There are limited studies available on the effects of different types of nuts on CVD risk; three studies showed an association of peanut consumption with a lower risk of CHD mortality. Two of those studies also showed an inverse association of peanut consumption with CVD mortality, whereas one study found an inverse association with total CHD with similar relative risk estimates of those found in the meta-analysis for all nuts. Walnuts were also associated with a lower risk of all-cause and CVD mortality (Mayhew *et al.*, 2016). Acute and chronic consumption of walnuts (42.5-85 g/d) has been reported to lower total and LDL-C concentrations by 9-16%, decrease diastolic BP by 2-3 mm Hg, improve endothelial function, decrease both oxidative stress and markers of inflammation, and increase cholesterol efflux (Kris-Etherton, 2014). It needs to be mentioned that walnuts have the highest ALA (C18:3n-3) content of all nuts. Soybeans and flaxseed also supply ALA, a shorter-chain n3PUFA with cardio-protective effects on its own. Conversion of ALA to EPA in the body is poor, leading to modest increases of EPA, but not DHA, in plasma and cell pools (Sala-Vila *et al.*, 2015). Long-term consumption of walnuts is recommended as an important dietary strategy for improving heart health (Kris-Etherton, 2014). The consistency of findings in all prospective and RCT studies strongly suggests a causal association between nut consumption and its cardio-protective benefits which has prompted the inclusion of this food group in many guidelines for lifestyle management as a component of healthy diet to reduce cardiovascular risk.

8.2.4 Fruit and vegetable

One dietary factor that should be considered in the primary prevention of CVD is fruit and vegetable intakes. The evaluation of effects of fruits and vegetables on health outcomes is complicated because there is a large variability at the global level; effects of fruits are not necessarily similar to those of vegetables; cooking for many vegetables may change their composition, and there are multiple possibilities of interactions among them (Sala-Vila *et al.*, 2015). Many observational studies have shown that high consumption of fruits and vegetables can have a protective role for some chronic diseases including CVD. It has been hypothesized that by increasing fruit and vegetable consumption to 600 g/day, the worldwide burden of IHD and ischemic stroke could be reduced by 31 and 19%, respectively (Bhupathiraju and Tucker, 2011). A meta-analysis of 20 observational studies showed a linear dose-response relationship for risk reduction of stroke by 32% (CI=18-44) for each 200-g/day increment in consumption of fruits and by 11% (CI=2-19) for a daily increase in the same amount of vegetables. Another meta-analysis of 16 prospective cohort studies found that each additional serving/day was associated with a 4-5% reduced risk of all cause and CVD mortality (Sala-Vila *et al.*, 2015). A meta-analysis of nine studies, showed risk reduction of CHD by 4% (RR=0.96; CI=0.93-0.99, *P*=0.003) for each additional portion of fruit or vegetable intakes and by 7% (RR 0.93; CI: 0.89-0.96) for fruit intake. Another meta-analysis of 12 studies showed that individuals consuming 3-5 or >5 servings/day of fruit and vegetable, compared to <3 servings/day, had 7% (CI=0.86-1.00) and 17% (0.77-0.89) lower CHD risk, respectively. In a Swedish study, men with high intakes of dairy fat and daily consumption of fruit and vegetable intake had lower 12 year risk of CHD (OR=0.39, CI=0.21-0.73) (Bhupathiraju and Tucker, 2011).

In addition to absolute quantity, frequency of fruit and vegetable intakes has been associated with lower CVD risk. The prospective epidemiological Study of MI in men, aged 50–59 years from France and Northern Ireland found that frequency of citrus intake, but not other fruits, was associated with a 5 year lower incidence of acute coronary events (tertile 3 vs 1; RR=0.64, CI=0.41-0.99) (Bhupathiraju and Tucker, 2011). In contrast, several studies found no significant protective effects for fruit and vegetables on mortality, although study of adults in Maryland, the Kuopio IHD Risk Factor study among middle-aged Finnish men, and the Adventist Health Study showed protective trends. These studies might have insufficient power, or inadequate ranges of intakes to determine significant effects (Bhupathiraju and Tucker, 2011).

In an analysis of ten trials, six of which examined the effectiveness of increasing fruit and vegetable consumption as a single intervention without the influence of other dietary patterns or other lifestyle modifications in healthy adults and those at high risk of CVD for the prevention of CVD was reviewed; their results showed no strong evidence in favor of the effects of fruit and vegetable provision on CVD risk factors; however, the trials were heterogeneous and short term. Four trials examined increasing fruit and vegetable consumption through dietary advice. These trials provided some evidence of the favorable effects of increased fruit and vegetable consumption on BP and to a lesser extent on LDL-C at six months. Note that that few trials contributed to each analysis and the majority of trials were conducted in developed countries. Considering the

shortage of RCTs that examine solely the effects of increasing fruit and vegetable consumption suggests implementing long-term interventions to determine the sustainability of such behavioral change, and to examine effects on CVD outcome events (Hartley *et al.*, 2013).

The mechanisms by which fruit and vegetables exert their protective effects are not entirely clear but this may be because these plant foods are high in fiber, low in sodium and high in potassium (Sala-Vila *et al.*, 2015); and most likely have components with antioxidant and anti-inflammatory effects. Greater variety in fruit and vegetable intakes is associated with lower circulating concentrations of CRP, a marker of systemic inflammation. Several bioactive components in fruits and vegetables such as carotenoids, vitamin C, fiber, magnesium, and potassium act synergistically or antagonistically to promote a holistic beneficial effect (Bhupathiraju and Tucker, 2011). Grapes and other berries are abundant in flavonoids, which are inversely associated with many risk factors that in turn caused cardiovascular health (Wightman and Heuberger, 2015).

The totality of the evidence supports current dietary guidelines to increase fruit and vegetable consumption; which recommends the consumption of at least five portions of fruit and vegetables per day.

8.2.5 Dairy

Natural dairy products contain relatively high fat, high SFAs and cholesterol. In recent years, several studies have examined the association of dairy foods and CVD risk; most observational studies failed to find an association between the intake of dairy products and increased risk of CVD, CHD, and stroke, regardless of milk fat levels (Berciano and Ordovas, 2014). For example, in a prospective cohort study of Swedish adults, 33,636 women were followed for 11.6 years to examine the association between total, as well as specific, dairy food intakes and incidence of MI. Comparing the highest quintile with the lowest, total dairy food intake was associated with a 23% decreased incidence of MI (HR=0.77 (CI=0.63-0.95, $P<0.05$)) (Sikand *et al.*, 2015). The results of a meta-analysis show that dairy consumption (high vs low intake) may be associated with reduced risk of CVD, CHD and stroke (Alexander *et al.*, 2016). Interestingly, the results indicate that cheese intake lowers LDL-C, compared with butter of equal milk fat content (Berciano and Ordovas, 2014). Among specific dairy food products, a 26% decreased incidence of MI (HR=0.74, CI=0.60-0.91, $P<0.01$) was observed in highest versus lowest quintile of cheese intake, while a 17% reduction in incidence of MI (HR=0.83, CI=0.68-1.01, $P=0.03$) was observed when comparing the highest versus lowest quintile of full fat cheese (Sikand *et al.*, 2015).

Based on meta-analyses, the highest levels of undefined milk consumption may significantly reduce the risk of CVD by a maximum of 16% (Fardet and Boirie, 2014). Results of another meta-analysis suggest a reduction in risk in subjects with the highest dairy consumption relative to those with the lowest intake by 8% for IHD (RR=0.92, CI=0.80-0.99) and by 21% for stroke (RR=0.79, CI=0.68-0.91). Similarly, a dose-response meta-analysis of over 600,000 multi-ethnic adults reported an inverse association between milk intake and CVD risk, with a 6% decreased risk associated with each 200 ml/day of milk consumed. In a prospective cohort study (n=26,445)

examining the association between intake of milk, cheese, cream and butter, and incidence of CVD, total dairy consumption was associated with a 12% decreased risk of CVD (HR=0.88, CI=0.77-1.02, *P*=0.05) (Sikand *et al.*, 2015).

There is an inverse association or no association between high-fat dairy consumption and metabolic health in studies (Berciano and Ordovas, 2014). These inconsistencies between studies as well as within studies between different dietary sources of dairy fat may be due to residual confounding of associated dietary factors. Also consumption of dairy fat of specific foods may have different health effects. The potential mechanisms by which dairy fat may exert beneficial effects on cardio-metabolic risk may be through reducing chronic inflammation and lipid peroxidation.

In particular, dairy fat is a rich source of butyric acid (C4:0), cis- and trans-palmitoleic acid (C16:1), and the branched-chain phytanic acid (C20:0); there are inconclusive findings about their significant effects on end points relevant to chronic disease in the amounts present in dairy fat. Existing evidence suggests that CLA in the amounts and form present in dairy fat is unlikely to have a significant effect on end points relevant to chronic disease; however, there has been much interest in CLA as a potential beneficial fatty acid in dairy fat (Kratz *et al.*, 2013).

Overall, it can be concluded that consumption of dairy products is either protective against CVD or has no adverse effects. Most dietary guidelines recommend the consumption of fat-reduced milk and dairy products as an important part of a healthy, well-balanced diet.

8.2.6 Fast food

There is no agreement on the definition of fast food; however, it is mainly defined in dictionaries and encyclopedias as 'easily prepared processed food served in snack bars and restaurants as a quick meal to be taken away'; industrial foods such as canned foods or snacks may also be considered as fast foods. A sharply growing trend of fast food consumption along with an alarming trend of cardio-metabolic disorders is a major global health problem. Out-of-home meals and fast foods are rich in highly processed meat and refined carbohydrate, sodium, total fat, saturated and trans-fatty acids, cholesterol, and poor in essential nutrients and dietary fibers. Higher consumption of fast foods and higher exposure to multiple sources of accessible, cheap, energy-dense fast foods have also been accompanied with a 56-162% increased risk of CHD mortality. Most fast foods have an extremely high energy density, approximately 158 to 163 kcal per 100 gram of food; a fast food meal typically has an energy density twice that recommended for a healthy diet. High energy density of foods may have adverse effects. High-fat content and inappropriate composition of fatty acids of fast foods is a main dietary risk for chronic disease. Mean total fat percentage of beef hamburgers, chips, chicken hamburgers and hot dogs has been reported to be 35.8±10.7, 35.8±8.7, 23.0±5.1, and 34.0±13.5%, respectively, with 28-52% of total fat estimated as saturated fat. Large portion sizes, high amount of refined carbohydrates, added sugar, and high glycemic load are other characteristics that could explain the threatening properties of fast food meals. The higher content of industrially produced trans-fatty acids in

fast foods is an important component leading to weight gain, abdominal fat accumulation, development of insulin resistance and CVD events. Furthermore, the sodium content of fast foods is often higher than recommended amounts; in some common fast food meals, salt content was reported to range from 4.4 to 9.1 gram per meal; a high-salt diet besides increasing BP, also aggravates insulin resistance and MetS features (Bahadoran *et al.*, 2015). Harmful additives such as nitrites, nitrates, and nitrosamines in processed meats, e.g. sausages, salami, and bacon, are consistently reported to be associated adversely with cardiovascular outcomes and mortality (Sala-Vila *et al.*, 2015).

The prospective approach of TLGS also showed that the risk of MetS in the highest compared with the lowest quartile of fast foods increased by 85% (OR=1.85, CI=1.17-2.95); in this study, the adverse effects of fast food consumption were more pronounced in younger adults (<30 years) (Bahadoran *et al.*, 2013). The rate of accessibility to fast food services has been reported as a risk factor for CVD; risk-adjusted outcomes in regions with high, compared to low accessibility to fast food services, were greater for mortality (OR=2.52, CI=1.54-4.13) and acute coronary hospitalizations (OR=2.62, CI=1.42-3.59) (Bahadoran *et al.*, 2015).

Existing evidence on the association between fried food consumption and the risk of CVD is limited and conflicting; however, Guallar-Castillón and colleagues reported that consumption of fried foods was not associated with risk of CHD after 12 years of follow-up in a prospective study of 40,757 adults, aged 29-69 years, free of CHD; neither was any association found between intakes of fried fish, fried meat, fried potatoes, fried eggs and CHD risk. A case-control study from Costa Rica, including 485 survivors of a first acute MI and 508 controls, found that increasing the frequency of fried food consumption from 4.57 to 9.75 servings/day was not associated with nonfatal acute MI. Data from the Nurses' Health Study, the Health Professional Follow up Study and the INTERHEART study showed that frequent fried food consumption was significantly associated with a higher risk of CAD and acute MI. Results from the Cardiovascular Health Study showed that fried fish consumption is associated with trends toward higher risk of death due to IHD (Gadiraju *et al.*, 2015).

Inconsistencies in results may be due to the quality of fried foods; the fatty acid composition of the final fried product largely depends on multiple factors, including the actual composition of the food being fried, the type of oil used and frying conditions (temperature, duration) (Gadiraju *et al.*, 2015).

Regarding the high prevalence of fast food consumption, food policies with an emphasis on providing healthy foods, and providing nutritional information of fast-foods at restaurants may encourage consumers to order more healthful or lower-calorie foods (Bahadoran *et al.*, 2015).

8.2.7 Coffee

Coffee is one of the most widely consumed beverages in the world. The best characterized compound in coffee is caffeine, which contains chlorogenic acid, flavonoids, melanoidins, and

various lipid-soluble compounds that have antioxidant properties. Caffeine is also found primarily in tea, cocoa products, cola beverages and 'energy' drinks. There is a possible bias in comparing caffeinated and decaffeinated coffee. In the last few years there is extensive data suggesting no harm, and often indicating even a protective association between moderate coffee drinking (3-4 cups/day providing 300-400 mg of caffeine) and CHD morbidity and CVD mortality. However there are no results from RCTs reporting its beneficial effects. Some groups, including people with hypertension, children, adolescents, and the elderly, may be more vulnerable to the adverse effects of caffeine. Furthermore, based on available evidence, pregnant women should limit coffee consumption to 3 cups/day providing no more than 300 mg/day of caffeine (Eilat-Adar *et al.*, 2013).

8.2.8 Tea

Tea has been one of the most popular beverages for 4,000 years and is consumed in different parts of the world as green, black, or Oolong tea. Green and black teas are processed differently during manufacturing. A glass of black tea beverage (190 ml) contains 15-24 mg caffeine. A meta-analysis conducted on 18 studies including 13 on black tea and 5 studies on green tea; for black tea, no significant association with the risk for developing CAD was seen. In a meta-analysis of 194,965 participants in nine studies, individuals consuming ≥3 cups of tea per day had a 21% lower risk of stroke than those consuming <1 cup per day (absolute risk reduction, 0.79, CI=0.73-0.85). In a meta-analysis, black tea consumption increased systolic BP (5.69 mm Hg; CI=1.52-9.86) and diastolic BP (2.56 mm Hg; CI=1.03-4.10), whereas long-term consumption did not seem to affect BP (Eilat-Adar *et al.*, 2013).

A meta-analysis of five studies on green tea showed a significant negative association between green tea consumption and risk of CAD, particularly one cup per day was associated with a 10% reduction in CAD risk (RR=0.90, CI=0.82-0.99) (Sikand *et al.*, 2015). Over 50% of randomized controlled trials have reported the beneficial effects of green tea on CVD risk profiles. In a meta-analysis of 133 trials, green tea did not appear to affect BP, but reduced LDL-C levels (-9 mg/dl; CI=4.6-13.1; 4 studies) (Eilat-Adar *et al.*, 2013).

The cardio protective compounds of green tea include polysaccharides and polyphenols; catechins, EGCG, in particular, exert their effects via multiple mechanisms including antioxidant, anti-hypertensive, anti-inflammatory, anti-proliferative, anti-thrombogenic, hypolipidemic and anti-diabetic effects (Sikand *et al.*, 2015).

Epidemiological, clinical, and experimental evidence supports the role of green tea in preventing CVD.

8.2.9 Chocolate

Cocoa like green tea is rich in polyphenols including catechins, epicatechins, and procyanidins which exert antioxidant and anti-inflammatory effects by scavenging of ROS, Fe^{2+}, and Cu^{+}

chelation, inhibition of key enzymes, activation of nitric oxide, and promoting antioxidant defenses (Eilat-Adar *et al.*, 2013). Chocolate and cocoa are two different products; cocoa is the non-fat component of cocoa liquor that is used in chocolate making or as cocoa powder for cooking and drinks, while the major components of chocolate are fat and sugar, which have high caloric content (Sikand *et al.*, 2015).

Evidence on the cardio-preventive properties of polyphenol-rich cocoa products has been reported in meta-analyses. Results of a meta-analysis of seven observational studies showed a positive association between higher levels of chocolate consumption and the risk of CVD; the highest levels of chocolate consumption were associated with an adjusted lower risk for CVD (RR=0.63; CI=0.44-0.90) and a 29% reduced risk of stroke, compared to lowest consumption levels. Consumption of cacao and green tea improved endothelial function in several studies, an effect that appears to be at least partly mediated by their flavan-3-ol components (catechins and EGCG) (Landberg *et al.*, 2012). Several studies indicated the inability to distinguish between milk and dark chocolate as a limitation.

8.2.10 Fish

Seafood is the main source of LCn3PUFA, viz. EPA, C20:5n3 and DHA, C22:6n3 acids. The effects of LCn3PUFA on plasma lipids and vascular function are only seen at pharmacological doses (>3 g/day); however protection against sudden cardiac death due to its antiarrhythmic effect can be observed at regular intakes of 250 mg/day; this amount is easily achievable by meeting the American Heart Association recommendation to consume at least two servings/week of fish, preferably fatty fish. A meta-analysis of 17 prospective studies in cohorts without prior CAD reported that, compared to individuals with the lowest consumption, those who consumed fish 1/week showed a 16% (CI=5-25) lower risk of fatal CHD. In a dose–response analysis of data from eight prospective studies each additional 100-g serving of fish/week was associated with a 5% reduced risk of acute coronary syndrome. The results of several RCTs showed no cardio-protective effects of LCn3PUFA since 2010; this may be due to interplay of bioactive compounds found in the fish, either beneficial (LCn3PUFA, iodine, taurine, peptides) or harmful (polychlorinated biphenyls and heavy metals) that need to be focused on in research. In addition methodological issues in these trials, including the length of intervention, background diet, and drug use make it impossible to draw firm conclusions (Sala-Vila *et al.*, 2015).

Collectively, the epidemiologic research to date clearly demonstrates a beneficial relationship between fish or fish oil intake and CHD mortality, including CHD death and sudden death.

8.2.11 Meat

Recent evidence from epidemiological studies shows that the consumption of processed meat consistently relates to adverse cardiovascular outcomes and mortality; however, there are neutral or weak direct associations between red unprocessed meat consumption and the risk of CAD, stroke, diabetes, or cardiovascular and all-cause mortality. The lack of harmful effects

of unprocessed meat may be due to its high content of SFA, regarding that intake of SFAs is considered to be neutral for CVD risk in recent evidences. Besides a null effect on cardiovascular health outcomes, (lean) red meat has little effect on lipid profiles, BP, or body weight. As a whole, current evidence suggests that moderate consumption of lean red meat is not harmful for CVD or diabetes risk (Sala-Vila *et al.*, 2015).

8.3 Dietary patterns

Meals consist of multiple foods in which the nutrients of all foods have combined effects, making it difficult to investigate the separate effects of individual foods or nutrients simultaneously. Dietary pattern analysis focuses on the entire diet rather than on just one food or nutrient; this analysis provides an additional aspect for examining the relationship between diet and disease risk and suggests a more comprehensive approach to disease prevention or treatment. There are two different ways to define dietary patterns: 'a priori', a score-based approach (dietary indices), based on published dietary recommendations and focusing on the construction of patterns that reflect hypothesis-oriented combinations of foods and nutrients. The interpretation of this approach is easy because dietary behaviors summarize into a single score. Two common scores that have been used to examine risk of CVD and dietary pattern are the MD and the DASH diet.

The second approach to define dietary patterns is 'a posteriori', which builds on exploratory statistical methods and uses the documented dietary data in order to extract dietary patterns. The two most commonly used approaches include PCA and cluster analysis. PCA is a form of factor analysis that reduces data into patterns based on inter-correlations between data. Cluster analysis maximally separates individuals into different food groups to identify those consumed together by the same subsets of individuals.

Both ways have beneficial and adverse effects; 'a priori' methods or predefined diet quality indices determine adherence to a desirable pattern, based on current nutritional knowledge. On the contrary, focusing on 'a posteriori' dietary patterns prevent increasing heterogeneity; using dietary data in-hand might be debatable in relating diet and disease, since the extracted dietary patterns may have little relation to morbidity and mortality when nutrients or foods relevant to the etiology of diseases are not included in each pattern (Rodriguez-Monforte *et al.*, 2015).

There are many ways of describing dietary patterns according to key characteristics or culture, such as Mediterranean (characterizing foods from that region), prudent (implying wise, judicious food choices), and western (reflecting certain aspects of food industrialization in Western culture) (Jacobs and Tapsell, 2015). The following section reconsiders diet, focusing on dietary patterns and long term CVD outcomes.

8.3.1 Mediterranean dietary pattern

The MD, first described in Crete and Italy, is identified by a relatively high fat intake (40%–50% of total daily calories), of which SFA comprises ≤8% and MUFA 15%-25% of calories. It is specified by a high omega-3 fatty acid intake from fish and plant sources and a low omega-6:omega-3 ratio of 2:1-1:1 (Eilat-Adar *et al.*, 2013). Also it has been characterized as: (1) daily consumption of unrefined cereals and cereal products, vegetables (2-3 servings), fruit (4-6 servings), olive oil, dairy products (1 or 2 servings), and red or white wine (1-2 wine glasses); (2) weekly consumption of potatoes (4-5 servings), fish (4-5 servings), olives, pulses, and nuts (more than 4 servings), eggs and sweets (1-3 servings); and (3) monthly consumption of red meat and meat products (4-5 servings) (Bhupathiraju and Tucker, 2011).

Several indices have been developed to describe the MD and these have been used frequently in relation to CHD events and CHD mortality. Consistent epidemiological and clinical trial evidence supports the role of the MD in the prevention of CHD. A meta-analysis of 8 prospective studies, representing 514,816 participants and 33,576 deaths, showed that a two-point increase in the adherence score was associated with a 9% lower risk of CVD mortality (pooled RR=0.91; CI=0.87-0.95) (Bhupathiraju and Tucker, 2011). When 7 new studies were added to the previous meta-analysis and overlapping data were excluded, a new random-effect meta-analysis with 16 estimates showed that each 2-point increment in a 0- to 9-point score of adherence to the MD was associated with a 10% relative reduction in CVD (risk ratio 0.90; CI=0.86-0.94). After removing studies that only assessed fatal CVD, the inverse association became stronger (risk ratio 0.87; CI=0.85-0.90), with no evidence of heterogeneity. A systematic review of 32 candidate dietary factors associated with CHD ranked the MD first as the most likely dietary model to provide causal protection (Ros *et al.*, 2014; Sofi *et al.*, 2013, 2014).

A review on 26 studies which evaluated the relationship between the adherence to the MD and CVD showed that the MD is a useful tool to reduce the risk of CVD (D'Alessandro and De Pergola, 2015).

The Lyon Diet Heart Study reported the benefits of a MD on the secondary prevention of CVD in 605 volunteers who had suffered a first MI (Fito and Konstantinidou, 2016). The PREDIMED, a multicenter, randomized, nutritional intervention trial aimed at assessing the long-term effects of the MD on incident CVD in men and women at high cardiovascular risk and was performed in Spain from 2003 to 2011(Ros *et al.*, 2014).

Another review on 11 RCTs (15 papers) assessed the effects of dietary advice regarding MD on healthy adults or people at increased risk of CVD to prevent the occurrence of CVD and to reduce the risk factors associated with it. Small reductions in total cholesterol levels as well as in the harmful LDL-C concentrations were found. The reductions in total cholesterol were greater in the studies that themselves provided a MD (Rees *et al.*, 2013).

A meta-analysis of 50 prospective studies and RCTs suggested that adherence to the MD was associated with a 50% reduction of MetS (Salas-Salvado *et al.*, 2016); it furthermore, had a beneficial effect on individual components of MetS, including waist circumference (-0.42 cm, CI=-0.082, -0.02), HDL-C (1.17 mg/dl, CI: 0.38, 1.96), TG (-6.14 mg/dl, CI=-10.35, -1.93), systolic (-2.35 mm Hg, CI=-3.51, -1.18) and diastolic BP (-1.58 mm Hg, CI=-2.02, -1.13), and FBG (-3.89 mg/dl, CI=-5.84, -1.95) (Shen *et al.*, 2015). Intervention studies show that a MD may reduce CIMT progression, especially in those with higher CIMT (Petersen *et al.*, 2014).

Investigating diet modulation of the genetic variation has led to evidence regarding the effectiveness of personalized nutrition as a more adequate tool for prevention of chronic diseases than the traditional one-size-fits all recommendations. Our genetic predisposition is responsible for a percentage of CVD risk that varies among people. Genetic predisposition could explain a great part of the different responses observed in individuals after the same dietary interventions. Nutrigenetic studies could help health professionals further individualize their recommendations. The results of the PREDIMED study identified the notion that individual genetic predisposition toward CVD risk could be influenced by dietary components, mainly by stricter adherence to the MD, results demonstrating interactions between a MD and cyclooxygenase-2, interleukin-6, apolipoprotein A2, cholesteryl ester transfer protein plasma, and transcription factor 7-like 2 gene polymorphisms in relation to CVD risk (Fito and Konstantinidou, 2016; Ros *et al.*, 2014). Lopez-Guimera *et al.* (2014) analyzed the effect interaction of emotional eating behavior and the same CLOCK 3111 T/C polymorphism on the effectiveness of a weight-loss program. In a 30-week follow-up in a Mediterranean population, they reported that the CLOCK 3111 T/C SNP interacted with emotional eating behavior to modulate total weight loss (Fito and Konstantinidou, 2016). As a whole, genetic predisposition can be useful in the management and control of CVD risk factors and their subsequent consequences in its development. Moreover, the MD is increasingly being recommended in non-Mediterranean countries. Quantity of foods forms the basis for most a priori Mediterranean dietary pattern scoring criteria since it appears to impact health outcomes. Meta-analytic results show that individuals with high intakes of vegetables, fruits/nuts, legumes, cereals and fish, high MUFA:SFA ratio and lower intakes of dairy and meat/poultry and consuming moderate amounts of ethanol have better cardiovascular and cognitive health (Davis *et al.*, 2015).

However, Adherence to the MD, according to MD score, could not predict MetS components and MetS incidence after 3 years of follow-up in Iranian adult populations (Mirmiran *et al.*, 2015). More studies in non-Mediterranean countries are needed to investigate the applicability of the MD and its benefits to the prevention of metabolic abnormalities.

The mentioned evidences are also corroborated by the biological plausibility of several mechanisms in explaining the beneficial effect of the MD, as a whole, and of its individual components on cardiovascular health; the higher dietary intakes of inorganic nitrates and MUFA, as well as ω-3 PUFAs and olive oil polyphenols substantially contribute to the protection provided by the traditional MD against chronic degenerative diseases, including cancer and CVDs. MD polyphenols possess anti-microbial, anti-inflammatory, anti-angiogenic and anti-proliferative

activity, improve vascular function, and reduce intermediate clinical markers of CVDs (Casas *et al.*, 2014; Scoditti *et al.*, 2014; Smidowicz and Regula, 2015).

Adoption of a Mediterranean-style diet found to be important for the primary and secondary prevention of MetS and its individual components. Moreover, it has been proven to reduce CVD incidence, reoccurrence, and mortality (Shen *et al.*, 2015).

Some aspects and components of a Mediterranean-style diet are already included in scientific and clinical guidelines for the prevention of CVD; e.g. guidelines on consumption of at least five portions of fruit and vegetables per day (Department of Health 2010) (Rees *et al.*, 2013).

8.3.2 Dietary approaches to stop hypertension dietary pattern

This pattern is rich in fruits, vegetables, and low-fat dairy products, also includes whole grains, poultry, fish, nuts, and limits SFA, red meat, sweets, and sugar containing beverages. Several prospective cohort studies have examined associations between adherence to a DASH dietary pattern and BP or incident CVD events (Bhupathiraju and Tucker, 2011). This diet appears to have beneficial effects on several CVD risk factors, including total cholesterol, LDL-C, inflammation, and homocysteine (Bhupathiraju and Tucker, 2011). The PREMIER trial combined the DASH diet with a lifestyle program to reduce overweight, increase physical activity, and restrict sodium and alcohol intake; systolic and diastolic BP was reduced by 14.2 and 7.4 mm Hg, respectively in subjects with hypertension. A decrease in BP was observed in normotensive participants as well. The theoretical decrease in the Framingham risk score for CHD was 12% greater when adding lifestyle changes to the DASH diet (Eilat-Adar *et al.*, 2013).

Consumption of a healthy dietary pattern including MD, Nordic diet, Tibetan diet, and the DASH diet was associated with significant reductions in CRP as an inflammatory biomarker (weighted mean difference, -0.75 [-1.16, -0.35]; P=0.0003). Non-significant changes were found for all other biomarkers (Neale *et al.*, 2016).

Epidemiological evidence shows that increased intakes of fruits, whole grains and soluble fiber and lower consumption of SFA in favor of PUFA are associated with lower CIMT. Observational data suggest that CIMT may be lower when >93 g/day of fruit, >0.79 gram/day of whole grains and >25 g/day of fiber, predominantly in the soluble form, is consumed. In addition, SFA is positively associated with CIMT progression, and for every 10 g/day increase in SFA, CIMT is 0.03 mm greater. There is some evidence that olive oil is inversely associated with CIMT; a beneficial effect has been observed when consumption was >34 g/day (Petersen *et al.*, 2014).

Results of a systematic review of 20 RCTs that investigated the effect of dietary patterns on BP, a major risk factor for developing CVD, in adults aged >19 years, but results of the subgroup analysis suggested that only the DASH, MD, and Nordic diets significantly reduced 4.25 mm Hg (CI=25.37, 23.13) in systolic BP and 2.27 mm Hg (CI=23.07, 21.48) in diastolic BP (Ndanuko, 2016).

The association of adherence to DASH diet with development of MetS in Tehranian children and adolescents was assessed and adherence to recommendations of the DASH eating pattern reduced prevalent MetS incidence. The OR (95% CI) of developing MetS in the highest, compared with the lowest quartile of DASH score was 0.36 (0.14, 0.94) with a linear decreasing trend (*P* for trend=0.02). Also, incidence of hypertension, high FBG, and abdominal obesity decreased with higher adherence to DASH diet (Asghari *et al.*, 2016).

The Nordic diet, consumed in Nordic countries, includes foods of Nordic origin such as whole grains, rapeseed oil, berries, fruit, vegetable, fish, nuts, and low-fat dairy products; one of its characteristics is that it is rich in berries. Animal studies have shown that Nordic wild blueberries lead to a reduction in BP (Ndanuko, 2016).

As a whole, evidence for the protective role of the DASH dietary pattern in prevention of CVD is strong. It is noteworthy that the DASH dietary pattern is consistent with current USA dietary guidelines for CVD risk reduction.

8.3.3 Priori dietary patterns

The HEI determines adherence of diets according to the major recommendations of the Dietary Guidelines for Americans and the food guide pyramid. The first edition of HEI has 10 components (grains, vegetable, fruits, milk, meat, total fat, saturated fat, cholesterol, sodium, and variety); the minimum and maximum scores of each component are between 0 and 10 points (overall scoring range = 0 to 100) (Schwingshackl and Hoffmann, 2015).

The HEI-2005 has 12 components: total fruit; whole fruit; total vegetables; dark green and orange vegetables and legumes; total grains; whole grains; milk; meat and beans; oils; saturated fat; sodium; and calories from solid fats, alcoholic beverages (i.e. beer, wine, and distilled spirits) and added sugars (overall scoring range = 0 to 100) (Schwingshackl and Hoffmann, 2015).

The HEI-2010 has 12 components: total fruit; whole fruit; total vegetables; greens and beans; whole grains; dairy; total protein foods; seafood and plant proteins; fatty acids (PUFA+MUFA-to-SFA ratio); refined grains; sodium; and empty calories from solid fats, alcoholic beverages (i.e. beer, wine, and distilled spirits) and added sugars (overall scoring range=0 to 100) (Schwingshackl and Hoffmann, 2015).

AHEI has nine components (vegetables, fruit, nuts and soy protein, ratio of white to red meat, cereal fiber, trans fat, PUFA-to-SFA ratio, duration of multivitamin use, and alcohol). The overall scoring range was 2.5 to 87.5 (Schwingshackl and Hoffmann, 2015).

AHEI-2010 has eleven components: vegetables, fruit, whole grains, sugar-sweetened beverages and fruit juice, nuts and legumes, red/processed meat, trans fat, LCn3PUFA (EPA and DHA), PUFA, sodium, and alcohol (overall scoring range=0 to 110) (Schwingshackl and Hoffmann, 2015).

The diet quality index is a measure of adherence to 8 food groups and nutrient-based recommendations from the committee on diet and health of the National Research Council Food and Nutrition Board (Bhupathiraju and Tucker, 2011).

The highest association of diet quality as assessed by the HEI, AHEI, or DASH score was significantly associated with a reduced risk of all-cause mortality (RR=0.78; CI=0.76-0.80; $P<0.00001$), cardiovascular mortality or incidence (RR=0.78; CI=0.75-0.81; $P<0.00001$), using a random effects model. Subgroup analysis suggested that all diets that scored high on the included dietary indices (HEI, AHEI, and DASH score) were associated with a reduced risk of CVD (Schwingshackl and Hoffmann, 2015).

In the 3 dietary pattern methods project, cohorts of women and the 2 cohorts of men, consistent inverse associations of each of the 4 measures of diet quality, as characterized by HEI-2010, AHEI-2010, alternate MD, and DASH scores, with all-cause, CVD, and cancer mortality. In women, high diet quality was associated with an 18–26% lower risk of all-cause mortality, a 19-28% lower risk of CVD mortality. In men, high diet quality was associated with a 17-25% lower risk of all-cause mortality, a 14-26% lower risk of CVD mortality (Liese *et al.*, 2015).

In Tehranian adolescents, adherence to multiple indices based on dietary guidelines recommendations were examined to determine which index can better demonstrate the risk of obesity associated phenotypes. Our findings indicate that participants who had high adherence with HEI-2010 had a lower risk of general and central obesity (Mohseni-Takalloo *et al.*, 2016).

This type of congruence of observational research and previous RCTs findings can be considered as the basis for some of the strongest recommendations in an evidence-based review such as the one conducted for the establishing Dietary Guidelines (Liese *et al.*, 2015).

8.3.4 Posteriori dietary patterns

In most evidence available on dietary patterns and CHD risk, healthy/prudent dietary patterns were characterized by high consumption of vegetables, fruits, whole grains, olive oil, fish, soy, poultry and low fat dairy. Some studies labeled it as 'prudent', 'simplified food', 'low SFA and high fruit and vegetable', 'DASH', 'Mediterranean', 'vegetable and fruit', and 'Japanese pattern'. These dietary patterns were extracted by PCA and/or factor analysis. Strong evidence was found a decreased risk of CHD in the highest compared with the lowest categories of healthy/prudent dietary patterns (OR=0.67; CI=0.60-0.75; $P<0.00001$). Unhealthy/Western-type dietary patterns were characterized by high consumption of red and/or processed meat, refined grains, sweets, high-fat dairy products, butter, potatoes, and low intakes of fruit and vegetables. Some studies termed this pattern as 'Western' or 'Animal foods'. There was evidence of an increased risk of CHD in the highest compared with the lowest categories of unhealthy/Western-type dietary patterns (OR=1.45; CI=1.05-2.01; $P=0.02$), when all studies were combined in the random-effects model (Zhang *et al.*, 2015).

Another meta-analysis estimating the association between empirically derived dietary patterns and CVD, showed that in a comparison of the highest to the lowest category of prudent/healthy dietary patterns, the pooled RR for CVD, CHD and stroke was 0.69 (CI=0.60-0.78), 0.83 (CI=0.75-0.92) and 0.86 (CI=0.74-1.01), respectively in cohort studies. In case–control studies, the pooled RR for CHD was 0.71 (CI=0.63-0.80). The pooled RR for CVD, CHD and stroke in a comparison of the highest to the lowest category of western/unhealthy dietary patterns in cohort studies was 1.14 (CI=0.92-1.42), 1.03 (CI=0.90-1.17) and 1.05 (CI=0.91-1.22), respectively. The pooled RR for CHD in case–control studies was 1.61 (CI=1.17-2.21), with statistically significant heterogeneity between studies.

Evidence on Asian countries extracted dietary patterns different from those of Europe or America. Studies from China or Japan defined dietary patterns as normal for the general population; for example, Chen *et al.* (2013) explained a pattern named 'gourd and root vegetable' in China and Shimazu *et al.* (2007) extracted a Japanese dietary pattern defined by high intake of soyabean products, fish, seaweed, vegetable and green tea (Rodriguez-Monforte *et al.*, 2015).

Effects of the interaction between MC4R polymorphisms 12970134 and dietary factors on MetS were investigated in subjects of the nested case-control study in the framework of TLGS. Two dietary patterns were extracted. Among A allele carriers, being in the highest quartiles of western dietary pattern scores had an increased risk of MetS, compared to those in the lowest quartile (P trend = 0.007) (Koochakpoor *et al.*, 2016).

Taken together, the posteriori approach has been widely used in epidemiological studies to evaluate the relationship between dietary patterns and several outcomes such as cardiovascular incidence and mortality, risk factors for CVD; it could be a useful tool to better understand the effectiveness of dietary patterns in managing CVD risk since it may easily be translated into public health action for primary prevention of CHD. Healthy dietary patterns are associated with reduced risk of CVD and its risk factors, while unhealthy/Western-type patterns are associated with an increased risk of CVD.

8.3 Conclusions

Based on results of observational studies coupled with the RCT findings, we assert that certain dietary patterns can prevent or delay CVD. A modest long-term consumption of plant centered dietary patterns rich in vegetables, fruits, beans, lentils, nuts, seeds, and whole grains and at the same time minimizing intake of nutritionally poor plant foods such as sugar, refined grains and highly processed and very salty foods could prevent CVD, which supports recently published guidelines. These effects are attributed to the functional or harmful components of different foods on risk markers related to the initiation and progression of cardio-metabolic diseases.

Acknowledgements

The authors would like to acknowledge Iran Science Elites Federation for their enthusiastic support. We would also like to acknowledge Mrs. Nilufar Shiva for critical editing of English grammar and syntax of this manuscript.

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9. Estimating changes in cardiovascular disease burden through modelling studies

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Abstract

Cardiovascular diseases (CVD) comprise disease of the heart, vascular disease of the brain and diseases of the blood vessels. The predominant risk factors in promoting atherosclerosis are tobacco use, physical inactivity, unhealthy diet and harmful use of alcohol, high blood pressure, high blood sugar, high blood lipids, being overweight and obesity, amongst others such as poverty and low educational status, advancing age, gender, genetic disposition and psychological factors. Modelling studies are a different group of studies that use estimates of the effects of risk factors and treatment to predict, using modelling, changes in CVD mortality. Although mortality models have been used for a long time, more sophisticated methods have been developed over the last 15 years, and there are now different approaches used to forecasting mortality. The aim of this chapter is to present CVD trends in the past and future, to explore a potential mortality reduction using a modelling approach and to describe some ways to tackle the risk factors for CVD development.

Keywords: coronary heart disease, stroke, risk factors

Ronald Ross Watson and Sherma Zibadi (eds.) Handbook of nutrition and heart health
Human Health Handbooks no. 14 - DOI 10.3920/978-90-8686-853-7_9, © Wageningen Academic Publishers 2017

Key facts

- Cardiovascular disease (CVD) is the leader in noncommunicable disease (NCD) mortality, responsible for around 46% of all NCD deaths.
- Around 32% of all deaths in 2012 was related to CVD, claiming 17.5 million lives. 7.4 million were due to ischaemic heart disease (IHD) and 6.7 million to strokes.
- The top three causes of *Years of Life Lost* (YLLs) due to premature mortality are IHD, stroke and lower respiratory infections.
- In 2012, coronary heart disease (CHD) and strokes were the main causes of CVD, 46% and 26% respectively, in the UK.
- In the USA, between 2000 and 2011, the annual rates of CVD, IHD and strokes declined by 3.79%, 3.69% and 4.53% respectively.

Summary points

- The future burden of CVD mortality on the USA may have been overestimated in some modelling studies using conventional methods.
- CVD mortality in high-income countries and in some medium-high-income countries in Latin American has decreased in recent decades.
- CHD mortality rates for each gender and at all ages follow a continuously downward trend, which slows by 2030 in England and Wales.
- In nine European countries, future policy scenarios estimate 10.8%, 20.7% and 29.1% fewer CHD deaths in 2020 under conservative, intermediate and optimistic scenarios, respectively.
- In Turkey, the reductions in CHD deaths by 2025 should be approximately 16% and 30% for modest and ideal scenarios, respectively.
- The Global Action Plan has targets in relation to risk factors for CVD for the period 2013-2020, focusing on a 25% reduction in the risk of premature mortality from CVD, cancer, diabetes or chronic respiratory diseases.

Abbreviations

AHA	American Heart Association
BAPC	Bayesian age-period-cohort
BMI	Body mass index
BP	Blood pressure
CHD	Coronary heart disease
CVD	Cardiovascular disease
DPPs	Deaths prevented or postponed
F&Vs	Fruits and vegetables
HD	Heart disease
IHD	Ischaemic heart disease
LCF	Living Cost and Food Survey
MONICA	Multinational Monitoring of Trends and Determinants in Cardiovascular Disease
NCD	Noncommunicable disease
POP	Point-of-purchase
SDI	Socio-demographic index
TC	Total cholesterol
YLLs	Years of life lost
WHO	World Health Organization

9.1 Introduction

CVDs comprise disease of the heart, vascular disease of the brain and diseases of blood vessels. CVDs can be divided as due to atherosclerosis and others. Within the atherosclerosis group are IHD or coronary artery disease (e.g. heart attack), cerebrovascular disease (e.g. stroke) and diseases of the aorta and arteries, including hypertension and peripheral vascular disease. Other types of CVDs are congenital heart disease, rheumatic heart disease, cardiomyopathies and cardiac arrhythmias (Mendis *et al.*, 2011).

The risk factors for promoting atherosclerosis include: behavioral risks, such as tobacco use, physical inactivity, unhealthy diet and harmful use of alcohol; metabolic risks, such as high blood pressure (hypertension), high blood sugar (diabetes), high blood lipids (e.g. cholesterol), being overweight and obesity; and others, including poverty and low educational status, advancing age, gender, genetic disposition and psychological factors (e.g. stress and depression) (Mendis *et al.*, 2011; Tzoulaki *et al.*, 2016).

In China, Li *et al.* (2016) observed that high blood pressure, high low-density lipoprotein cholesterol, high blood glucose and high BMI were the main metabolic risk factors associated with CHD and strokes. Smoking, high sodium intake and physical inactivity were also associated with CVD events in the Chinese population. Critchley *et al.* (2004) tried to explain, using a model, as to how CHD mortality in Beijing was increasing between 1984 and 1999. The authors

also observed that TC levels, diabetes and obesity were increasing in the population, although blood pressure decreased slightly. 77% of additional deaths were related to high cholesterol, 19% to diabetes, 4% to BMI and 1% to smoking.

Li *et al.* (2016), using a random effect model, projected values for the period 2012 to 2031 in relation to some risk factors and CVD in China. If the increase in BMI, decline in physical activity and increase in consumption of unhealthy food all persist, additional CVD events will be occurring associated with these risk factors over the following 20 years. On the other hand, reduced rates of smoking and increased consumption of dietary fiber, fruit, nuts and omega-3 fatty acids may attenuate the increase in CVD. Some risk factors, such as tobacco consumption, have decreased, but others, such as obesity, have increased (Wang *et al.*, 2016). Furthermore, updated data in China shows a clear trend in decreasing risks of CVD across all age groups since 2010 (Wang *et al.*, 2016).

In Finland, there was a decline in CHD mortality based on the decrease in some risk factors. This was observed in a study developed between 1972 and 2007. The reduced risks of diastolic blood pressure, cholesterol levels and smoking combined were responsible for a 60% reduction in CHD mortality. The cholesterol lowering effect was mainly related to a dietary awareness of the population, reducing the consumption of saturated fats during this period (Vartiainen *et al.*, 2010). However, one of the great problems nowadays in the world is increasing obesity, probably related not only to energy increases in food intake, but also the reduction of physical activity.

NCDs are responsible for 38 million deaths per year accounting for 68% of all causes of deaths worldwide in 2012 (WHO, 2014). Most deaths by the leading NCDs have increased between 1990 and 2013, but age-standardized mortality rates have fallen (Wang *et al.*, 2016). CVD is the leader of NCD mortality, accounting for about 46% of NCDs deaths and about 32% of all deaths, claiming 17.5 million lives; 7.4 million due to IHD and 6.7 million to strokes (WHO, 2014). Between 1990 and 2013, age-standardized death rates from NCDs fell by 18.6%; a fall of 22% for cardiovascular and circulatory diseases. Global age-standardized death rates have fallen by more than one-fifth for IHD (-22.3%) and strokes (-22.5%) (Vos *et al.*, 2015).

A recent analysis of the Global Burden Disease Study 2015 showed that the top three causes of YLLs due to premature mortality are IHD, stroke and lower respiratory infections. There were reductions in age-standardized rates between 2005 and 2015, but minimal change in rankings. Age-standardized mortality rates for IHD decreased by 12.8% between 2005 and 2015 and strokes decreased by 21%, both driving the reduction in 15.6% in CVD. IHD and strokes were responsible for 15.2 million deaths in 2015, equating to 85.1% of all deaths provoked by CVD (Wang *et al.*, 2016).

According to the sustainable development goals, a target has been set to reduce premature mortality from NCDs by one third, through prevention and treatment as well as promoting mental health and well-being (WHO, 2016a).

In the USA, Sidney *et al.* (2016) reported that the decline in CVD, HD and stroke mortality decelerated dramatically between 2011 and 2014. From 2000 to 2014, the rate of decline was observed to be 35.6, 35.2 and 40.1% for CVD, HD and strokes, respectively. The annual decline rates from 2000 to 2011 were 3.79% (CVD), 3.69% (HD) and 4.53% (stroke). The rates for 2011-2014 were 0.65, 0.76 and 0.37%, respectively.

In the UK, CVD continue to be the most prevalent cause of death amongst women (28% of all deaths), however, for men, cancer (32%) has overtaken CVD (29%) to become the major cause of death. In 2012, CHD and strokes were the main causes of CVD, 46 and 26%, respectively (Bhatnagar *et al.*, 2015).

In the eastern Mediterranean, comprised of 22 countries, Mokdad *et al.* (2016) compared the leading causes of death between 1990 and 2013. In 2013, IHD was the leading cause with 90.3 deaths per 100,000 people, which has increased by 17.2% since 1990. There has been a change in the causes of death in this region, from communicable diseases to non-communicable. High blood pressure was the leading risk factor for disability-adjusted life-years in 2013.

According to Saidi *et al.* (2013), in Tunisia, a North African country, between 1997 and 2009, the age specific CHD mortality rate increased by 11.8% in men and 23.8% in women. Rises in cholesterol and systolic BP were responsible for 51% and 31% respectively of CHD mortality among men. The rise in BMI and diabetes accounted for 26% of the increase in CHD deaths, highlighting the importance of prevention strategies.

Rastam *et al.* (2012) observed CHD mortality trends in Syria between 1996 and 2006, and it increased by 64%, resulting in 6,370 additional CHD deaths in 2006 compared with 1996. Changes in risks factors were responsible for 80.7% of these deaths. Approximately 52% were due to increases in systolic BP, 19% to increases in cholesterol, 15% to increases in diabetes, 9% to increases in BMI and 4% to increases in smoking. Drug treatment was responsible for a reduction of 34% in overall CHD.

CVD mortality in high-income countries and in some medium-high-income countries in Latin American has decreased in recent decades and some risk factors have decreased (blood pressure, serum cholesterol and smoking) in some countries and others have increased (obesity and smoking) in some countries (Ezzati *et al.*, 2015; Tzoulaki *et al.*, 2016). Under current trends the high-income countries are projected to have a 29% reduction in premature CVD mortality, although the number of deaths as a whole is projected to rise by 0.2 million between 2010 and 2025 (Tzoulaki *et al.* 2016). According to recent analysis by Global Burden Disease 2015, the YLLs rates for IHD were the lowest in countries with the lowest socio-demographic indices, however for strokes and overall CVD, YLLs rates decrease gradually at higher levels of SDI and then fall sharply for the highest SDI populations. It is theorized that mid-sociodemographic people survive long enough to develop IHD, but do not have access to treatment (Wang *et al.*, 2016).

Contrary to what has been observed in developed and developing countries, where CVD deaths has declined over the years, in sub-Saharan Africa this trend has not been observed as demonstrated by (Mensah *et al.* 2015). There have been more deaths amongst women than men and more deaths from strokes than IHD (Mensah *et al.*, 2015).

In order to evaluate trends in CHD and stroke mortality and morbidity, trends in known risks factors in different countries over ten years were analyzed by the MONICA study, and coordinated by the WHO (Kuulasmaa and Tolonen 2016). The MONICA project monitored classic risks factors for CHD in 38 populations from 21 countries from the mid-1980s to the mid-1990s. Smoking rates decreased in most populations, mean blood pressures, cholesterol concentrations and body mass indices increased, while overall coronary rates decreased (Kuulasmaa *et al.*, 2000).

What about the future trends? Pearson-Stuttard *et al.* (2016) modelled the CVD mortality in USA using scenarios, firstly based on constant age-period-cohort effects (conventional) and secondly using projections incorporating expected trends in age-period-cohort effects (trend-based). The conventional model projected increases from 2012 to 2030 in total USA CHD and stroke deaths by approximately 18% and 50% respectively, due to population growth and aging. In comparison, the decline in total USA CHD was 27% using the 'trend-based' model. For strokes, the decline was non-significant. The 'trend-based' model suggests that the potential growing burden of projected population growth and demographic changes could be tackled with a rapid decline in CVD mortality rates. The model also suggests that the future burden of CVD mortality in the USA may have been overestimated by conventional methods.

In the same way, Guzman-Castillo *et al.* (2014) predicted a decline in CHD mortality in England and Wales, by comparing different forecasting methods (conventional, Lee-Carter model and BAPC model). Basically, the differences between these methods is that, in the first one, the forecast uses the assumption that the mortality rates hold constant into the future, based on the first year of the forecast period. However, using the Lee-Carter and BAPC models, it was assumed that the declines in CHD mortality rates would continue.

According to the BAPC model (it had better predictive performance), CHD mortality rates for each gender and across all age groups follow a continuously downward trend, but decreasing by 2030. The future decline is faster at ages 75-84 and older, especially in males, nevertheless, for the middle age groups (45-54, 55-64 and 65-74) the model predicts a clear slowing in mortality decline. Using the conventional model, due to population aging, which increases the overall number of elderly people, the total number of CHD deaths will increase by 62%, 67% for men and 54% for women. However, if the declines persist, under the BAPC model, it was suggested that the total number of deaths would decrease by 56% (-49% for men and -66% for women) (Guzman-Castillo *et al.*, 2014).

9.2 Exploring potential mortality reduction using modelling approaches

Modelling studies are a different group of studies which use estimates of the effects of risk factors and treatment to predict, using modelling, changes in CVD mortality (Ezzati *et al.*, 2015). A model is, 'a logical mathematical framework that permits the integration of facts and values to produce outcomes of interest to clinicians and decision makers' or, alternatively: 'an analytical methodology that accounts for events over time and across populations based on data drawn from primary or secondary sources'(Weinstein *et al.*, 2003).

To understand modelling, it is important to understand the concept of causal attribution of health outcomes or states that can be traditionally attributed to categorical attribution or counterfactual analysis. In categorical attribution, an event such as death is attributed to a single cause (such as a disease or risk factor) according to a set of rules. The categorical attribution ignores the fact that many diseases have multiple causes. In the counterfactual analysis the contribution of one or a group of risk factors to disease or mortality is estimated by comparison of the current or future disease burden with the magnitude that would be expected in some alternative hypothetical scenario (referred to as the counterfactual) (Mathers *et al.*, 2002). Using the counterfactual analysis there is the advantage of providing potential gains of view on population health by consistently reducing the risk of exposure to risk factors (Ezzati *et al.*, 2002).

Counterfactual analysis of summary measures may be used for the assessment of specific policies or actions and more general assessments of the contribution of diseases, injuries or risk factors. In addition, in intervention analysis, the change in a summary measure, resulting from the application of a specific intervention, may be estimated (Mathers *et al.*, 2002).

Although mortality models have been used for a long time, over the last 15 years more sophisticated methods have been developed, according to (Booth and Tickle, 2008). Some recent modelling studies and their implications for CVD mortality are presented below.

A reduction in CHD mortality might occur if some changes in risk factors were possible in the future. On this perspective, O'Flaherty *et al.* (2016) proposed three scenarios to attack dietary, smoking and physical inactive people in nine European countries to predict the impact of future policy scenarios. They are: a conservative scenario (S1) (10% decrease in current salt intake and a 5% decrease in the prevalence of smokers and physically inactive people), an optimistic scenario (S3) (a salt intake reduction of 30%, a 15% decrease in the prevalence of smoking and a 15% decline in physical inactivity, and an absolute decrease of 5% in energy from saturated fats) and an intermediate and plausible scenario which involves reductions between S1 and S3 (S2). They estimated 10.8, 20.7 and 29.1% fewer CHD deaths in 2020 under the conservative, intermediate and optimistic scenarios, respectively.

Sahan *et al.* (2016) modeled some risk factors for CHD in Turkey. The authors created two scenarios, a modest one and an ideal one. The modest scenario comprises a 3% reduction in smoking, a 40% reduction in dietary salt intake, a 2% reduction in recent diabetes trends, a 2.3%

of reduction in recent BMI trends, a 1% reduction in dietary saturated fat intake, a 40% increase in fruit and vegetable consumption and a 3% reduction in the prevalence of physical inactivity. In the ideal scenario, the changes were 6, 70, 4, 4.6, 2, 100 and 6%, respectively, for each risk factor. The reductions in CHD deaths by 2025 should be approximately 16 and 30% for the modest and ideal scenarios, respectively. The largest contributions to mortality reduction were associated with dietary changes, such as salt intake, energy intake from saturated fat, and fruit and vegetable consumption. Together, they account for a 68-63% reduction in deaths in Turkey by 2025.

Likewise, Moreira *et al.* (2015) explored the potential mortality reduction associated with substantially reducing ultra-processed food intake in the UK. The authors used the IMPACT food policy model to estimate reduction in CVD mortality. Both the ideal and feasible scenarios suggested a substantial reduction in CVD mortality, preventing or postponing approximately 17,000 to 22,000 CVD deaths in the UK by 2030.

Bandosz *et al.* (2015) developed a model to evaluate the changes in cholesterol in Poland related to statin or dietary changes. It was observed that if the change attributed to statin use was subtracted, the changes in cholesterol should be lower. The model compared the TC levels from two studies in 2002 and 2011. A fall in TC, mainly in older adults, was also observed, although there wasn't a considerable drop in the consumption of saturated fats, according to fat supply per capita, a statistic provided by the Food and Agriculture Organization. However, it is known that reducing salt and saturated fat intakes have effects on blood pressure and TC levels (Sahan *et al.*, 2016).

Allen *et al.* (2016) developed a model focusing on smoking and they modelled an increase in each component of the Tobacco Control Scale in England, where the retail price would increase by 20%, and reductions would be observed in the prevalence of smoking in smoke-free places, public information campaigns, advertising bans, health warnings and treatment stratified by socio-economic circumstances. The authors observed a reduction of approximately 3.0% on overall smoking prevalence and a reduction of 5.8% among the most deprived quintile, estimating the prevention or postponement of approximately 3,300 CHD premature deaths. They concluded that a decline in smoking prevalence would lower premature CHD mortality and that the more deprived socio-economic groups would benefit more.

A remarkably wide variety of CHD policy models exist as has been shown above. Here we present a basic and simplified version of the cell-based IMPACT food policy model developed by O'Flaherty *et al.* (2012) which can be easily implemented for quick analyses. The model is used here to quantify changes in CHD mortality in England and Wales from 2020 to 2030 as consequence of reducing dietary risk factors.

To estimate the current levels of saturated fats, salt and sugar present in the England and Wales diet, the LCF was used (DEFRA, 2012). This survey is based on a sample of roughly 6,000 households and it is routinely carried out by the Office for National Statistics. The LCF allows the estimation of the average quantity of food and drink purchased per person per day. Additionally, the LCF uses the official UK dietary risk factors conversion table supplied by the Department

of Health to convert data on food intake into quantitative measures of the nutritional content. Table 9.1 shows sex- and age-specific levels of saturated fats, salt and sugar taken directly from the LCF. The content of trans fats in the diet was also estimated from Food Standard Agency (Bates *et al.*, 2012).

International guidelines have set optimal levels for these risk factors: the AHA (2016a) has set an ideal sodium daily intake of 1,500 milligrams, equivalent to 3.75 g/day of salt. Also, the intake of saturate fats should be limited to less than 7% of the total daily energy intake according to (AHA, 2016b). The WHO guidelines recommend that less than 5% of total daily energy should ideally come from sugars. WHO also advocates towards the total elimination of industrial fats (i.e. trans fats set to 0% of the daily energy intake) (WHO, 2015a).

The aim of this modelling exercise is to quantify the potential reductions in CHD mortality up to 2030 as the result of achieving recommended levels in saturate fats, salts, trans-fats and sugar by 2020. The primary outcome measure is the total number of DPPs that can be attributed to population-level dietary risk factor changes in saturated fats, salts, trans fats and sugar. To calculate CHD DPPs as the result of changes in saturated fat, trans fat, salt and added sugar, the IMPACT food policy model uses a regression approach, where the projected number of CHD deaths between 2020 and 2030 is multiplied by the absolute change in the dietary risk factor level (absolute difference between the current level of dietary risk factor and the recommended level) and a regression coefficient quantifying the estimated relative change in CHD mortality that would result from a one-unit change in dietary risk factor level.

Table 9.1. Sex- and age-specific content of saturated fats, sugar, salt and trans fats in the England and Wales diet.

Gender	Age group	Saturated fats as % of total daily energy intake	Sugar as % of total daily energy intake	Salt g/day	Trans fats as % of total daily energy intake
male	35-39	13.49%	22.53%	5.19	1.08%
male	40-49	13.53%	22.84%	5.66	1.08%
male	50-64	14.03%	22.35%	6.86	1.08%
male	65-74	14.55%	23.83%	7.40	1.05%
male	>75	14.91%	24.72%	6.74	1.05%
female	35-39	13.49%	22.53%	5.19	1.16%
female	40-49	13.53%	22.84%	5.66	1.16%
female	50-64	14.03%	22.35%	6.86	1.16%
female	65-74	14.55%	23.83%	7.40	1.15%
female	>75	14.91%	24.72%	6.74	1.15%

For example, 29,500 CHD deaths will occur among men aged 65-74 years between 2020 and 2030 (Guzman-Castillo *et al.*, 2014). The mean current salt intake level for this group is 7.4 g/day. To achieve the AHA recommendation of salt intake, the current levels need to fall by 3.7 g/day.

The largest meta-analysis reports an estimated age-sex specific reduction in CVD mortality of 17% for every 5 g/day reduction in salt intake (Strazzullo *et al.*, 2009), yielding a regression coefficient of 0.034 (i.e. a 3.4% reduction for every 1 g/day reduction in salt intake).

The subsequent reduction in CHD deaths between 2020 and 2030 was then estimated as:

DPPs = projected deaths × salt effect

salt effect = absolute salt reduction × salt regression coefficient

DPPs = 29,500 × 3.7 × 0.034 ≈ 3,700

Table 9.2 contains the projected number of CHD deaths and Table 9.3 contains the regression coefficients used in the model.

CHD deaths are usually caused by multiple risk factors acting simultaneously. Hence, part of the effect of one risk factor may be mediated through another. It is therefore recommended that mortality benefits attributable to risk factors which may be causally related, or which overlap in population groups, should not be combined by simple addition. Ideally, their effects should instead be jointly estimated (Danaei *et al.*, 2009). One approach commonly used is to calculate the cumulative risk-reduction (Wald and Law, 2003). This approach accounts for risk factor prevalence overlap but assumes independence of effects (Taylor *et al.*, 2006).

Table 9.2. Predicted number of coronary heart disease (CHD) deaths between 2020 and 2030 as reported by Guzman-Castillo *et al.* (2014).

Gender	Age group	CHD deaths between 2020-2030
male	35-39	4,100
male	40-49	4,000
male	50-64	12,600
male	65-74	29,500
male	>75	164,100
female	35-39	750
female	40-49	750
female	50-64	2,700
female	65-74	8,700
female	>75	100,600

Table 9.3. Regression coefficient quantifying the estimated relative change in coronary heart disease mortality that would result from a one-unit change in dietary risk factor level.

Gender	Age group	Saturated fats (Jakobsen *et al.*, 2009)	Sugar (Yang *et al.*, 2014)	Salt (Strazzullo *et al.*, 2009)	Trans fats (Mozaffarian and Clarke, 2009)
male	35-39	0.078	0.014	0.048	0.163
male	40-49	0.078	0.014	0.048	0.115
male	50-64	0.074	0.013	0.048	0.082
male	65-74	0.052	0.016	0.034	0.060
male	>75	0.032	0.020	0.021	0.058
female	35-39	0.074	0.014	0.048	0.163
female	40-49	0.074	0.014	0.048	0.115
female	50-64	0.074	0.013	0.048	0.082
female	65-74	0.052	0.016	0.034	0.060
female	>75	0.032	0.020	0.021	0.058

Therefore, the total DPPs from the combined (or cumulative) effect of dietary risk factors can be expressed as:

DPPs = projected deaths × (1 – saturated fats effect) × (1 – salt effect)
× (1 – sugar effect) × (1 – transfats effect)

Then, the calculations are repeated for each age-sex group.

Between 2020 and 2030, approximately 327,900 CHD deaths could be expected. However, if optimal levels of dietary risk factors are achieved by 2020 according to the international guidelines, CHD mortality could be reduced by 8.1%. This equates to approximately 26,700 fewer deaths than from original projections. See Table 9.4 for sex- and age-specific results.

The highest effect on CHD mortality comes from reductions in salt intake, followed by sugar, saturated fats and trans fats (Figure 9.1). The highest reductions could be possible for men, reflecting the higher burden of disease among this group.

The modelling exercise presented here shows a simple tool to evaluate policy interventions or hypothetical scenarios to reduce the future burden of disease. The results suggest that there is potential for substantially reducing the CHD burden through overall improvements of the England and Wales diet. Reducing consumption of saturated fat, trans fat, salt and added sugar, according to the recommended international guidelines could result in an 8% decrease in deaths from CHD.

Table 9.4. Deaths prevented or postponed between 2020-2030 as the result of achieving recommended levels in saturated fats, salts, trans-fats and sugar.

Gender	Age group	Deaths prevented or postponed between 2020-2030
male	35-39	330
male	40-49	430
male	50-64	2,000
male	65-74	3,900
male	>75	11,600
female	35-39	65
female	40-49	84
female	50-64	443
female	65-74	1,200
female	>75	7,200

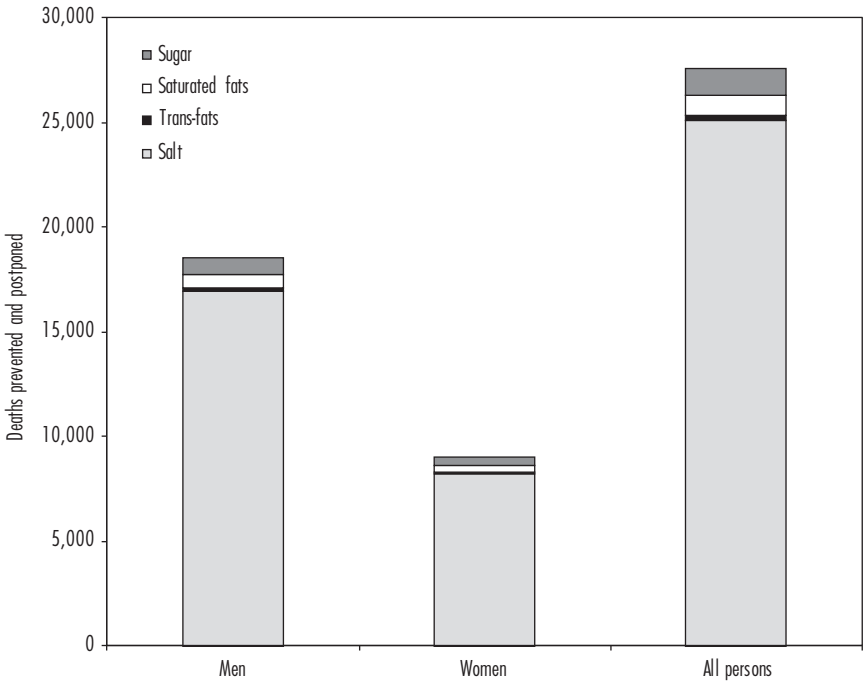

Figure 9.1. Deaths prevented or postponed (DPPs) between 2020 and 2030 as the result of achieving recommended levels in saturated fats, salts, trans fats and sugar.

As with any other modelling exercise, the results should be interpreted in light of the modelling assumptions. In this exercise, no 'lag time' between achieving optimal risk factor levels and mortality reduction was assumed. However, lag times for CHD have been proved to be short and considerable mortality declines can occur rapidly after individual or population-wide dietary changes (Capewell and O'Flaherty, 2011).

This modelling exercise does not incorporate any trend effect in risk factors that might additionally influence their future levels. This might result in over or under estimation of the potential reduction.

This modelling exercise also has important limitations: the LCF survey from which the data on salt, sugar and saturated fats consumption was obtained, reports on food expenditure rather than nutrient intakes. The survey is generally viewed as being statistically representative, reflecting its large sample size and frequency. However, the mixture of food acquisition and consumption data can lead to the overestimation or underestimation of true consumption (Vandevijvere *et al.*, 2013).

Predictions of any type are subject to uncertainty. However, to keep the simplicity of this exercise, uncertainty was not modelled. Incorporating and reporting uncertainty are distinctive marks of good modelling practice. The ISPOR task force report presents a good guide on how to incorporate and report uncertainty along with other good research practices for modelling studies (Caro *et al.*, 2012).

9.3 How to tackle the risk factors for the development of cardiovascular diseases

Trends in the number of global deaths are being affected by population growth, aging populations and changes in age-related mortality rates. Others factors that are related to changes in global death numbers are urbanization, modernization and changes in lifestyles where people are following unhealthier diets and are physically inactive.

The Global Action Plan for the prevention and control of NCDs has targets in relation to risks factors related to CVD for 2013-2020: a 10% relative reduction in the prevalence of insufficient physical activity, a 30% relative reduction in the mean intake of salt/sodium, a 30% relative reduction in the prevalence of current tobacco use, a 25% relative reduction in the prevalence of high blood pressure, halt the rise of diabetes and obesity and at least 50% of eligible people receive drug therapy and counseling to prevent heart attacks and strokes, focusing on a 25% reduction in the risk of premature mortality from CVD, cancer, diabetes or chronic respiratory diseases (WHO, 2013).

To achieve these targets some measures are being implemented in different countries. In relation to taxation, some studies have shown that tax increases are effective for the control policies of

some risk factors, such as tobacco. People who are more deprived tend to reduce consumption with rising prices and this reduces rates of CVD, as demonstrated by Allen *et al.* (2016). In Finland, 0.5% of the tobacco tax is designated for tobacco control (Vartiainen *et al.*, 2010). In some countries the power of the tobacco industry is stronger than the tobacco control units, as in Syria (Rastam *et al.*, 2012).

Meanwhile, since 2008, WHO has introduced a package of measures under the acronym of MPOWER (Monitor, Protect, Offer, Warn, Enforce, Raise). According to the last WHO report, more than half of the world's countries, containing 40% of the world's population, have implemented at least one MPOWER measure at the highest level. The tobacco control policies principally include smoke-free environments, cessation programs, pack warnings, mass media, advertising bans and taxation. The most effective measures in tobacco control, recognized by WHO member states, are higher pricing and taxing, particularly affecting young persons (WHO, 2015b).

Together, different sectors of society can work towards fighting the risk factors for CDV with promising results, as in Finland. A successful project was developed in Finland (North Karelia Project) and the disease prevention strategies were applied to the whole country, involving health services, industry, employers, non-governmental organizations, decision makers and the media, resulting in an 80% reduction in CHD (Vartiainen *et al.*, 2010). In Tunisia, the NCD strategy involves controlling diabetes and stimulating physical activity, based on multi-sectorial, effective and evidence-based interventions (health promotion, fiscal measures, market control and community participation) (Saidi *et al.*, 2013).

Strategies are being implemented to reduce salt consumption in many countries. These strategies include reducing the amount of salt in bread, the quantity of processed foods and ultra-processed foods, educational campaigns to avoid placing salt on tables and campaigns to promote healthy eating (MOH, 2011).

For the WHO (2016b), the strategies for salt reduction include:

- government policies – including appropriate fiscal policies and regulation to ensure food manufacturers and retailers produce healthier foods or make healthy products available and affordable;
- working with the private sector to improve the availability and accessibility of low-salt products;
- consumer awareness and empowerment of populations through social marketing and mobilization to raise awareness of the need to reduce salt intake consumption;
- creating an enabling environment for salt reduction through local policy interventions and the promotion of 'healthy food' settings such as schools, workplaces, communities, and cities;
- monitoring of population salt intake, sources of salt in the diet and consumer knowledge, attitudes and behaviors relating to salt to inform policy decisions.

According to the guideline for sodium intake for adults and children, WHO recommends a reduction in sodium intake to reduce blood pressure and the risk of CVD, strokes and CHD. Recommended sodium level intakes are <2 g/day and 5 g/day for children and adults respectively (WHO, 2012).

In relation to promoting healthy eating, there are some places where fruit and vegetables are more accessible and cheaper. Glanz and Yaroch (2004) described four types of grocery-store-based interventions to increase fruit and vegetable intake, such as POP information, reduced prices, coupons, increased availability, variety, and convenience, and promotion and advertising. POP is the use of shelf labels and/or signage that specifies healthy food choices. This strategy has used posters, brochures, and shelf labels and most have focused on decreasing high-fat food choices. In Turkey, they are using this strategy (Sahan *et al.*, 2016). Reducing prices and coupons are used for healthy choices and F&V programs adapt these offers to fruit and vegetables (Glanz and Yaroch, 2004). Increased availability, variety and convenience mean providing more easy-to-use or -eat F&Vs, and/or making F&V easier to locate in stores, as well as the using advertisements, posters, games, and multimedia sources to encourage purchases of F&Vs (Glanz and Yaroch, 2004).

9.4 Conclusions

Models have been increasingly used in all areas of science including public health. Models are useful because they permit policy makers to simulate the effects of different scenarios within a population and hence examine future policy options to the extent that clinical trials would never do (Unal *et al.*, 2006). It is imperative for governments and health authorities to be aware of the information provided by the models to monitor the trends and determinants of CDVs and assess progress in prevention and control.

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10. Advances of effects of copper on cardiovascular health

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Abstract

Coronary heart disease is a major cause of death in developed countries. The disease is primarily caused by severe atherosclerosis (AS). According to the 'response to injury' hypothesis, AS is initiated by series of inflammatory events and eventual damage to the endothelium. As a main regulator of hemodynamic homeostasis, the endothelium in the vasculature functions as a physical barrier, as well as an 'endocrine depot' for the synthesis of vasoactive agents that exert cardioprotective activities. The intrinsic ability to sense humoral and hemodynamic stimuli endows the endothelium with a broad range of bioactivities, including, the local modulation of vascular tone and structure, the control of proliferation and migration of vascular smooth muscle cells, and the dynamic regulation of the adhesion and extravasation of leukocytes. Accordingly, impairment of endothelial cell function and their enhanced permeability to atherogenic lipoproteins are major pathological events that initiate AS. Atherosclerotic lesions are characterized by chronic inflammation of the vessel wall initiated by leukocyte recruitment, adhesion, and enhanced retention of low-density lipoprotein (LDL) within activated endothelium. Migration and aggregation of LDL into the subendothelium coupled with subsequent lipid accumulation within macrophages support foam cell formation. Elucidating molecules and pathways essential for the preservation of vascular endothelial integrity and function will provide insights that could lead to discovery of efficient therapeutic targets for the treatment of atherosclerotic vascular disease. This chapter will explore the contributions of copper in controlling vascular endothelial cell activation and inflammation. Evidences on cardioprotection by copper and its association with cuproenzymes that control mitochondrial energy generation, collagen and elastin cross-linking, iron-transport, and degradation of superoxide will be summarized.

Keywords: copper, endothelium, atherosclerosis, ROS/RNS

Key facts

- Cardiovascular disease is the major cause of death worldwide.
- Maintaining copper homeostasis is critical to health of vascular endothelial tissues.
- Copper deficiencies limit the function of superoxide dismutases (SODs) and allow excess reactive oxygen species (ROS) to react with nitric oxide (NO) thus diminishing its effect as a vasodilator.
- ROS are metabolic products that have oxidizing capacity which can be harmful to the cell. ROS include superoxide and hydrogen peroxide, among others.
- There are three forms of superoxide dismutase. Cu-Zn SOD/SOD1 is found in the cytoplasm of cells, manganese-SOD/SOD2 is found in mitochondria, and ecSOD/SOD3 is found in the extracellular environment.

Summary points

- Copper is used as a cofactor in the antioxidant enzymes Cu-Zn SOD and ecSOD which help regulated intra and extracellular ROS.
- Copper can form free radicals and therefore must be tightly regulated in the body via chaperones and transporters.
- NO is a potent signaling molecule in blood vessels. It induces vasodilation which is pertinent for healthy relaxation of blood vessels. NO must be protected from reactions with oxidative molecules.
- Increased pro-inflammatory cytokines are responsible for the upregulation of endothelial nitric oxide synthase.

Abbreviations

AS	Atherosclerosis
ATP7A	ATPase copper transporting alpha
ATP7B	ATPase copper transporting beta
CCSs	Copper chaperone for superoxide dismutases
Cu(I)	Cuprous
Cu(II)	Cupric
Cu,Zn SOD	Copper-zinc superoxide dismutase
CVD	Cardiovascular disease
ecSOD	Extracellular superoxide dismutase
Fe(II)	Ferrous
GPI	Glycophosphatidyl inositol
LDL	Low-density lipoprotein
NO	Nitric oxide
NOS	Nitric oxide synthase
$ONOO^-$	Peroxynitrite
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SCO1 and SCO2	Cytochrome c oxidase assembly proteins
SOD	Superoxide dismutase
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
VEGFR-1 and -2	VEGF receptors-1 and -2

10.1 Introduction

Maintaining copper homeostasis is critical to health of vascular endothelial tissue. Copper deficiencies limit the function of SODs and allow excessive ROS to react with NO thus diminishing its effect as a vasodilator. Thus, the intricacies of copper and endothelial cell health need to be understood. This chapter will explore the impact of copper in controlling vascular endothelial cell integrity and in regulating inflammatory responses within the cardiovascular system.

10.2 Copper in heart health

Copper is an essential transition metal that exhibits two oxidation states, Cu(I) and Cu(II). The redox potential between these ionic forms is exploited by both mitochondrial and cytosolic cuproenzymes. The catalytic activity relies on its capacity to cycle between Cu(I) and Cu(II) states enabling it to donate or accept electrons accordingly (Kalinowski *et al.*, 2016). This feature provides a facile redox system that underlies its biological reactivity. The Cu(I) state in cuproenzymes is especially critical for reducing superoxide and molecular oxygen with concomitant formation

of H_2O_2 or hydroxyl ion. In either case, copper is oxidized to Cu(II) and requires ascorbic acid or (Fe(II)) ion to reduce it back to its (Cu(I) state for reactivation (Lieu *et al.*, 2001). It should be noted that this same property which allows transfer of free electrons to oxygen leads to generation of ROS such as superoxide and the hydroxyl radical. As will be discussed later, these highly reactive products can induce cell death by initiating reactions with proteins, lipids and nucleic acids resulting in damage to mitochondria, plasma membranes and DNA (Cai *et al.*, 2005; Denoyer *et al.*, 2015; Dizdaroglu and Jaruga, 2012; Stadtman, 1990). Thus, the role ROS plays in control of cell signaling are particularly important in oxygen-demanding tissues such as skeletal and cardiac muscle. In terms of the importance of redox signaling in the heart, ROS and redox signaling are critical factors that enable the cardiovascular system to adapt to both physiological and pathological stresses. Compared to endothelial cells within the vasculature, cardiomyocytes exhibit the highest oxygen consumption and consequently must endure ROS generation during mitochondrial reduction of molecular oxygen to water within the electron transport chain. In view of the continuous generation of ROS during regular metabolic processes, cardiac tissue is able to acutely adapt to meet contractile performance and chronically remodel to meet demands of prolonged workloads. Cardiac hypertrophy is a major component of chronic cardiac remodeling which involves enlargement of cardiomyocytes and increased thickness of ventricular walls (Santos *et al.*, 2011). During sustained cardiac stress irreversible structural and contractile abnormalities will lead to complete cardiac dysfunction. During these adaptive phases that evolve to eventual pathologies, redox signaling pathways play critical roles both in acute cardiac adaptations and in chronic cardiac remodeling that can lead to heart failure. In the next section, we will focus on ROS and copper in heart health.

10.3 Copper, superoxide dismutase and redox homeostasis

Copper is essential for the transformation and maintenance of ROS. While ROS are common products of metabolism, their disposition as oxygen-containing molecules with unpaired electrons can be harmful to macromolecules. ROS are critical factors in controlling cardiovascular health. ROS in particular superoxide and hydrogen peroxide are coupled to many physiological processes that not only cause oxidative damage to endothelial cells but are critical to initiating cell signaling. The superoxide radical is a byproduct of oxidative phosphorylation within the mitochondria and is the target of antioxidant cuproenzymes, SOD. Three isoforms of SOD (Cu,Zn-SOD1, manganese-SOD2, and a secretory form, extracellular Cu,Zn-SOD3) are found in all tissue types throughout the body and are particularly important in prevention of CVD. One primary function of SOD is to protect NO, a potent vasodilator, by degrading superoxide before it can react with NO to form $ONOO^-$. If SODs were not present to intercept and convert superoxide into a less harmful oxidant, hydrogen peroxide, superoxide would inactivate NO thus facilitating risk of developing hypertension and cardiovascular dysfunction in general.

While the aforementioned oxidants can be harmful to intracellular metabolites and pathways, they are essential for initiating cell signaling and proper cell function (Tullio *et al.*, 2013). For instance, H_2O_2 is a known regulator of gene expression as it selectively activates gene transcription.

Hydrogen peroxide has a number of diversified functions that include causing angiotensin II-associated vascular hypertrophy and functioning as an endothelium-derived hyperpolarizing factor and endothelium-derived relaxing factor. Even across numerous species such as insects, birds, and mammals, a positive correlation exists between the amount of mitochondrial H_2O_2 and longevity, bolstering the importance of H_2O_2 in healthy cellular functions. Studies support this idea given that ROS are instrumental in adaptation to hypoxia, immunity, and regulation of the cell cycle involving replication, signaling, and apoptosis. Maintaining a balance in ROS and RNS is particularly cardioprotective during ischemia/reperfusion injury. Both ROS and RNS have been identified as essential signaling molecules during pre- and post-conditioning processes affiliated with preservation of myocardiocytes during ischemic episodes (Pagliaro *et al.*, 2011; Zhao *et al.*, 2003). Given the dichotomous nature of ROS, a delicate balance exists between antioxidant cuproenzymes and formation of ROS in order to control gene expression and maintain cell homeostasis.

As mentioned earlier, three isoforms of SOD are found in all tissue types. This review will consider the role of Cu,Zn-SOD1 and esSOD3 and not the mitochondrial manganese-SOD in cardiovascular health (Afolayan *et al.*, 2016; Guo *et al.*, 2016). Cu,Zn-SOD1 is found in the cytoplasm of cells and plays a key role in vascular relaxation and health. Use of the copper chelator, diethyldithiocarbamate, to inhibit Cu,Zn-SOD, results in an increase in concentration of superoxide and impediment in vasodilation. Interestingly, maintaining adequate concentrations of Cu,Zn-SOD1 are essential for protecting nitric oxide, since the affinity of superoxide to react with NO is three-fold faster than the reactivity of superoxide with Cu,Zn-SOD1 (Kelm *et al.*, 1997). Numerous studies have shown that sufficient concentrations of Cu,Zn-SOD1 are needed to maintain vascular health (D'Alessandro and Zolla, 2011). Mice with deficiencies in Cu,Zn-SOD1 demonstrate with increased myogenic tone, cardiovascular hypertrophy, and vascular permeability followed by ischemic episodes. In a study of genetically modified mice with 7-fold increase in Cu,Zn-SOD1 activity, mice were protected from several implements of vascular dysfunction including cerebral hemorrhage, hypoxia with reoxygenation, and exposure to ceramide, lipopolysaccharide, and beta-amyloid protein precursor (Wang *et al.*, 1998). In addition, overexpression of Cu,Zn-SOD1 has been shown to reduce inflammation by inhibiting the effects of monocyte chemoattractant proteins and monocyte infiltration into vessel walls (Groleau *et al.*, 2010). In an animal study of diabetes and atherosclerosis, overexpression of Cu,Zn-SOD1 using adenovirus vector was accompanied by a marked decrease in superoxide levels and improved endothelial functions, and protected autoregulation of the heart was observed when animals were challenged by a fluid compression injury model (Neves *et al.*, 2012).

Extracellular (ec)-SOD, also known as SOD3, is highly expressed in the lung and vasculature and is a major contributor to the regulation of redox balance, preventing vascular inflammation, and maintenance of vascular tone. Following its secretion from endothelial and smooth muscle cells, a portion of ec-SOD translocates to the vascular circulation, while a major portion is anchored to extracellular matrix proteins such as type-I collagen or the proteoglycan, heparan sulfate (Hartney *et al.*, 2014). Studies show that ec-SOD3 provides optimal protection when localized to the compartment subjected to extracellular oxidative stress. Ec-SOD3 is normally concentrated

in the space between the endothelia and vascular smooth muscle cells where endothelial-derived NO must diffuse in order to achieve vascular relaxation. Redistribution of ec-SOD3 from lung and pulmonary circulation to the extracellular fluids is beneficial in alveolar lung disease but detrimental in pulmonary vascular disease (Adachi *et al.*, 1996). Accordingly, a single-nucleotide polymorphism that substitutes glycine for arginine-213 (R213G) in the C-terminal region of ec-SOD results in diminished binding affinity of ec-SOD to heparan within the lung and vasculature but does not influence overall enzyme activity. This mutation shifts the distribution of ec-SOD from the lung and vascular tissue to the extracellular fluid volume and paradoxically increases risk of CVD and diminishes risk of lung disease (Adachi *et al.*, 1996). These findings account for the discrepant risk associated with R213G polymorphism in humans with lung diseases compared to those with CVDs (Hartney *et al.*, 2014). Thus, the homozygous R213G mutation results in marked increases in the plasma ec-SOD3 levels which contributes to increased risk of myocardial infarction and decreased risk of developing pulmonary disorders such as chronic obstructive pulmonary disease.

Extracellular-SOD3 modulates blood pressure by lowering levels of superoxide that inactivate the gaseous vascular signaling relaxant, NO, to produce $ONOO^-$. $ONOO^-$ is itself a potent oxidant that contributes to lipid peroxidation and membrane damage. Nitric oxide is synthesized from the amino acid, L-arginine, by three isomeric forms of NOS (Bruckdorfer, 2005). $ONOO^-$ can directly affect NOS by oxidizing tetrahydrobiopterin, the cofactor of NOS (Harrison *et al.*, 2010). This subsequently results in the uncoupling of NOS reaction, redirects electron transfer to generate superoxide rather than NO, and decreases bioavailability of NO for arterial vasodilation.

Vascular endothelia use NO to signal adjacent intimal smooth muscle cells to relax thus causing vasodilation and improving vascular perfusion. In addition to regulation of blood pressure, NO also exhibits anti-inflammatory and anticoagulant properties by blocking activation of blood platelets. Following its entry into smooth muscle cells, NO activates the heme-enzyme, guanylyl cyclase, which amplifies signaling through the second messenger, cyclic GMP. Other signal transduction systems modified by NO include cyclic adenosine monophosphate, G-protein, for Janus kinase/signal transducer and activator of transcription and mitogen-activated protein kinase dependent pathways (Guzik *et al.*, 2003). In addition, the interaction between NO and superoxide anion to produce $ONOO^-$ can modify proteins by direct nitrosation of tyrosine and nitration of cysteinyl thiol moieties (Parodi *et al.*, 2007). These post-translational modifications produce myriad effects on target proteins to cause diverse and subtle changes in cellular reactivity such as those observed in normal immune responses and in allergic inflammatory reactions. In total, disproportionate production of $ONOO^-$ through imbalanced interaction between nitric oxide and superoxide contributes to the pathogenesis of disease conditions such as inflammation, hypertension, AS and ischemic injury, hallmarks of CVD (Bauersachs and Widder, 2008; Brüne and Zhou, 2007; Ginnan *et al.*, 2008; Majid and Kopkan, 2007; Salvemini and Cuzzocrea, 2002). Most studies reveal that the balance between nitric oxide and superoxide is more important than the absolute levels of either substance alone. Thus, the compartmentalization of SOD cuproenzymes and the availability of copper at the active site are indispensable for normal

function of the enzyme. In the following section, we further explore the role of cuproenzymes in heart health using cytochrome c oxidase.

10.4 Cytochrome c oxidase and mitochondrial function

Both epidemiological data and animal models show that functional impairment of cuproenzymes contributes to pathological states that are associated with CVDs (Elsherif *et al.*, 2003; Mandinov *et al.*, 2003; Mao *et al.*, 1998; Prohaska and Heller, 1982). Of particular note, hallmark symptoms of deficiency of mitochondria-derived cytochrome c oxidase alone include hypertrophic cardiomyopathy, muscular hypotonia and lactic acidosis (Baertling *et al.*, 2015). Thus, animal models of dietary or genetically imposed copper deficiency develop severe cardiovascular dysfunction resulting in aneurysm, cardiac hypertrophy and other functional defects in the cardiovascular system (Cheung *et al.*, 2015; Prohaska and Heller, 1982).

Copper is required for the function of cytochrome c oxidase, the terminal, rate-limiting enzyme of the respiratory chain. The enzyme complex is localized in the inner mitochondrial membrane and catalyzes the transfer of reducing equivalents from cytochrome c to molecular oxygen. Dietary or transport deficiencies in copper or isolated cytochrome c oxidase deficiency initiate an array of mitochondrial abnormalities that predominantly affect tissues with high energy demands, especially brain, skeletal muscle and heart (Elsherif *et al.*, 2004). The clinical manifestations are variable but primarily result in encephalopathy and hypertrophic cardiomyopathy.

Copper deficiencies in animals produce predominantly a concentric hypertrophic morphology of heart muscle characterized by increased cardiac wall thicknesses with a corresponding decrease in lumen size. This type of morphology is also typically observed in patients with hypertension to compensate for wall stress in view of elevated blood pressure. Pressure overloads also lead to decreases in copper concentration and decreased cytochrome c oxidase activities in heart (Jiang *et al.*, 2007). Animal studies have shown that copper supplementation can remediate cytochrome c oxidase activity and reverse the cardiac hypertrophy (Zhou *et al.*, 2008). In addition, recovery from cardiac hypertrophy depends on the extent of angiogenesis (Bharathi Devi *et al.*, 2016). Copper has been shown to promote angiogenesis by activating critical factors in the angiogenic process such as angiogenin, heparan, collagenase, TNF-α and VEGF. Thus, the development of cardiac hypertrophy that occurs in response to increased workload must be accompanied by enhanced vascular growth to meet nutrient demands for expansion of myocardial size and performance. Studies have shown that copper regulates dichotomously VEGF receptors-1 and -2 (VEGFR-1 and -2) which exhibit opposite responses when acted upon by VEGF in cardiomyocytes. VEGFR-2 is critical for the hypertrophic response of cardiomyocytes and VEGFR-1 promotes regression of cardiomyocyte hypertrophy. Thus, homeostatic control of copper could affect pathological outcomes of cardiac hypertrophy by switching cardiomyocyte responses from VEGFR-2 dominant to VEGFR-1 dominant. Investigators have speculated that control of these two signaling factors by target-specific delivery of copper could influence clinical outcomes of heart disease (Wang *et al.*, 2014; Zheng *et al.*, 2015).

In particular, deficits in cytochrome c oxidase compromise the proton gradient involved in mitochondrial ATP synthesis; decreases in superoxide dismutase activity affect cellular and organelle integrity by preventing elimination of superoxide; dopamine-β-hydroxylase deficits affect norepinephrine synthesis in sympathetic nerves; loss of lysyl oxidase activity abates compliance of elastin and collagen fibrils; and low levels of ceruloplasmin diminish hemoglobin formation and oxygen supply. In the last section, we will discuss copper transporters in heart health and its association with dopamine-β-hydroxylase, lysyl oxidase and ceruloplasmin.

10.5 Copper transporters

Extracellular copper transporters and intracellular chaperone proteins are essential for precise delivery of copper to all cuproenzymes. Specific ligand-exchange reactions enable copper to be transferred from metallochaperone proteins to their client cuproenzymes. This direct exchange prevents copper ions from engaging in deleterious reactions while en route through plasma membranes, the endoplasmic reticulum and especially the mitochondria. The initial import of copper into eukaryotic cells occurs via a transmembrane human copper transporter protein. Once captured within the cytosol, Cu(I) ions become apportioned to families of soluble Cu(I)-binding proteins referred to as metallochaperones. Chaperone proteins make the final delivery of copper to their client proteins via direct protein-protein interactions. Each family of cuproenzyme has a specific chaperone protein to conduct metalation of their active sites. Copper transported to SODs involves CCSs (copper chaperone for superoxide dismutases) which transfer copper to SODs following their synthesis and passage to the cytoplasmic compartment. In a similar fashion, the antioxidant 1 copper chaperone transfers Cu(I) to the membrane-bound, metal transporting ATPases, Menkes ATP7A and Wilson disease ATP7B. Mutations in these copper-transporting P-type ATPase lead to deficiency of copper and excessive copper accumulation, respectively (Migocka, 2015). Critical to mitochondrial membrane integrity, SCO1 and SCO2 control transport of copper needed for assembly of cytochrome c oxidase in mitochondria. In humans, SCO1 and SCO2 are needed for viability. Deficiencies in copper, errors in cytochrome oxidase synthesis and assembly into the electron transport chain, and hypertrophic cardiomyopathies have been associated with mutations in SCO1 and SCO2. It is important to consider that proper transport of copper is critical to organelle, cellular, and ultimately cardiovascular health. Thus, deficiency or mutation in copper transporters leads to a multitude of illnesses, many of which include CVD. Proper regulation and transportation of copper is essential to heart health (Urso and Maffia, 2015).

10.6 Dopamine-β-hydroxylase and sympathetic function

In patients with Menkes disease, mutations in ATPase 7A copper transporter results in diminished delivery of copper to cuproenzymes. The importance of copper transport proteins and intracellular chaperones for timely delivery of copper to cuproenzymes is critical. In particular, decreased activity of dopamine β-hydroxylase which catalyzes hydroxylation of

dopamine to norepinephrine plays a predominant role in the pathogenesis of Menkes disease due to poor delivery of the copper cofactor (Kaler and Holmes, 2013). Norepinephrine is the primary β-adrenergic response hormone that functions as a key mediator of stress response and sympathetic nervous system activity in mammals. Studies in mice that target disruption of the dopamine β-hydroxylase gene lead to a loss of norepinephrine response and embryonic lethality due to heart failure (Baker *et al.*, 2015; Thomas *et al.*, 1995). These studies suggest that norepinephrine plays a significant role to facilitate the metabolic shift of cardiac mitochondria away from glycolysis and toward aerobic oxidative phosphorylation during embryonic to fetal development. Impaired norepinephrine response in copper-deficient animals translates into altered catecholamine metabolism in cardiac tissue deficient in copper. Deficiencies of both copper and dopamine β-hydroxylase in heart muscle results in diminished norepinephrine sympathetic response and normal sympathetic and parasympathetic cholinergic response. In this manner, dysregulation of autonomic regulation of cardiac function would predispose animals to orthostatic hypotension. In its totality, a deficiency of dopamine β-hydroxylase is characterized by severe hypotension, hypothermia, and hypoglycemia (Robertson and Garland, 2003). Thus, copper deficiency may evoke opposing actions on cardiac function by altering the ratio of sympathetic (norepinephrine) and parasympathetic (acetylcholine) responses in favor of bradycardia and decreased peripheral vascular resistance, both M3-type muscarinic acetylcholine receptor responses. These effects would increase venous filling and result in an increase in stroke volume, factors that can lead to cardiac hypertrophy (Olivas *et al.*, 2016; Saari *et al.*, 1999).

10.7 Lysyl oxidase and maintenance of elastin and collagen function

Lysyl oxidase is a rate-limiting copper-dependent enzyme involved in crosslinking of fibrillary collagens and elastin. The enzyme facilitates inter and intra-peptide crosslinking by catalyzing the oxidation of epsilon amino groups of lysyl moieties to form reactive aldehydes, allysine and hydroxyallysine. These post-translational protein modifications are precursors to the trifunctional, 3-hydroxypyridinium residues (desmosine and isodesmosine) that provide added rigidity and strength to collagen and elastin (Eyre *et al.*, 1984). Copper deficiency would compromise collagen crosslinking and would weaken blood vessels as well as the myocardium leading to greater workloads on the heart to maintain blood pressure. In similar fashion, heart valves which are rich in elastin would exhibit enhanced compliance coupled with diminished elasticity which would result in valvular regurgitation. In animals fed a copper deficient diet, diminished crosslinking of collagen and elastin causes these animals to succumb to vascular aneurysms and cardiac lesions within the myocardium. In conditions of Menkes disease which is characterized as an X-linked recessive disorder of mutations in ATP7A, a copper-transporting ATPase, vascular defects and connective tissue disorders are prevailing features (Grange *et al.*, 2005). Thus, studies in several animal species of copper deficiencies in association with losses of lysyl oxidase activity reveal a commonality of cardiovascular defects that include cardiac enlargement, aortic fissures and ruptures, coronary artery thrombosis and myocardial infarction.

10.8 Ceruloplasmin as antioxidant

A unique feature of ceruloplasmin in addition to its ferroxidase properties is its ability to exhibit superoxide dismutase-like properties. By contrast to superoxide dismutases, ceruloplasmin can react with superoxide and generate water rather than H_2O_2. In general, due to its multicopper complexes, ceruloplasmin can mediate four-electron reductions of molecular oxygen without generation of superoxide or hydrogen peroxide (Calabrese *et al.*, 1989). In patients with aceruloplasminemia, iron accumulates within hepatocytes, in pancreatic endocrine cells, as well as in astrocytes. The accumulation of iron results in extensive lipid peroxidation coupled with cell damage. Thus, ceruloplasmin appears to have a dual function in triggering either oxidative or anti-oxidative responses in cardiovascular tissue when coupled with its role in the mobilization and oxidation of iron (Kono, 2013).

As copper's major extracellular transport protein, ceruloplasmin has physiological functions that include defense of oxidant stress, angiogenesis, coagulation and iron homeostasis. Ceruloplasmin exists in both a secreted and a membrane bound glycophosphatidyl inositol (GPI)-linked isoform that contains 6 atoms of copper. As with most of the secretory cuproenzymes, copper is incorporated during the latter end of enzyme biosynthesis and concurrent with their secretory process (Hellman and Gitlin, 2002; Patel and David, 1997). Both *in vitro* and *in vivo* studies have indicated that ceruloplasmin is capable of catalyzing the oxidation of a number of different substrates such as iron and catecholamines as well as the reduction of others, for example, superoxide and hydrogen peroxide. This dichotomy has created some confusion as to the physiologic role of this copper transport protein with regard to CVD. Better understanding of the role of ceruloplasmin in CVD may require further insight into homeostatic control of the secreted and GPI-bound isoforms (Marques *et al.*, 2012).

Ceruloplasmin is an acute phase inflammation reactant which exhibits ferroxidase activity. Hallmarks of acute phase response to infectious and inflammatory agents are increases in plasma ceruloplasmin and decreases in ferric iron bound to transferrin, resulting in an increased retention of Fe(II) iron within hepatocytes. By contrast, and consistent with increases in intracellular iron in patients with aceruloplasminemia, serum ferritin concentrations are elevated (measure of intracellular iron) with concomitant decreases in iron bound to transferrin (i.e. increased total iron binding capacity with decrease percentage iron saturation) (Gitlin, 1998; Harris *et al.*, 1998; Roeser *et al.*, 1970). Thus, although ceruloplasmin has been widely accepted as a scavenger and quencher of reactive oxygen species, it nonetheless may function independently or together with other metalloproteins to generate oxidized LDL during acute phase responses. This paradoxical activity may be explained by findings that the secreted, but not the GPI-ceruloplasmin isoform is upregulated by proinflammatory cytokines. In context of hepatocytes, macrophages and intestinal cells, the GPI-isoform co-localizes with ferroportin within lipid raft domains on the outer membrane of the cells. Its major function may be in the conversion of Fe(II) ions to less toxic ferric ions for storage and transport (Marques *et al.*, 2016). Thus, the interplay between copper and iron occurs at several intra- and extracellular locations and involves redox changes that generate formation of ROS and RNS. Given recent insights on copper and iron homeostasis

and the physiological significance associated with their electron exchanges, both these elements must be considered in concert with regard to oxidative stress and CVD (Gulec and Collins, 2014).

In studies of acute phase responses in CVD patients (Ahmed *et al.*, 2012), ceruloplasmin levels have predicted multiple cardiovascular disorders that include abdominal aortic aneurysm (Powell *et al.*, 1987), vasculitis and peripheral arterial disease (Belch *et al.*, 1989), arteriosclerosis (Salonen *et al.*, 1991a) and unstable angina (Jayakumari *et al.*, 1992). These studies have concluded that substantial hypercupremia occurs after myocardial infarctions and that serum ceruloplasmin can independently predict cardiovascular events (Adelstein *et al.*, 1956; Bustamante *et al.*, 1976). Prospective studies have also verified that serum copper concentration can serve as an independent risk factor for ischemic heart disease (Salonen *et al.*, 1991b).

Ceruloplasmin levels and CVD should be viewed in association with LDL levels and the susceptibility of these lipid vesicles to oxidative stress. Studies show that phospholipid content of arterial LDL particles is lower than that of circulating plasma LDL (Tailleux *et al.*, 1993). Isolation of LDL from atherosclerotic lesions of hyperlipidemic animals reveal marked increases in lysophosphatidyl choline and decreases in content of phosphatidylcholine, differences consistent with oxidation of phospholipid content (Ylä-Herttuala *et al.*, 1989). This observation was later supported by findings that arterial LDLs react with antibodies generated against lipid peroxidation products, malondialdehyde and 4 hydroxynonenal, and that atherosclerotic plaque contains oxidized LDL components consistent with exposure to ROS and copper ions (Cabassi *et al.*, 2014; Leeuwenburgh *et al.*, 1997). Thus, oxidation of LDL has been widely accepted as playing a critical role in the pathogenesis of AS and that oxidized LDL in plasma serves as a major risk factor for human CVDs (Yang *et al.*, 2012).

In addition to exposure to copper ions, a number of inflammatory cytokines such as TNF-α, IL-1 and IL-6 can induce synthesis of ceruloplasmin by activating macrophages which contribute to cell-mediated LDL oxidation (Mackiewicz *et al.*, 1988; Smith *et al.*, 1992; Ziakas *et al.*, 2009). In addition to activated macrophages that contribute to the inflammatory response, the liver is the primary organ responsible for changes in plasma proteins that accompany the acute phase response. Thus, hepatic ceruloplasmin is among the group of proteins responding positively to inflammation and infection in terms of increased synthesis and secretion. Other acute response proteins involved with copper and iron metabolism include ferroportin which decreases in acute phase response and causes accumulation of iron within mucosal cells, macrophages and hepatocytes (Ahmad *et al.*, 2014; Conley *et al.*, 2005).

Thus, the dichotomy in ceruloplasmin expression is evident during hypoxia and hyperbaric oxygen exposure as both conditions enhance ceruloplasmin levels. Exposure to hyperbaric oxygen has the potential of generating ROS as do conditions of hypoxia especially during disorders characterized by ischemia/reperfusion (e.g. myocardial infarction, stroke, and peripheral vascular disease). It appears that ceruloplasmin responds during either condition and serves to mitigate oxidative insult.

10.9 Conclusions

The effects of copper on the cardiovascular system appear paradoxical as copper exhibits both anti- and pro-atherogenic effects. Numerous experimental and clinical studies also suggest positive correlations between elevated serum copper and CVD. Thus, while low copper intake causes deficits in mitochondrial energy generation, collagen and elastin cross-linking, superoxide degradation leading to development of atherosclerotic coronary disease, other studies have focused on high serum copper being associated with endothelial damage and an initiator of atherosclerosis. Thus, the intricacies of copper and endothelial cell health need to be understood. In this chapter, we cover copper regulation, regression of pathological cardiac hypertrophy, and the potential clinical application of copper in heart disease. In addition, we also review the overall importance of copper, cuproenzymes and copper transporters in CVDs.

Acknowledgments

Stephanie Brown and Jennifer Madrid were participants in STAR (Summer Trainees in Academic Research), a summer program at New York Medical College aimed at enhancing the research experiences of students.

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Dietary supplements, herbs and foods in health

11. Taurine exposure affects cardiac function and disease

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Abstract

Taurine (2-aminoethanesulfonic acid) is a β-amino sulfur amino acid found abundantly in many human tissues, particularly brain, heart, and muscle. Taurine is an abundant free amino acid found in the heart and possesses many physiological functions from conception to adult life. Taurine is involved in cardiac growth, metabolism, ionic balance, excitation-contraction process, and oxidative activity, all of which contribute to cardiac function in health and disease. Further, taurine also influences neural and hormonal control of the heart and blood pressure. High taurine diets or taurine supplementation are reported to prevent or improve cardiovascular diseases particularly hypertension and the ischemic heart disease. In cardiac ischemia/reperfusion (IR), taurine supplementation before, during, and after IR decreases cardiac damage, dysfunction, and remodeling. Taurine reduces cardiac reactive oxygen species production, Ca^{2+} accumulation, mitochondrial permeability transition pore, and apoptosis following IR. Other than its cellular beneficial activity, taurine treatment also reduces inflammation, blunted baroreflex sensitivity, increased sympathetic nerve activity, and renin-angiotensin system overactivity following IR. These adverse effects of cardiac IR are more severe in men than premenopausal women and perinatal taurine depletion exacerbates these adverse effects in adult male than female animals, particularly followed by a high sugar. Fortunately, several lines of evidence report that taurine supplementation is clinically important in cardiac IR prevention and treatment in both sexes. However, a clinical guideline for taurine treatment strategy in heart diseases is still not accepted worldwide. Further use in treatment will require more data from human clinical trials.

Keywords: cardiac ischemia/reperfusion, cardiac work and control, heart disease, inflammation, oxidative stress, perinatal growth, renin-angiotensin system, taurine

Key facts

- Taurine is found abundantly in the heart.
- Taurine plays many physiological roles.
- Cardiac ischemia/reperfusion (IR) causes cardiac damage and dysregulation.
- Cardiac IR results in death.
- Taurine supplementation improves cardiac IR effects.

Summary points

- Taurine affects cardiac growth, differentiation, and function from conception to adults.
- Perinatal taurine imbalance has long-term effects on cardiac structure and function.
- Cardiac IR is the leading cause of death and disability.
- Cardiac injury and malfunction following cardiac IR involves several factors including oxidative stress and neurohormonal disturbances.
- Taurine supplementation or diets high in taurine prevent or improve cardiac IR adverse effects, particularly via its anti-oxidative activity, inhibition of sympathetic activity and renin-angiotensin system, and cardiac calcium mobilization.
- The incidence and severity of cardiac IR are sex dependent and estrogen rather than testosterone displays a protective effect. Surprisingly, taurine supplementation is clinically important in both sexes.
- Although a clinical guideline for taurine treatment strategy in heart diseases is still not accepted worldwide, many human clinical trials have been increasingly reported.

Abbreviations

AKT	Protein kinase B
AMPK	Adenosine monophosphate-activated protein kinase
IR	Ischemia/reperfusion
MAPK	Mitogen-activated protein kinase
mTOR	Mammalian or mechanistic target of rapamycin
ROS	Reactive oxygen species
SHR	Spontaneously hypertensive rats
SL	Sarcolemma
TauTKO	Taurine transporters knockout

11.1 Introduction

Taurine appears to play an important role in cardiovascular control, including the response of the organism to cardiac ischemia. The cardiovascular system supplies blood to all organs, and thus, any abnormality in the cardiovascular system can critically affect the organism as a whole. Epidemiological studies demonstrate that cardiovascular diseases (especially, cardiac IR, coronary vascular disease, stroke, and hypertension) are the leading causes of morbidity and mortality around the world (Mozaffarian *et al.*, 2016). Cardiac IR results from a period of ischemia followed by restoration of blood flow (reperfusion), resulting in injuries that cause both reversible and irreversible physiological, structural, and biochemical changes (Kalogeris *et al.*, 2012). The severity of IR-induced myocardial injury depends, at least in part, on the duration of both ischemia and reperfusion, and both sex and age also contribute to the severity (Murphy and Steenbergen, 2007). Myocardial ischemia has a close relation to autonomic nervous system dysregulation, particularly since cardiac ischemia leads to heightened sympathetic nerve activity to the heart before and during an ischemic attack, following reperfusion, and during recovery. Renin-angiotensin system overactivity is also reported to contribute to the severity of IR-induced cardiac injury and arterial pressure dysregulation (Schaffer *et al.*, 2014). In addition, perinatal environment (e.g. nutrition, stress, maternal care, and hormonal imbalance) programs cardiovascular function and disease in adults (Roysommuti and Wyss, 2014). This chapter reviews the evidence that taurine exposure can critically alter cardiac IR injury and arterial pressure regulation. Both perinatal and adult effects of taurine on cardiac function and control are discussed.

11.2 General taurine function in the heart

Taurine (2-aminoethanesulfonic acid) is a β-amino sulfur amino acid found abundantly in many human tissues, particularly brain, heart, liver, muscle, and kidney. Plasma taurine concentration (40-100 μM) is relatively low when compared to tissue taurine levels, including blood cells (white blood cells and platelets 5-20 mM; red blood cells <0.1 mM (De Luca *et al.*, 2001; Learn *et*

al., 1990) and heart (2-40 μg/g wet weight). Myocardial taurine content is species dependent and correlated to heart rate (Schaffer *et al.*, 2010). Taurine plays diverse physiological functions beginning at conception and continuing throughout life (Roysommuti and Wyss, 2014). These effects include promotion of growth and development, regulation of cell volume, production of cell and mitochondrial energy, antioxidation, modulation of immune function, adjustments to brain and autonomic function, regulation of hormones, and modification of renal excretory function and cardiac contractility. The heart is the first organ that completely forms and continuously works until the end of life. Thus, it is not surprising that taurine affects cardiac function from the fetal to aged life.

In the normal heart, taurine affects several aspects of cardiac excitation and contraction process. Myocytes' volume is regulated by ionic and solute transport across cell membrane. Taurine is the main organic osmolyte in cardiac cells. In a hypertonic environment, taurine is transported into cells via a Na^+-dependent taurine transporter, whereas in a hypotonic environment, it is released from cells via a non-selective anionic channel and a Na^+/taurine symporter (Schaffer *et al.*, 2010, 2014). This process is regulated in part by protein kinase C and AKT, which regulate several intracellular phosphorylating and dephosphorylating cascade reactions. Alterations of cytosolic sodium ion concentration also alter a Na^+/Ca^{2+} exchanger, a bidirectional transport. Thus, taurine's cell volume regulation affects the distribution of calcium, sodium, and potassium ions across the myocytes' cell membrane, i.e. affecting the cell excitability. Further, taurine moderates cell calcium homeostasis by controlling the calcium-induced calcium release from the sarcoplasmic reticulum, which is a key process in cardiac excitation-contraction coupling. Ryanodine receptors are the site of taurine action to increase calcium release from sarcoplasmic reticulum. To maintain a low cytosolic calcium ion concentration during diastole, taurine increases phosphorylation of phospholamban, the protein that activates sarcoplasmic reticulum Ca^{2+}ATPase, and increases sarcolemma Ca^{2+}ATPase activity. This process helps to maintain rapid contraction and relaxation during cardiac cycle.

In the contraction process, binding of calcium ion to troponin C is the key point of starting myosin-actin binding and the cross-bridge cycling process of thick and thin filaments. Taurine increases phosphorylation of troponin I, and that increases the sensitivity of troponin C binding of calcium ions (Galler *et al.*, 1990). Further, taurine controls mitochondrial ATP production by maintaining proper reactions in the electron transport chain. Lack of taurine (e.g. through β-alanine treatment) induces a low energy state in the myocyte (Schaffer *et al.*, 2016). Thus, the contraction and relaxation of the heart in the taurine-depleted condition is impaired similar to what occurs in cardiac hypoxia and ischemia.

Taurine also possesses anti-oxidative activity by its reaction with hydrochlorous acid to form taurochloramine, a lesser oxidant than the hydrochlorous acid. This taurine activity helps to maintain the cardiac oxidative balance and prevent cell injury and rapid apoptosis (Roysommuti and Wyss, 2014). Further, taurine inhibits the renin-angiotensin system. In case of increased cardiac preload and/or afterload (e.g. congestive heart failure and hypertension), the heart becomes hypertrophic, at least in part, relating to cardiac renin-angiotensin system overactivity.

Either taurine or angiotensin converting enzyme inhibitors/angiotensin receptor blockers prevent or decrease cardiac hypertrophy in many animal models of IR. In Japan, taurine is recommended for patients with heart failure to improve cardiac contractility and hypertrophy (Ito *et al.*, 2014). In addition, taurine increases glucose transport across cardiac cell membrane and improves insulin sensitivity in diabetic patients (Lewis *et al.*, 2014). The beneficial effects of taurine and its possible mechanisms of action on the heart are summarized in Table 11.1.

11.3 Perinatal taurine exposure and cardiac function

Cell growth and proliferation are regulated by genes that are epigenetically regulated by several factors. Among them, a cell protein named mTOR and its related pathways have been reported to play an important role in the control of cell growth, metabolism, and autophagy (Kim *et al.*, 2013). mTOR is regulated by at least three main pathways. First, the alpha-amino acids, particularly leucine and arginine, stimulate mTOR, likely via activation of Rag GTPases. This amino acid pathway appears to monitor sufficient amino acid availability for protein synthesis with respect to cell energy status. Thus, maternal protein restriction retards fetal growth in utero. Second, the energy status or ATP/AMP ratio affects mTOR activity via the AMPK pathway. Low ATP/AMP (e.g. low glucose uptake or hypoxia) causes AMPK stimulation of mTOR and vice versa. However, a sufficient energy condition is also necessary for the assembly of a functional, dimeric mTOR via AMPK-independent pathways. Third, many growth factors (particularly growth hormone and insulin/insulin-like growth factor) act on specific receptors and thereby activate many intracellular cascade reactions (particularly through protein kinase B or AKT) to stimulate mTOR activity. The interaction of amino acids, energy, and growth factor pathways determines mTOR's ability to promote cell growth and proliferation or induce autophagy.

Taurine stimulates AKT and is necessary for mitochondrial production of adequate ATP by respiratory chain reactions (Schaffer *et al.*, 2010). Thus, prenatal taurine deficiency induces low birth weight newborns, similar to the effects of maternal protein restriction (Roysommuti and Wyss, 2014). Further, both perinatal taurine depletion and intrauterine growth restriction have long-term effects on adult offspring. Thus, taurine not only controls cell growth and development (at least in part via the mTOR signaling pathway in the early life), but it may also program gene expression during later life via the mTOR pathway or other yet to be identified mechanisms.

Taurine transporters are the key process in the maintenance of taurine balance in animals and humans. TauTKO animals are deficient in taurine. Further, a variety of taurine analogues, especially β-alanine, has been used to induce a taurine-depleted condition in animal models and *in vitro* experiments. TauTKO mice display cardiac injury and remodeling in adult life (Ito *et al.*, 2010). Heart weight, myocardial mass, and systolic and diastolic contraction are markedly decreased in these animals. Mitochondrial energy production is also impaired, leading to an energy deficiency in these KO animals. Increases in superoxide production and decreases in anti-oxidative enzyme activity are also present in cardiac tissues of these animals (Ito *et al.*, 2010; Schaffer *et al.*, 2016). Further, continuous taurine supplementation improves cardiac damage

Table 11.1. General functions of taurine in the cardiac tissue.[1]

Function	Action	Possible mechanism
Growth	Perinatal growth stimulation	Cell osmoregulation and AKT pathway
	Inhibit cardiac hypertrophy	Inhibition of renin-angiotensin system
Energy production	Increase	Mitochondrial respiratory chain reaction
		AMPK pathway
Oxidative stress	Decrease	Taurochloramine formation
		Decrease ROS production
Ionic balance	Myocytes' Na^+, K^+, Ca^{2+} balance	SL Na^+/taurine symporter coupled with Na^+/H^+, Na^+/K^+, and Na^+/Ca^{2+} exchanger
		SR Ca^{2+} release and uptake
Cell volume	Regulatory volume increase and decrease	Main organic osmolyte in myocyte
L-type Ca^{2+} channel	Increase	Stimulation of channel phosphorylation
		Increase ATP production and protein synthesis
SR Ca^{2+} release	Increase	Stimulation of Ca^{2+}-induced Ca^{2+} release channel
		Ryanodine receptor phosphorylation
		Increase ATP production and protein synthesis
SL Ca^{2+}ATPase	Increase	Increase ATP production and protein synthesis
SR Ca^{2+}ATPase	Increase	Phospholamban phosphorylation
SL Na^+/Ca^{2+} exchange	Increase in low taurine	SL Na^+/taurine symporter coupled with Na^+/H^+, Na^+/K^+, and Na^+/Ca^{2+} exchanger
	Decrease in high taurine	
Ca^{2+}-troponin C binding	Increase	Phosphorylation of troponin I
Cross-bridge cycling	Increase	Increase ATP production
		Increase troponin C sensitivity to Ca^{2+}
Glucose uptake	Increase	Glucose transporter 4 synthesis and translocation to sarcolemma
Apoptosis	Decrease	Antioxidative activity
		AKT pathway
Inflammation	Decrease	Taurochloramine formation
		Inhibition of proinflammatory cytokine synthesis and action
Angiotensin II	Decrease	Inhibit angiotensin II-induced Ca^{2+} accumulation, apoptosis, and expression of atrial natriuretic peptide, transforming growth factor-beta, c-fos, and c-jun
Catecholamine effects	Decrease	Inhibit NE-induced Ca^{2+} accumulation, NADPH oxidase activation, ROS production, activation of calpain (a calcium-dependent protease), and apoptosis

[1] AKT = protein kinase B; AMPK = AMP-activated protein kinase; NADPH = nicotinamide adenine dinucleotide phosphate; NE = norepinephrine; ROS = reactive oxygen species; SL = sarcolemma; SR = sarcoplasmic reticulum.

and function, particularly if treatment is performed early in life. These adverse effects of taurine depletion are also observed in adult animals perinatally depleted of taurine. These findings strongly suggest that taurine is necessary for the growth and development of the heart.

Perinatal taurine depletion also affects the cardiovascular control mechanisms in adults. The perinatal taurine depletion alone does not affect mean arterial pressure, heart rate, and baroreflex sensitivity but decreases sympathetic and parasympathetic nerve activity in adult male rats. Further, high sugar intake since weaning increases mean arterial pressure and sympathetic nerve activity but decreases parasympathetic nerve activity and baroreflex control of heart rate and renal nerve activity in these rats, while heart rate is not disturbed (Roysommuti *et al.*, 2009). These effects of perinatal taurine depletion followed by a high sugar diet also occur in adult female rats, though the perinatal taurine depletion alone does not affect mean arterial pressure, heart rate, baroreflex sensitivity, or autonomic function. In the female rats, the adverse effects of perinatal taurine depletion followed by the high sugar intake is abolished by a short-term inhibition of renin-angiotensin system (Thaeomor *et al.*, 2010). Renal nerve activity spectral analysis indicates that renal renin release is increased in male rats perinatally depleted of taurine followed by a high sugar diet (Rakmanee *et al.*, 2017). Thus, the renin-angiotensin system overactivity may underlie the adverse effect of taurine and sugar on the neural and hormonal control of the heart and blood vessels both in males and females. Perinatal taurine depletion may disturb growth and development of central nervous system in such a way that some brain areas-related to autonomic control of the heart and circulation are sensitive to high sugar and/or insulin. Further experiments are needed to clarify this hypothesis.

11.4 Pathophysiology of cardiac IR

Among the cardiovascular diseases, cardiac IR is the leading cause of death and disability. The heart contracts and relaxes continuously to maintain adequate blood flow to all organs, and the work and the energy consumption of the heart are dependent on preload, afterload, contractility, and heart rate. Adequate coronary blood flow is necessary to continuously supply oxygen and nutrients for cardiac energy production and utilization. Following cardiac ischemia, the oxygen supply to the heart decreases leading to low ATP production by mitochondrial respiratory chain reactions (Schaffer *et al.*, 2014), and the accumulation of reducing equivalents (NADH and $FADH_2$) limits β-oxidation of fatty acids and conversion of pyruvate to acetyl-CoA (Figure 11.1). Thus, the tricarboxylic acid cycle is inhibited. Lactate dehydrogenase conversion of pyruvate to lactate to produce ATP also increases. However, anaerobic glycolysis not only produces a very low ATP level, but it also increases cytosolic H^+. To maintain the cell acidity, H^+ is extruded and Na^+ is moved into cells. Along with a low energy state, active transport is decreased, particularly for Na^+/K^+ATPase and SL and sarcoplasmic reticulum Ca^{2+}ATPase. Thus, both Na^+ and Ca^{2+} accumulate inside cardiac cells causing a cell volume increase. A regulatory volume decrease is then activated, and taurine is extruded out via a Na^+/taurine symporter. Myocardial taurine content is thus low in the ischemic phase, a condition that further exacerbates ischemia-induced cardiac injury.

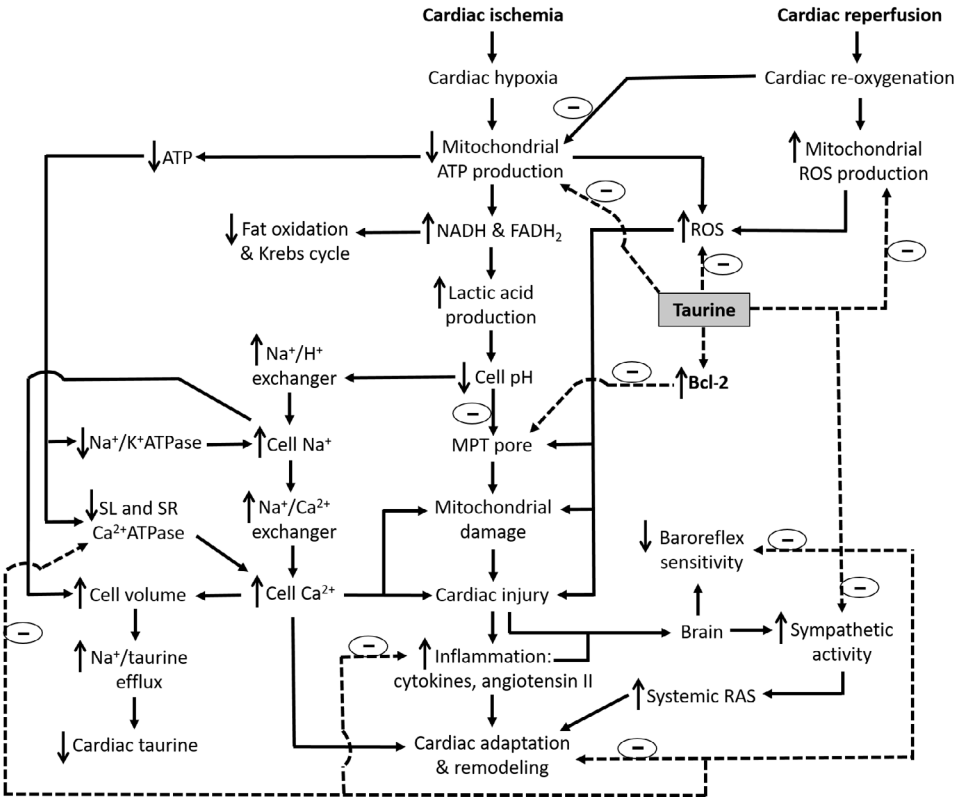

Figure 11.1. Pathophysiology of cardiac ischemia/reperfusion (solid lines) and the possible protective role of taurine (dash lines) (MPT = mitochondrial permeability transition; ATP = adenosine triphosphate; Bcl-2 = B-cell lymphoma 2; $FADH_2$ = flavin adenine dinucleotide; NADH = nicotinamide adenine dinucleotide; RAS = renin-angiotensin system; ROS = reactive oxygen species; SL = sarcolemma; SR = sarcoplasmic reticulum).

IR also increases ROS (particularly from complex I and complex II of the mitochondrial electron transport chain) (Schaffer *et al.*, 2014), and low taurine content amplifies this oxidative stress. In addition, high cytosolic Ca^{2+} and low taurine increase mitochondrial permeability transition pore causing mitochondrial swelling and damage. The severity/infarct size of cardiac injury is dependent on the severity and duration of ischemia and the subsequent reperfusion parameters.

When the ischemia is followed by reperfusion, the cardiac injury is initially worsened, instead of improvement. On reperfusion, the high oxygenation causes the mitochondria to generate more ROS by the cytochrome C oxidase in complex IV of electron transport chain (Schaffer *et al.*, 2014). Increased oxidative stress and Ca^{+} overload and low taurine and energy availability can act via many cell mediators including AMPK, protein kinase C, AKT, and Bcl-2 to cause cell injury and death. A rapid correction of cell pH by reperfusion abolishes the protective role of H^{+} on ischemia-related mitochondrial permeability transition pore, causing mitochondrial injury

and cell death. Thus, the cardiac injury is exacerbated in the reperfusion phase. In the late phase of injury (hours to days), inflammation is activated to induce cell apoptosis and the healing of the infarcted area and cordoning off the tissue that cannot be repaired. The recruitment of inflammatory monocytes involves cardiac angiotensin II and monocyte chemotactic protein-1 (Tsujioka *et al.*, 2009). Many cytokines from inflammatory lymphocytes are also involved (Wang *et al.*, 2016). Although inflammation helps to repair the myocardium, excess inflammatory responses can cause target cell injury and death. Clinically, a slow reperfusion after cardiac surgery is used to reduce the severity of cardiac IR injury, but cardiac IR injury is still present. Thus, many therapeutic agents including angiotensin II antagonists and taurine supplementation have been employed to minimize IR injury (see below).

It is well known that acute cardiac IR induces depressed baroreflex sensitivity and heightened sympathetic nerve and renin-angiotensin system activity (Ando *et al.*, 2002; Huang *et al.*, 2007; Jones *et al.*, 2008), but the mechanisms underlying these alterations have not been fully clarified. Sensory afferents appear to be sensitized by chemicals released at or pH changes in the infarct site, and this may play a prominent role in the resultant damage (Longhurst *et al.*, 2001). The severity of baroreflex and autonomic dysfunction is directly related to the infarct size and/or the severity of myocardial injury (Jones *et al.*, 2008). However, the infarct size is in turn increased by sympathetic efferent nerve overactivity and decreased by cardiac cholinergic stimulation (De la Fuente *et al.*, 2013).

Myocardial infarction patients experience increased sympathetic nerve activity beginning a few days after ischemia (2-4 days) and lasting for 3-6 months (Graham *et al.*, 2002). Baroreceptor reflex control of heart rate is also blunted or reset in these patients. In ischemic patients, a reduction in vagal activity is almost always accompanied by a concomitant increase in sympathetic activity sufficient to facilitate cardiac death (La Rovere *et al.*, 1998). Thus, β-blockers are often prescribed for the treatment of patients with chronic heart failure and myocardial infarction. Increased sympathetic and decreased parasympathetic nerve activity in cardiac IR conditions may initially result from an ischemic hypotension induced baroreceptor reflex response and cardiac sensory c-fiber activation (Morrey *et al.*, 2010). The later autonomic responses may be due to central autonomic resetting induced by neurohormonal factors, particularly the renin-angiotensin system.

In female rats, we found that mild cardiac IR induced by asphyxia does not affect baroreflex sensitivity, autonomic control of the heart, or plasma and cardiac angiotensin II levels, despite increased mean arterial pressure and heart rate. Further, short-term taurine supplementation a week before and after cardiac IR induction decreases cardiac injury, but does not improve any other parameters (Kulthinee *et al.*, 2015, 2017). When these rats are fed a high sugar diet since weaning, cardiac injury markers, mean arterial pressure, and heart rate after IR are not altered, and the baroreflex control of the heart and parasympathetic activity decrease, and sympathetic activity and cardiac but not plasma angiotensin II concentrations increase. Short-term taurine supplementation a week before and after cardiac IR induction decreases cardiac injury and restores the baroreflex sensitivity, autonomic function, and cardiac angiotensin II. This line of

evidence suggests that cardiac angiotensin II rather than the infarct size underlies the baroreflex and autonomic dysfunction after cardiac IR, at least in this female model. However, if the cardiac IR is very large, then the infarct size may have more influence on autonomic control of the heart. In dogs, blunted baroreflex sensitivity and increased sympathetic activity after cardiac IR appear to result from a sensitization of cardiac afferent fibers, while the arterial baroreceptor sensitivity seems to be preserved (Jones *et al.*, 2008).

11.5 Perinatal taurine exposure and cardiac IR

As mentioned earlier, TauTKO animals display cardiac damage and dysfunction. Thus, it is not surprising that cardiac injury and dysregulation after cardiac IR are more severe in these rats compared to normal control rats. Perinatal taurine exposure affects adult cardiac function and disease in the rat (Roysommuti and Wyss, 2014). Following IR, adult male rats perinatally depleted of taurine display increased cardiac injury and autonomic dysfunction compared to rats perinatally treated with normal or high taurine diets (Kulthinee *et al.*, 2010). Further, these adverse effects are exacerbated by high sugar intake since weaning, suggesting that a combination of high sugar intake and perinatal taurine depletion has a synergistic effect on IR-induced myocardial damage, as confirmed by elevated cardiac injury markers. The more severe cardiac IR responses in the perinatally taurine depleted rats (compared to controls) may be a consequence of their higher sympathetic and renin-angiotensin system responses. These effects also are observed in female rats, although compared to the males, in females, IR causes significantly less severe cardiac damage (Kulthinee *et al.*, 2015, 2017).

In female rats, perinatal taurine depletion alone does not alter cardiac injury and autonomic nerve activity, but it slightly depresses baroreflex function and significantly increases renal nerve activity after cardiac IR in adults compared to adult control diet rats (Kulthinee *et al.*, 2017). Further, high sugar intake since weaning increases IR-induced cardiac injury, elevates sympathetic nerve activity, decreases parasympathetic activity, and blunts baroreflex sensitivity. Both plasma and cardiac angiotensin II also are markedly elevated. These adverse effects, particularly the autonomic and baroreflex dysfunction, can be partially prevented by taurine supplementation starting a week before IR induction. Altogether, these data suggest that a combination of perinatal taurine depletion and high sugar intake since weaning exacerbates the myocardial dysfunction and arterial pressure dysregulation after cardiac IR in a gender specific manner related to the differential action of the renin-angiotensin system. Moreover, taurine supplementation can ameliorate post cardiac IR injury and arterial pressure dysregulation.

As mentioned earlier, acute cardiac IR commonly depresses baroreflex sensitivity and heightens sympathetic nerve and renin-angiotensin system activity, probably related to size or severity of the myocardial injury (Jones *et al.*, 2008). However, the effect of hypotension and hypoxemia resulting from cardiac IR injury and heart failure on nervous tissues may also contribute significantly. In addition, taurine supplementation partially improves the adverse effects of cardiac IR on cardiac injury, blunted baroreflex, and autonomic dysfunction in adult female rats perinatally depleted of

taurine followed by high sugar intake since weaning. This evidence supports a relation between cardiac infarct size and depression of baroreflex control of the heart, since taurine itself can act directly on both myocardial cells and brain areas controlling the heart.

Renin-angiotensin system overactivity is reported to underlie baroreflex and autonomic dysfunction after cardiac IR. Central and systemic inhibition of the renin-angiotensin system decreases cardiac injury, improves baroreflex sensitivity, and reduces autonomic dysfunction after cardiac IR (Huang *et al.*, 2007, 2013). Angiotensin type 1 receptors in paraventricular nucleus (Huang *et al.*, 2014) and rostral ventrolateral medulla (Shi *et al.*, 2009) seem to play a major role in these alterations. The renin-angiotensin system may be activated by increased renal sympathetic nerve activity during cardiac IR induction, due to hypoxemia and hypotension. Angiotensin II then may act centrally to depress baroreflex sensitivity and increase sympathetic nerve activity (Johns, 2005) and also directly increase cardiac injury and remodeling (Schaffer *et al.*, 2014). After cardiac IR, plasma angiotensin II increases only in the female rats perinatally depleted of taurine followed by a high sugar diet since weaning, while renal nerve activity increases in both control rats and rats perinatally depleted of taurine followed by a high sugar diet since weaning. Further, the circulating angiotensin II is completely normalized by short-term taurine supplementation, while the renal nerve activity and the baroreflex dysfunction after IR are only partially improved (Kulthinee *et al.*, 2017). These data suggest that the systemic renin-angiotensin system overactivity may partially contribute to the cardiac IR-induced baroreflex and autonomic dysfunction.

The cardiac renin-angiotensin system underlies cardiac oxidative stress and myocardial injury after cardiac IR. This is most strongly supported by *in vitro* experiments (Schaffer *et al.*, 2014). This adverse effect is abolished by taurine treatment before, during, and/or after cardiac IR induction. Our studies in female rats indicate that after cardiac IR, cardiac angiotensin II levels markedly increase in both control rats and rats perinatally depleted of taurine followed by a high sugar diet since weaning, while cardiac injury markers markedly increase only in female rats perinatally depleted of taurine followed by a high sugar diet since weaning. Further, taurine supplementation partially improves the cardiac IR injury and cardiac angiotensin II levels and abolishes a rise in plasma angiotensin II in these taurine-depleted rats. These data support the major role of cardiac rather than systemic renin-angiotensin system on cardiac IR injury, particularly in rats perinatally depleted of taurine followed by a high sugar diet since weaning.

Figure 11.2 summarizes the possible mechanisms of cardiac IR effects in adults perinatally depleted of taurine followed by a high sugar diet.

11.6 Sex differences in cardiac IR effects

Estrogen plays a protective role to healthy organs and it also blunts the development of several diseases, including cardiac IR injury (Ashraf and Vongpatanasin, 2006; Hay, 2016; Metcalfe and Meldrum, 2006). Estrogen treatment can directly decrease myocardial infarct size and cardiac

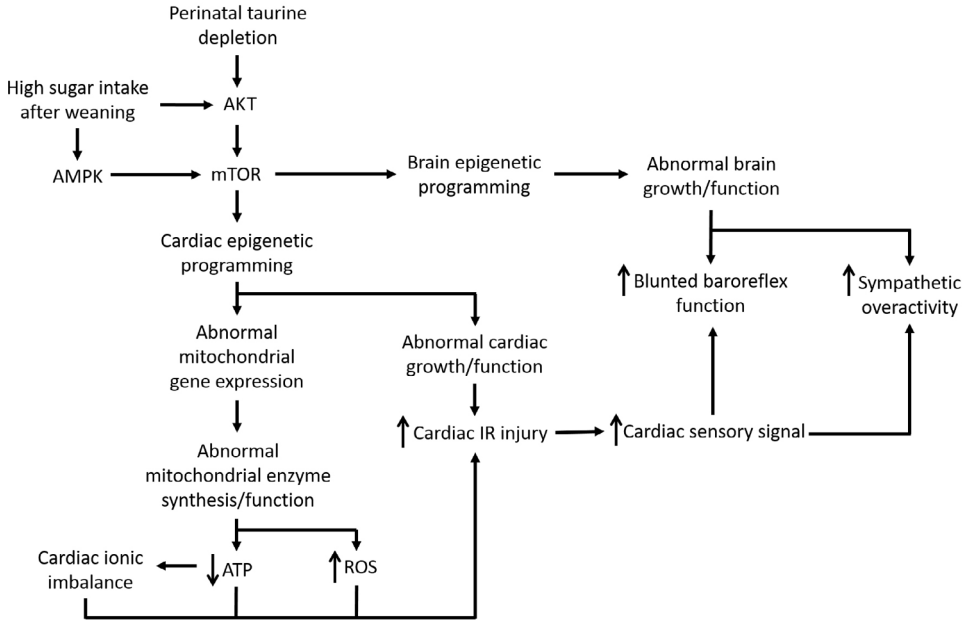

Figure 11.2. The possible pathways that perinatal taurine depletion followed by a high sugar diet after weaning exacerbates cardiac ischemia/reperfusion injury, baroreflex dysfunction, and sympathetic overactivity in adult animals (AKT = protein kinase B; AMPK = adenosine monophosphate-activated protein kinase; ATP = adenosine triphosphate; IR = ischemia/reperfusion; mTOR = mammalian or mechanistic target of rapamycin; ROS = reactive oxygen species).

arrhythmias induced by IR (Hale *et al.*, 1997), by acting on cardiac estrogen receptors (Deschamps *et al.*, 2010). The estrogen action is, at least in part, related to decreased cardiac oxidative stress (Deschamps *et al.*, 2010) and β_1-adrenergic receptor activity (Kam *et al.*, 2004). Estrogen also may modulate the adverse effects of p38 MAPK and AKT on cell growth, metabolism, ionic balance, and autophagy (Kher *et al.*, 2005). However, the beneficial effect of estrogen may be acute, and the chronic effects of estrogen treatment on ischemic heart disease appear more complex. Although estrogen decreases sympathetic nervous system activity and subsequently reduces renin-angiotensin system activity, it may also increase plasma renin activity and angiotensin II level by directly stimulating hepatic angiotensinogen synthesis (Oelkers, 1996). In female compared to male rats, the severity of myocardial damage, hypertension, blunted baroreflex sensitivity, and heightened sympathetic nerve activity following IR is lower (Kulthinee *et al.*, 2010, 2015). Further, after cardiac IR, plasma electrolytes and blood chemical parameters are relatively normal in the female compared to male rats. However, estrogen alone cannot completely prevent the adverse effect of perinatal taurine depletion and high sugar intake on cardiac IR in adult female rats.

The adverse effects of cardiac IR are more severe in males than females; however, in the recovery phase of cardiac IR, testosterone supplementation decreases cardiac injury and dysfunction similarly in both male and female animals (Bell *et al.*, 2011; Kher *et al.*, 2005). Further, testosterone decreases inflammation and increases myocardial contractility in males, and thus, castration and testosterone receptor blockade exacerbate cardiac IR injury. Castration decreases the myocardial expression of β-adrenergic receptor, L-type Ca^{2+} channel, and Na^{+}/Ca^{2+} transporter, while androgen supplementation reverses these effects. Testosterone also affects MAPK activity (Kher *et al.*, 2005). Thus, androgens may increase cardiac contractility through these mechanisms. Nevertheless, testosterone increases apoptosis in cardiac myocytes, vascular endothelial cells, and renal tubular cells. Testosterone is reported to upregulate Fas, Fas ligand, and Fas-associated death domain. Further, inhibition of caspase-3, caspase-8, or casepase-9 decreases testosterone-induced apoptosis. In addition, testosterone decreases Bcl-2 expression, which inhibits mitochondrial permeability transition pore. These diverse actions of testosterone and other anabolic androgens have often led to a lack of consideration of testosterone's cardio-protective effect. Testosterone may play a protective role in mild to moderate cardiac IR injury, while in more severe IR, testosterone may exacerbate the adverse effects of cardiac IR (Bell *et al.*, 2011).

11.7 Beneficial effects of taurine in cardiac IR

Plasma taurine levels increases in patients with acute myocardial infarction and heart failure (Schaffer *et al.*, 2014). In contrast, cardiac taurine content and taurine transporter expression decrease. Brief ischemia prior to cardiac surgery, known as ischemic preconditioning, decreases the post-surgery cardiac injury and accelerates cardiac recovery. For decades, it was postulated that a reduction of cell taurine content played the key role in this procedure, such that a low cell taurine facilitated Na^{+}/Ca^{2+} exchanges and other pathways. To date, cumulative data indicate that independent on taurine, preconditioning can modulate many cellular pathways to help cardiac myocytes resist IR injury. Further, several lines of evidence in the past decade support the beneficial effect of taurine supplementation before, during, and after cardiac IR insults. In addition, the taurine supplementation and diets high in taurine are recommended to prevent and improve cardiovascular disease in humans (Roysommuti and Wyss, 2014).

Addition of taurine to the drinking water of rodents 6 months prior to an IR insult protects the heart against ROS generation (Hanna *et al.*, 2004). Clinical studies indicate that patients receiving a rapid intravenous infusion of 5 g of taurine before bypass surgery exhibit fewer necrotic cells and less lipid peroxidation damage than patients infused with medium lacking taurine (Milei *et al.*, 1992). In a related study, arrested hearts stored in St. Thomas's cardioplegic solution containing 10 mM taurine are more resistant to storage-induced ischemic injury than arrested hearts stored for 6 hours in cold cardioplegic solution lacking taurine (Oriyanhan *et al.*, 2005). In rats, taurine feeding (200 mg/kg/day) diminishes elevations in cardiac oxidative stress, inflammation, and swelling during 5 hours of cold isotonic storage (Sahin *et al.*, 2011). In addition, taurine treatment at the time of reperfusion protects the ischemic heart against reperfusion injury, including contractile dysfunction, creatinine kinase release, and lipid peroxidation (Ueno *et al.*,

2007). This advantage of taurine may be due to the fact that taurine possesses many activities, especially antioxidation, cell volume regulation, and inhibition of renin-angiotensin system and sympathetic nerve activity.

Although taurine possesses many beneficial effects related to cardiovascular function, some disadvantages and limitations have been reported. Taurine supplementation fails to attenuate salt-induced hypertension in SHR (Dawson Jr. *et al.*, 2000). Further, the taurine supplementation rapidly increases nighttime but not daytime arterial pressure in adult SHR (Suwanich *et al.*, 2013). Taurine in drinking water for 4 weeks significantly increases arterial pressure in female, but not male, Long-Evans rats (El *et al.*, 2013). In addition, perinatal taurine excess may cause arterial pressure and renal dysregulation in adult animals; however, compared to taurine deficiency, the adverse effect of taurine supplementation is relative low, while its beneficial activity is well recognized, especially in ischemic heart disease.

11.8 Concluding remarks

Taurine is an abundant free amino acid found in the heart and possesses many cardio-protective actions. Taurine is involved in cardiac growth, metabolism, ionic balance, and oxidative activity, all of which contribute to cardiac function in health and disease. Taurine also influences neural and hormonal control of the heart. High taurine diets or taurine supplementation are reported to prevent or improve cardiovascular diseases including the ischemic heart disease. In IR, taurine supplementation before, during, and after IR decreases cardiac damage and dysfunction. Other than its cellular beneficial activity, taurine also reduces inflammation following IR. Taurine treatment also reduces blunted baroreflex sensitivity, increased sympathetic nerve activity, and renin-angiotensin system overactivity following IR. Although the adverse effects of cardiac IR are more severe in men than women, taurine supplementation is clinically important in cardiac IR prevention and treatment in both sexes. However, a clinical guideline for taurine treatment strategy in heart diseases is still not accepted worldwide. Further use in treatment will require more data from human clinical trials.

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12. Environmental causes of cardiovascular disease

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Abstract

One in three deaths in the USA can be attributed to cardiovascular disease (CVD), making it the leading cause of death, outnumbering those due to cancer and respiratory disease. There are multiple risk factors for CVD from the environment, including secondhand smoke, thirdhand smoke and other environmental pollutants such as air and noise pollution. Limiting exposure to these risk factors can be achieved through the implementation of smoking bans in homes, vehicles, and public places, as well as through public health interventions to reduce air and noise pollution.

Keywords: secondhand smoke, thirdhand smoke, air pollution, noise pollution

Key facts

- Cardiovascular disease (CVD) causes one in three death in the United States.
- Secondhand smoke may pose nearly the same amount of risk as active smoking in developing CVD.
- Thirdhand smoke (THS) can be summarized with the use of the three R's: residual tobacco that remains on surfaces or in the air and is re-emitted either in gas phase or via certain chemical reactions
- Although air pollution is not considered a main risk factor for CVD, the American Heart Association reported that exposure to fine particulate matter is associated with CVD morbidity and mortality in susceptible individuals
- Noise pollution can contribute to CVD via a stress-mediated response

Summary points

- CVD remains a significant cause of morbidity and mortality in the USA, and environmental hazards due to second hand smoke, third hand smoke, and noise and air pollution play an integral role in increasing the risk of CVD.
- With the knowledge that environmental risk factors contribute to CVD reducing exposure to environmental pollutants should be a top priority among all individuals.
- Limiting exposure can be achieved through the implementation of smoking bans in homes, vehicles, and public places, as well as through public health interventions to reduce air and noise pollution.
- Along with diet, exercise, and genetics, environmental hazards play a large role in the development of CVD.
- Avoiding contact with secondhand and THS can be challenging, but there are a number of ways to reduce or even eliminate exposure. Methods include prohibiting others from smoking in the house or vehicle, demanding smoke free workplaces, and using business that offer smoke free facilities, such as smoke free restaurants or smoke free hotels

Abbreviations

CVD	Cardiovascular disease
HONO	Nitrous acid
MI	Myocardial infarction
NNA	1-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridinyl)-4-butanal
NNK	4-(*N*-nitrosomethylamino)-1-(3-pyridinyl)-1-butanone
NNN	*N*-nitrosononicotine
SHS	Secondhand smoke
THS	Thirdhand smoke

12.1 Introduction

One in three deaths in the USA can be attributed to CVD, making it the leading cause of death, outnumbering those due to cancer and respiratory disease (Anonymous, 2016). Along with diet, exercise, and genetics, environmental hazards play a large role in the development of CVD. This chapter will focus on three key environmental risk factors that contribute to CVD: second hand smoke, third hand smoke, and environmental pollutants. Briefly, SHS is any smoke that is emitted by burning tobacco products or exhaled by an individual using a tobacco product, while third hand smoke is considered to be any remaining toxicants after smoke has cleared on surfaces, such as clothes, window dressings and wallpaper. Environmental pollutants associated with CVD include air pollution and noise pollution. Each of these three environmental risk factors and their mechanism of action will be discussed in detail during this chapter.

12.2 Secondhand smoke

A major environmental risk factor for CVD is cigarette smoke. Chemicals (tar, nicotine, carbon monoxide, acetaldehyde, formaldehyde, and lead, among others) in cigarette smoke cause endothelial cells that line blood vessels to be swollen and inflamed, leading to narrowed vessels. Narrowed vessels encourage plaque build-up in arteries and veins, which leads to clots and decreased blood flow throughout the body; clots and decreased blood flow then result in severe cardiovascular consequences such as coronary heart disease, stroke, and aneurysms. Studies show that the risk of CVD increases with the number of cigarettes smoked per day, and increases with the number of years spent smoking.

SHS, a mixture of toxic gases and particles from exhaled smoke as well as burning cigarettes, cigars, and tobacco, is often involuntarily inhaled and difficult to avoid. Studies demonstrate exposure to secondhand smoke is a significant risk factor for developing heart disease in both smokers and non-smokers. In fact, non-smokers who are exposed to SHS increase their risk of developing heart disease by 25 to 30 percent, an effect larger than one would expect based on

the cardiovascular risk associated with active smoking and the relative doses of tobacco smoke delivered to smokers versus nonsmokers (Anonymous, 2010, 2014).

Other studies further define the health risks of second hand smoke. In 2015, a meta-analysis of 24 articles, using a random effects model to quantify the effects of second-hand smoke on heart disease outcomes. Their results showed significant effect sizes in both males and females, with the relative risk for both sexes 1.35 (95% CI: 1.22-1.50) for stroke and 1.27 (95% CI: 1.10-1.48) for ischemic heart diseases (Fischer and Kraemer, 2015).

Moreover, studies show that SHS may pose nearly the same amount of risk as active smoking in developing CVD. Researchers from the Center for Tobacco Control Research and Education, Division of Cardiology, University of California, San Francisco reviewed all research on SHS and active smoking published since 1995 in order to compare the effects of SHS with the effects of active smoking. This study demonstrated that the effects of even short exposures – minutes to hours – to SHS are often almost as large (averaging 80% to 90% as large) as chronic active smoking. In people who already have heart disease, these brief exposures to SHS puts them at an even greater risk of adverse cardiovascular effects (Barnoya and Glantz, 2005). These effects of SHS are not surprising, given that SHS contains some of the same components as cigarette smoke and air pollution. There is more and more evidence that the cardiovascular system is extremely sensitive to the toxins in SHS. Data from cellular experiments and experiments in animals consistently demonstrate that SHS, like active smoking, leads to inflammation, increased blood clots, arterial stiffness, atherosclerosis, oxidative stress, inflammation, heart rate variability, energy metabolism, and increased infarct size. Low levels of exposure to SHS contribute to CVD

Over 33,000 nonsmokers each year die in the USA from coronary heart disease caused by exposure to SHS (Anonymous, 2014). Beginning with surgeon general Dr C. Everett Koop's report (U.S. Department of Health and Human Services, 2006) on the dangers of SHS and his support for warning labels on cigarettes, there have been many attempts to try and enforce smoking bans in public spaces, in an effort to reduce involuntary exposure to SHS in the USA. These laws have allowed us to study the effects of bans on CVD from a population perspective.

In 2008, the Institute of Medicine assessed the relationship between exposure to secondhand-smoke and effects on the heart (Anonymous, 2010). The committee reviewed 11 key studies regarding the effects of smoking bans; all 11 studies showed a decrease in MIs after the implementation of smoking bans. Of these, two studies looked at changes in hospitalization rates in non-smokers, demonstrating that decreased SHS exposure due to implementation of smoking bans directly lead to decreased heart attacks in nonsmokers. The nine other studies examining the effect of smoking bans provide indirect evidence of an association between SHS exposure and heart attacks. Based on its review of available literature, the committee concluded that there is a causal association between both smoking bans and SHS, and decreased MI (Anonymous, 2010). This study supports the surgeon general's report in 2006, which stated 'the evidence is sufficient to infer a causal relationship between exposure to SHS and increased risks of coronary heart disease among both men and women' (Anonymous, 2006).

There are many recent clinical trials supporting the conclusion that SHS is a hazard to public health, through increasing the risk of various CVDs. For instance, a 10-year longitudinal study from 2004 to 2014 among cardiovascular patients from 6 hospitals in Greece showed that patients who reported being exposed to SHS had a 33% (95% CI: 1.12, 1.60, $P = 0.01$) higher risk of having recurrent acute coronary syndrome events. The study reported that second-hand smoke among cardiac patients represented a substantial clinical burden in this population (Notara *et al.*, 2015). One final study quantified the impact of SHS on ischemic heart diseases, chronic obstructive pulmonary diseases, and stroke in Germany. The group performed a health impact assessment using a Markov model, which estimated that 687,254 ischemic heart disease cases and 288,015 stroke cases in Germany in 2014 were attributable to SHS exposure (Fischer and Kraemer, 2016).

Avoiding contact with SHS can be challenging, but there are a number of ways to reduce or even eliminate exposure. Methods include prohibiting others from smoking in the house or vehicle, demanding smoke free workplaces, and using business that offer smoke free facilities, such as smoke free restaurants or smoke free hotels (Anonymous, 2006).

12.3 Thirdhand smoke

THS, is known as the residual toxicants or contamination that remain after active smoke from cigarettes is removed (Winickoff *et al.*, 2009). In other words, it is an 'invisible' form of smoke that may have insidious primary and secondary effects on those with prolonged exposure. THS can be summarized with the use of the three R's: residual tobacco that remains on surfaces or in the air and is re-emitted either in gas phase or via certain chemical reactions (Burton, 2011).

The concept of second-hand smoke is well established (see prior section) and was identified by the surgeon general in 2006 as a major health concern even at minimal levels (Anonymous, 2007). SHS includes over 4,000 chemicals and encompasses side stream smoke released from the cigarette, as well as exhaled mainstream smoke from the lungs of the smoker (Acuff *et al.*, 2016). The key here is that SHS is delivered to the recipient via inhalation. THS was only officially recognized in the medical literature in 2009 (Winickoff *et al.*, 2009) and gained more public exposure after articles published in the lay press. While SHS is understood to be immediate exposure to smoke by those in proximity to active cigarettes or tobacco, exposure to THS may be significantly prolonged. THS may deposit on various house surfaces, textiles, furniture, carpeting and flooring, and even in the car. It may persist in air and as dust particles. It may also be adsorbed dermally via hands or any exposed skin and can accumulate.

Infants and children are at particular risk for the negative consequences of THS due to increased contact with contaminated surfaces (Matt *et al.*, 2011a,b). Some evidence has found remnants of THS on tiles even after 30 years (Schick, 2011). During this period of prolonged exposure, the chemicals of THS may react with common atmospheric components to result in other secondary toxicants and possible carcinogens (Martins-Green *et al.*, 2014; Sleiman *et al.*, 2010a,b). There is evidence of other negative biological influences affecting the cardiovascular,

pulmonary, endocrine, and even neurological systems (Martins-Green *et al.*, 2014). THS may disproportionately affect low-income areas, particularly in high occupancy complexes (Acuff *et al.*, 2016). Research also indicates that general cleaning, ventilation, and physical separation of smoke and occupants only partially removes THS, and thus, may not do enough to prevent evolution of toxicants. This may result in problems for future occupants of housing, automobiles, or public spaces previously exposed to cigarette smoke (Matt *et al.*, 2011a,b).

12.3.1 Toxicant mechanisms

The International Agency for Research on Cancer identified at least 60 carcinogen components of mainstream smoke, and SHS contains many of these as well (Hang *et al.*, 2013). THS contains at least 11 compounds considered highly carcinogenic such as arsenic, carbon monoxide, hydrogen cyanide, and butane (Rabin, 2009). SHS substances that are adsorbed to surfaces contain chemicals such as nicotine, aldehydes, or polycyclic aromatic hydrocarbons which can be re-emitted into the air and result in additional exposure to non-smokers over an extended period of time (Burton, 2011). The insidious nature of THS may stem from further breakdown of second-hand smoke components through reaction with atmospheric gases.

Specifically, components of SHS such as nicotine are oxidized and age as a result of interaction with atmospheric oxidants or HONO. Atmospheric ozone or other oxidants may be present in sufficient amounts indoors to react with tobacco remains on various surfaces. Furthermore, nitrous components, or HONO, may be common in apartments with combustion processes or exhaust from improper venting or appliance use. Upon interaction with tobacco residue, HONO results in conversion of SHS into tobacco-specific nitrosamines which may serve as a secondary carcinogen. The most common of these in NNA, NNN and NNK (Sleiman *et al.*, 2010a,b). Both NNN and NNK are found in emitted tobacco smoke, while NNA is specific to THS as a secondary tobacco-specific nitrosamine. The World Health Organization identified NNK and NNN as carcinogens, and there is some evidence that NNA may also be carcinogenic (WHO, 2006). THS can be inhaled or delivered as irritants or as ultrafine particles dispersed in living spaces, and NNA and NNK may thus affect various biological systems such as lung development (Rehan *et al.*, 2011). Finally, evidence of genotoxicity of THS exists which may result in irregular cell apoptosis or DNA damage or interference with replication and transcription (Hang *et al.*, 2013).

12.3.2 Exposure to thirdhand smoke

Various studies have measured different tobacco chemicals and exposure levels such as nicotine or cotinine in urine, which has a longer half-life (Benowitz, 1994, 1996). For THS, researchers have looked at NNK urine metabolites such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol as a potential marker, which has a longer half-life than cotinine. Levels have been found to be similar to infants with exposure to SHS and THS (Martins-Green *et al.*, 2014; Thomas *et al.*, 2014). It is challenging to separate THS from SHS in a living space, though markers of variable organic compounds have demonstrated the prolonged nature of THS on surfaces (Sleiman *et al.*, 2014). As THS is delivered via surface contact through dermal adsorption, through inhalation

via re-emission as a gas or as ultrafine particles, or through exposure to secondary toxicants from development of oxidative products, it is difficult to completely remove THS from a living space (Quintana *et al.*, 2013; Thomas *et al.*, 2014). As dust particles may harbor nitrosamines, it is also possible that these can be transmitted to non-smoking residences or living areas, resulting in remote toxicants and presenting an unknown threat (Ramirez *et al.*, 2014; Sleiman *et al.*, 2010a,b). Analysis of cars previously used by smokers or living spaces previously inhabited by smokers still pose a secondary threat long after the smoke has cleared (Matt *et al.*, 2011a,b). Typical ventilation or steam cleaning to remove the odor of tobacco is not sufficient, and acid wash may be necessary to completely disinfect surfaces (Goniewicz and Lee, 2015).

Part of the issue with the adsorption process of THS is that vulnerable populations such as infants or children may behaviorally experience increased dermal contact to contaminated or improperly cleaned surfaces through play, crawling, touching, or particularly orally (Matt *et al.*, 2004). There is evidence that protein mediums actually result in more efficient toxicant extraction from THS. Infant saliva, for example, thus would increase the toxicant process. Furthermore, even low levels of smoking over time may result in a cumulative effect of THS, meaning no amount of smoke is safe (Bahl *et al.*, 2016).

The rise of E-cigarettes more recently may also have a link to THS. Nicotine delivered from the device is still able to adsorb onto surfaces, though these vary depending on material. For example, tiled flooring and glass windows are particularly susceptible to nicotine build-up and thus the toxicant mechanism of THS (Goniewicz, 2015; Kuschner *et al.*, 2011).

12.3.3 Biological effects

In terms of infants and children, several studies have shown possible links between environmental tobacco smoke (including SHS) and childhood behavior problems, interruption with sleeping patterns, and even cognition development. These results have provided a basis for further analysis on possible biological effects of THS (Yolton *et al.*, 2005, 2008, 2010).

In addition to noted carcinogenic toxicants resulting from THS, there are possible links between THS and certain pathological effects. Smoking generally is discouraged prior to surgery due to known negative effects on vasculature with possible complications post-surgery. Based on animal models, THS may further impair the mechanism of wound healing as it results in angiogenesis, a delay in would closure and collagen deposits, as well as an interruption of the anti-inflammatory response at the site of injury or procedure. Other contributors to weakened tissue may include molecular and DNA damage from reduced antioxidants (Dhall *et al.*, 2016).

A broad study using animals was conducted in 2014 to look at the various pathological effects of THS on the liver, lung, skin healing, and behavior. Liver effects here included increased lipid levels from the accumulation of fat in hepatocytes resulting in steatosis and progression to possible non-alcoholic fatty liver disease leading to cirrhosis, fibrosis, and even cancer. The authors noted elevated triglycerides and low-density lipoprotein levels (bad cholesterol) with

links to CVD. THS may also result in fibrosis of the lung, possibly leading to scarring and poor oxygen diffusion with links to chronic obstructive pulmonary disorder and asthma. The study re-confirmed the presence of delayed wound healing resulting from excessive keratinization of the epithelium, a poor inflammatory response, and low collagen levels. In addition, animals demonstrated hyperactivity following exposure to THS, demonstrating a potential behavioral link (Martins-Green *et al.*, 2014).

Finally, a recent study with animal models showed a possible link between THS and enhanced platelet aggregation and integrin GPIIb-IIa activation. Disturbance of these processes resulted in shortened bleeding and occlusion time in a thrombosis model. These hyperactive platelets may thus interfere with thrombogenesis and hemostasis and related diseases (Karim *et al.*, 2015).

12.3.4 Public perception

Though research into THS is in its infancy, there has been a movement toward public awareness and methods to reduce or restrict exposure to THS and its toxicants. When Winickoff and colleagues officially coined the term 'THS' in 2009, the study showed that around 65% of nonsmokers compared to 43% of smokers believed that THS could have adverse effects on children. Furthermore, smoking prohibition was more prevalent in the homes of nonsmokers. The study concluded that across participants the belief of THS harming children was independently associated with home smoking bans (Winickoff *et al.*, 2009).

Two studies indicated that parents who received advice or information from health professionals regarding dangers of THS were more likely to agree that THS could be harmful and promote a smoke-free environment. One study did indicate that fathers and smokers were less likely to agree with this (Drehmer *et al.*, 2012, 2014). A target study on low-income individuals indicated unawareness of THS and its effects. However, with education, the population was more likely to support smoking prohibition in the home, particularly to protect children. Unfortunately, THS may have a disproportionate effect on low-income populations due to prevalence of multi-unit housing and the difficult of instituting total smoke-free bans in these areas (Escoffery *et al.*, 2013). As mentioned, physical separation from smoke and general cleaning are not sufficient to prevent THS (Matt *et al.*, 2011a,b). As a result, it will take time and effort to educate the public on the potential dangers associated with THS and to institute effective action to reduce exposure to THS or to properly decontaminate exposed surfaces or living areas.

12.4 Environmental pollutants as cardiovascular risk factors

12.4.1 Air pollution

Overview

Air pollution as a modifiable risk factor for CVD is a relatively new, and novel, concept (Brook *et al.*, 2010). Although air pollution is not considered a main risk factor for CVD, the American Heart Association reported that exposure to fine particulate matter is associated with CVD morbidity and mortality in susceptible individuals (Brook *et al.*, 2010). The impact of air pollution on the cardiovascular system is hypothesized to be of equal or greater magnitude than the impact of air pollution on the respiratory system (Brook *et al.*, 2010).

Particulate air pollution is classified by aerodynamic particle diameter, and is grouped into the following three classes: course (>2.5 μm), fine (<2.5 μm or $PM_{2.5}$), and ultrafine (<0.1 μm) particles (Brook *et al.*, 2010; Pope, 2000). The strongest evidence exists for the association of $PM_{2.5}$ and CVD morbidity and mortality (Brook *et al.*, 2010). In susceptible individuals (including the elderly and those with pre-existing cardiac conditions), acute inhalation of $PM_{2.5}$ air pollution can trigger the rapid development of cardiovascular events, including heart failure aggravation, acute myocardial infarction, stroke, and arrhythmia (Araujo and Brook, 2010; Brook *et al.*, 2010). These events have been shown to occur at an average relative risk of 1% per 10 $\mu m/m^3$ increase in $PM_{2.5}$ exposure, although this estimate varies significantly across studies (Araujo and Brook, 2010; Brook *et al.*, 2010; Newby *et al.*, 2015). Chronic $PM_{2.5}$ exposure has been associated with an approximately 10% increase in mortality due to CVD (Araujo and Brook, 2010; Hoek *et al.*, 2013), and can significantly reduce life expectancy by months to years. In sum, particulate matter can either trigger acute cardiovascular events or contribute to the development of chronic CVD years after exposure.

Combustion of fossil fuels through transportation, manufacturing, power generation, and other activities, is the main source of $PM_{2.5}$ (Brook *et al.*, 2010). However, the composition of particulate matter is complex and dependent on geographical location and time of day, and may include carbon, transition metals, nitrate, sulfate, organic gases, microorganisms, endotoxins, and pollen (Araujo and Brook, 2010; Newby *et al.*, 2015; Verrier *et al.*, 2002). The cardiovascular effects of particulate matter depend on the toxicity of its components, particle size (only particles <10 μm can fully penetrate the lungs), and surface area in relation to particle volume (Verrier *et al.*, 2002). Thus, $PM_{2.5}$ toxicity may vary significantly by time and location. The majority of $PM_{2.5}$ exposure occurs indoors, as this type of pollution is able to penetrate buildings. Inhalation of such pollutants may be an unremarkable event, yet exposure is nearly ubiquitous in the global population (Brook *et al.*, 2010).

Other air pollutants that may exacerbate CVD include combustible solid fuels, gases such as carbon monoxide, sulfur dioxide, ozone, nitrogen oxides, and volatile organic compounds (Brook *et al.*, 2010; Fatmi and Coggon, 2016; Mills *et al.*, 2009). However, the cardiovascular effects of

these gaseous pollutants are not as well described as those attributed to particulate matter and will not be discussed here in detail (Brook *et al.*, 2010; Mills *et al.*, 2009).

Proposed mechanisms

It is hypothesized that while particulate air pollution is a trigger for cardiovascular events in at-risk individuals, it may also play a role in the development of CVD (Forastiere, 2013). Particulate air pollution has been specifically associated with coronary artery disease, heart failure, MI, cardiac arrest, cerebrovascular disease, and venous thromboembolism. Evidence supporting the association between arrhythmias or venous thromboembolism and $PM_{2.5}$ air pollution is inconsistent (Newby *et al.*, 2015).

Although no single pathway definitively describing the effect of air pollution on CVD has been found, multiple mechanisms have been proposed (Araujo, 2010). Two pathways have been suggested as the principle pathophysiological mechanisms of CVD development. The classical pathway proposes that pulmonary exposure to particulate matter causes an inflammatory reaction via activation of alveolar macrophages and subsequent cytokine release (Mills *et al.*, 2009). The alternative pathway proposes that small, inhaled particles enter the systemic circulation across the blood-air barrier to directly interact with and penetrate the vascular endothelium (Mills *et al.*, 2009). Effects of the alternative pathway include increased inflammation, oxidative stress, and rupture of existing atherosclerotic plaques, leading to thrombosis and vascular damage (Koulova and Frishman, 2014; Mills *et al.*, 2009). An autonomic imbalance also appears to play a role in rapid $PM_{2.5}$-mediated CVD through activation of the sympathetic nervous system and resultant blood pressure elevation (Araujo and Brook, 2010).

A growing body of evidence supports the basis of these mechanisms. For example, studies have shown evidence of intima media thickening and coronary artery calcium deposit increases with elevations in $PM_{2.5}$ exposure (Liu *et al.*, 2015; Mills *et al.*, 2009). Further, exposure to particulate matter has been associated with increased platelet aggregation (Mills *et al.*, 2009). More evidence is needed to determine the interactions of these pathways and how the variable composition of $PM_{2.5}$ molecules contributes to the mechanism of CVD development (Araujo and Brook, 2010).

Prevention

Exposure to air pollution is universal and reducing this pollution could be important in preventing CVD (Koulova and Frishman, 2014). One study estimated that reducing pollution by 3.9 μm/m^3 could prevent up to 8,000 hospitalizations for heart failure per year and save considerable healthcare costs (Shah *et al.*, 2013). Past public health interventions and general reductions in air pollution have been associated with strong decreases in CVD morbidity and mortality, indicating that reducing air pollution has the potential to decrease CVD prevalence (Henschel *et al.*, 2012).

12.4.2 Noise pollution

Noise pollution can contribute to CVD via a stress-mediated response (Munzel *et al.*, 2014; Tetreault, 2013). Elevated corticosteroids, along with activation of the sympathetic nervous system, increase blood pressure, heart rate, and overall cardiac output (Munzel *et al.*, 2014). Individuals exposed to noise pollution are at an increased risk of hypertension, atherosclerosis, MI, and cerebrovascular events (Babisch, 2011; Munzel *et al.*, 2014). Noise does not have to be cognitively recognized or sustained at a high threshold to stimulate this cardiac response (Munzel *et al.*, 2014).

Sleep disruption due to noise pollution can be particularly harmful to health as compared to noise exposure during the day (Babisch, 2011; Munzel *et al.*, 2014). Sleep disruption due to noise exposure can cause blood pressure and heart rate elevation and changes in endothelial function along with transient catecholamine increases (Munzel *et al.*, 2014).

However, noise pollution studies involving traffic noise are potentially confounded by the effect of air pollution (Hoek *et al.*, 2013; Tetreault *et al.*, 2013). Noise pollution often accompanies the vehicular traffic that produces $PM_{2.5}$, and research does not reveal consistent independent effects of each type of pollution on CVD (Babisch, 2011; Munzel *et al.*, 2014).

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13. Bioactive nutrients potential impact on cardio-metabolic risk factors

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Abstract

The growing body of evidence suggests that aggressive treatment for the risk factor management will improve survival and reduce the cardiac events. It is well known Mediterranean diets enriched in plant foods are associated with positive health outcomes in reducing risk factors of cardiovascular disease (CVD). This review summarizes plant bioactive nutrients such as resistant starch, omega-3 fatty acids source from walnuts, avocados, coenzyme Q10, hawthorn berry fruit extract, curcumin/curcuminoids, lycopene, gugulipid and gotu kala plant sources impact on cardio-metabolic risk factors. These plant sources of bioactive nutrients exhibited protective effects against hyperglycemia, hyperlipidemia, inflammation and oxidative stress, known risk factors for CVD and diabetic complications, in cell culture, animal studies and some human studies. This review provides information on plant bioactive nutrients, with a particular focus on active nutrients involved in lipid, carbohydrate and metabolic pathways and their potential health benefits in relation to the prevention and treatment of risk factors for hypertension, endothelial function, insulin resistance, type 2 diabetes, weight management, microalbuminuria, hypercoagulability, inflammation, abnormal thrombolysis, increased oxidative stress and CVD.

Keywords: metabolism, cardiovascular disease, risk factors, bioactive nutrients

Key facts

- Bioactive components impart cardiovascular and metabolic health benefits or desirable physiological effects.
- Bioactive components act on different metabolic pathways to protect cardiovascular system and other organs.
- Promotion of health through many lifestyle factors, including the consumption of bioactive nutrients from plant sources reduce risk of cardio-metabolic risk factors.
- Plant bioactive nutrients neutralize free radicals that may cause damage to cells and reduce oxidative stress.
- Plant bioactive nutrients may reduce the risk of coronary heart disease by lowering blood cholesterol levels and increase antioxidants to reduce inflammatory factors.

Summary points

- As scientific and technological advances develop in the field of health and nutrition, more focus has been directed toward biologically active nutrients mainly from plants to reduce chronic risk factors.
- Bioactive nutrients from plants for health are an important part of an overall healthful lifestyle, balanced diet and physical activity. Cardio-metabolic syndrome is a progressive disorder and it is a better term to understand to treat its potential risk factors such as obesity, hypertension, dyslipidemia, impaired glucose tolerance, oxidative stress and endothelial dysfunction, inflammation (increase in C-reactive protein, cytokines, tumor necrosis factor alpha, interleukins 6 and 10).
- The growing body of evidence suggests that aggressive comprehensive treatment with plant bioactive nutrients with a balanced nutrition diet and regular physical activity for the risk factor management will improve health and wellness of people.

Abbreviations

AA	Asiatic acid
CMS	Cardio-metabolic syndrome
CoQ10	Coenzyme Q10 or ubiquinone
CRP	C-reactive protein
CV	Cardiovascular
CVD	Cardiovascular disease
HDL-C	High-density lipoprotein cholesterol
IL-6	Interleukin-6
LDL-C	Low-density lipoprotein cholesterol
RS	Resistant starch
TC	Total cholesterol
TG	Triglyceride
TNF-α	Tumor necrosis factor alpha

13.1 Introduction

The cardiovascular and metabolic disturbances individually and interdependently lead to a substantial increase in CVD morbidity and mortality, making the cardio-metabolic syndrome an established and strong risk factor for premature and severe CVD and stroke. Cardio-metabolic syndrome risk factors (Table 13.1) are associated with increased incidence of CVD mortality and morbidity. People with diabetes have higher incidence of CVD deaths. The non-modifiable risk factors are age, gender, height, race and family history. The modifiable risk factors include lipids and lipoproteins, diabetes, hypertension, obesity, smoking, alcohol intake, inflammatory markers and thrombotic factors.

13.2 Resistant starch

RS is a low-calorie, dietary fiber from starchy food grains. RS is classified into four types (RS1, RS2, RS3, and RS4), based on the indigestible properties. Englyst *et al.* (1992) reported that physical structure of both the starch and the food matrix indicate digestibility of the starch. A high dietary fiber intake may reduce postprandial glucose, blood lipids and inhibit vitamins and minerals. Several studies demonstrated significant decrease of malondialdehyde, glycosylated hemoglobin, insulin, TC, and non-HDL (Karimi *et al.*, 2016; Kwak *et al.*, 2012; Nichenametla *et al.*, 2014). These studies suggest RS may decrease glucose and lipids. Further long term studies are required in both health and disease conditions.

Table 13.1. Target risk factors of metabolic syndrome and cardiometabolic syndrome.[1]

Characteristics	Metabolic syndrome			Cardiometabolic syndrome[4]
	NCEP/ ATP III	AACE[2]	WHO[3]	
Plasma glucose, mg/dl		>140		IGT: 100-125 mg/dl
• Fasting	110-125		>110 and <126	HbA1c: <7%
• 120 min post-glucose challenge[5]	140-200		≥140 and <200	
TG, mg/dl	≥150	≥150	≥150	≥200; patients with very high TG should not consume alcohol. The use of bile acid sequestrant is relatively contraindicated when TG are >200 mg/dl
HDL-C, mg/dl				LDL-C: <100 mg/dl
• Men	<40	<40	<35	If TG are ≥200 mg/dl, non-HDL-C
• Women	<50	<50	<39	should be <130 mg/dl[6]
Blood pressure, mm Hg	≥130/85	≥130/85[7]	≥160/90	<140 mm Hg or <130 mm Hg if the patient has diabetes and kidney disease
Abdominal obesity or waist circumference		–	WHR:	body mass index: 18.5 to 24.9 kg/m^2
• Men	>102 cm		>0.90	waist circumference: <102 cm for
• Women	>88 cm		>0.85	men and <88 cm for women
Microalbuminuria urinary albumin excretion rate, mg/g	–	–	≥20	+
Smoking	–	–	–	complete cessation; no exposure to environmental tobacco smoke
Age, sex, family history, race	–	–	–	+
Inflammation and coagulation	–	–	–	+
Physical activity	–	–	–	30 minutes, 7 days per week (minimum 5 days per week)

[1] HbA1c = glycosylated hemoglobin A1c; HDL-C = high-density lipoprotein cholesterol; IGT = impaired glucose tolerance; LDL-C = low-density lipoprotein cholesterol; NCEP/ATP III = National Cholesterol Education Program/Adult Treatment Panel III; TG = triglycerides; WHR = waist hip ratio.
[2] Absence of diabetes-fulfillment of 2 of the risk factors.
[3] Syndrome present if two or more of the other components met.
[4] CMS is defined based on ACC/AHA/ADA recommendations.
[5] After a 75 g glucose load.
[6] Non-HDL-C = total cholesterol minus HDL-C; creatinine should be <2.5 mg/dl in men and <2.0 mg/dl in women; potassium should be <5.0 mEq/l.
[7] Current use of antihypertensive medication.

13.3 Walnuts source of omega-3 fatty acids

Walnuts are good sources of n-6 (linoleate) and n-3 (linolenate) polyunsaturated fatty acids, monounsaturated fatty acids, vitamin E, arginine, folate, fiber, tannins, and polyphenols (pedunculagin, an ellagitannin). Table 13.2 provides nutrient composition of walnuts from the USDA nutrient database (US Department of Agriculture, 2016). Ellagitannins have antioxidant and anti-inflammatory bioactivity properties. Dietary walnuts have been shown to improve antioxidant properties (McKay *et al.*, 2010), improve post prandial adiponectin (Lozano *et al.*, 2013), lower TG, LDL-C (by ≈9-16%), stimulate LDL receptor activity and promote LDL-C removal (Munoz *et al.*, 2001), TC (Kris-Etherton, 2014; Rajaram *et al.*, 2009), and lower blood pressure (diastolic blood pressure by ≈2-3 mm Hg) and cell adhesion molecules (Zhao *et al.*, 2007). There is growing evidence that walnuts can directly or indirectly influence and reduce cardio-metabolic risk factors. Walnuts (28 g/day) may improve endothelial function, diet quality in people with type 2 diabetes mellitus and improve plasma adiponectin, reduce CVD risk indices in diabetes (Katz *et al.*, 2012; Luc Djoussé *et al.*, 2016; Ma *et al.*, 2010). In another study, daily consumption of walnuts (43 g/d) for 8 weeks significantly reduced non-HDL-cholesterol and apolipoprotein-B in healthy Caucasian subjects (Wu *et al.*, 2014). Inclusion of walnuts significantly improved diet quality, endothelial function, TC and LDL-C, but had no effects on anthropometric measures, blood glucose level, blood pressure (Njike *et al.*, 2016). Endothelial function, TC and LDL-C improved significantly from baseline in the walnut-included diet. Body mass index, percentage body fat, visceral fat, fasting glucose, glycated hemoglobin, and blood pressure did not change significantly (Njike *et al.*, 2015). Inflammation is a major risk in CVD. CRP and IL-6 decreased in non-diabetic overweight/obese women in one year intervention trial with walnut-rich (18% energy) associated with weight loss comparable to a standard lower fat diet in a behavioral weight loss intervention trial (Rock *et al.*, 2016). Intake of walnut-derived fatty acids (40 g of daily walnut intake for 4 weeks) can favorably affect plasma epoxide production, resulting in improved microvascular function (Holt *et al.*, 2015). These results support the use of walnuts to improve heart health with a healthy diet along with daily physical activity.

13.4 Avocados

Avocados contain monounsaturated fatty acids (63%), saturated fatty acids (13.6%), polyunsaturated fatty acids (11.6%) dietary fiber (94%),4.2% sugars and 1.4% starch (31% carbohydrates), 7% protein and 6% micronutrients essential nutrients and phytochemicals (Table 13.3). Avocado consumption is associated with improved overall diet quality, nutrient intake, and reduced risk of metabolic syndrome (Fulgoni *et al.*, 2013; Alvizouri-Munoz *et al.*, 1992). Dreher and Davenport (2013) reported in a critical review that eight preliminary clinical studies (Carranza *et al.*, 1995; Carranza-Madrigal *et al.*, 1997; Colquhoun *et al.*, 1992; Jayaprakasam *et al.*, 2005; Lerman-Garber *et al.*, 1994; Lopez-Ledesma *et al.*, 1996; Pieterse *et al.*, 2005; Table 13.4) showing that avocado consumption helps support lowering cholesterol, TG and glycemic control to reduce risk of diabetes and cardiovascular health (Table 13.3 and 13.4). These results suggest healthy natural foods will contribute for overall diet quality and reduce risk of chronic conditions.

Table 13.2. Nutrient profile of walnuts (US Department of Agriculture, 2016).

Nutrient	Unit	Value per 100 g	1 cup, chopped, 117 g	1 cup, ground, 80 g	1 cup, in shell, edible yield (7 nuts), 28 g	1 cup shelled (50 halves), 100 g	1 cup pieces or chips, 120 g	1 oz (14 halves), 28.35 g
Proximates								
water	g	4.07	4.76	3.26	1.14	4.07	4.88	1.15
energy	kcal	654	765	523	183	654	785	185
protein	g	15.23	17.82	12.18	4.26	15.23	18.25	4.32
total lipid (fat)	g	65.21	76.3	52.17	18.26	65.21	78.25	18.49
carbohydrate, by difference	g	13.71	16.04	10.97	3.84	13.71	16.45	3.89
fiber, total dietary	g	6.7	7.8	5.4	1.9	6.7	8	1
sugars total	g	2.61	3.05	2.09	0.73	2.61	3.13	0.74
Minerals								
calcium, Ca	mg	98	115	78	27	98	1.18	28
iron, Fe	mg	2.91	3.4	2.33	0.81	2.91	3.49	0.82
magnesium, mg	mg	158	185	126	44	158	190	45
phosphorus, P	mg	346	405	277	97	346	415	98
potassium, K	mg	441	516	353	123	441	529	125
sodium, Na	mg	2	2	2	1	2	2	1
zinc, Zn	mg	3.09	3.62	2.47	0.87	3.09	3.71	0.88
Vitamins								
vitamin C, total ascorbic acid	mg	1.2	1.5	1	0.4	1.3	1.6	0.4
thiamin	mg	0.341	0.399	0.273	0.095	0.341	0.409	0.097
riboflavin	mg	0.15	0.176	0.12	0.042	0.15	0.18	0.043
niacin	mg	1.125	1.316	0.9	0.315	1.125	1.35	0.319
vitamin B6	mg	0.537	0.628	0.43	0.15	0.537	0.644	0.152
folate, DFE	mg	98	115	78	27	98	118	28
vitamin B12	mg	0	0	0	0	0	0	0
vitamin A, RAE	mg	1	1	1	0	1	1	0
vitamin, IU	mg	20	23	16	6	20	24	6
vitamin E (alpha tocopherol)	mg	0.7	0.82	0.56	0.2	0.7	0.84	
vitamin D (D2+D3)	mcg	0	0	0	0	0	0	0
vitamin D	IU	0	0	0	0	0	0	0
vitamin K	mcg	2.7	3.2	2.2	0.8	2.7	3.2	0.8
Lipids								
fatty acids, total saturated	g	6.126	7.167	4.901	1.715	6.125	7.351	1.737
fatty acids, total monounsaturated	g	8.933	10.452	7.146	2.501	8.933	10.72	2.53
fatty acids, total polyunsaturated	g	47.174	55.194	37.739	13.209	47.174	56.609	13.374
cholesterol	mg	0	0	0	0	0	0	0
Amino acids	mg	0	0	0	0	0	0	0
Other								
caffeine	mg	0	0	0	0	0	0	0

Table 13.3. Nutrient composition of avocados (09038, avocados, raw, California) (US Department of Agriculture, 2016).

Nutrient	Unit	1 value per 100 g
Proximates		
water	g	72.33
energy	kcal	167
protein	g	1.96
total lipid (fat)	g	15.41
carbohydrate, by difference	g	8.64
fiber, total dietary	g	6.8
sugars, total	g	0.3
Minerals		
calcium, Ca	mg	13
iron, Fe	mg	0.61
magnesium, Mg	mg	29
phosphorus, P	mg	54
potassium, K	mg	507
sodium, Na	mg	8
zinc, Zn	mg	0.68
Vitamins		
vitamin C, total ascorbic acid	mg	8.8
thiamin	mg	0.075
riboflavin	mg	0.143
niacin	mg	1.912
vitamin B6	mg	0.287
folate, DFE	µg	89
vitamin B12	µg	0
vitamin A, RAE	µg	7
vitamin A, IU	IU	147
vitamin E (alpha-tocopherol)	mg	1.97
vitamin D (D2 + D3)	µg	0
vitamin D	IU	0
vitamin K (phylloquinone)	µg	21
Lipids		
fatty acids, total saturated	g	2.126
fatty acids, total monounsaturated	g	9.799
fatty acids, total polyunsaturated	g	1.816
fatty acids, total trans	g	0
cholesterol	mg	0

Table 13.4. Avocado cardiovascular health clinical trial summary.[1]

Reference	Study design (n)	Study population	Dose	Results
Grant (1960)	open label study for 4 weeks (n=16)	normal/hypercholesterolemic male patients in Veteran's Administration Hospital	0.5-1.5 California avocados per day in addition to habitual diet	↓ TC by 9-43%
Colquhoun *et al.* (1992)	randomized, crossover study for 3 weeks (n=15)	females between 37 and 58 y of age	Two diets: (1) high MUFA primarily avocado diet (AE); or (2) high in complex carbohydrates low-fat diet (AHA III)	Both diets ↓ TC; AE was more effective, with an 8.2% ↓ (P<0.05) whereas AHA-III was associated with a 4.9% ↓ (NS). LDL-C and apolipoprotein B ↓ significantly on AE but not on AHA-III (P<0.05). The HDL concentration did not change on AE but ↓ 13.9% on AHA-III (P<0.01).
Alvizouri-Munoz *et al.* (1992)	randomized, crossover study for 2 weeks (n=16)	healthy volunteers	Four diets: (1) control, typical diet; (2) MUFA fat diets with avocado (75% from Hass avocados) (RMF); (3) habitual diet plus same level of Hass avocados as (2) (FME); (4) low-saturated diet (LSF)	In both RMF and LSF diets ↓ plasma TC and LDL-C levels. The levels of HDL-C significantly ↓ (P<0.05) after 2 weeks of the LSF and FME diets. The plasma triacyglycerol levels ↓after RMF and FME diets, while LSF diet ↑. In TC and in LDL-C levels, there were statistically significant differences between the FME and the LSF diet periods.
Lerman-Garber *et al.* (1994)	randomized, crossover study for 4 weeks (n=12)	women with type 2 diabetes; mean 56±8 years; BMI=28±4	Three diets: (1) control, American Diabetes Diet plan; 30% kcal from fat; (2) high MUFA diet with 1 avocado (Hass) and 4 teaspoons of olive oil; 40% kcal from fat (HMUFA); (3) high in complex carbohydrates 20% Kcal from fat (high-CHO)	Both diets had a minor hypocholesterolemic effect with no major changes in HDL-C. The HMUFA diet was associated with a greater decrement in plasma TG (20 vs 7% in the high-CHO diet). Glycemic control was similar with both diets.

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Table 13.4. Continued.

Reference	Study design (n)	Study population	Dose	Results
Carranza *et al.* (1995)	randomized crossover for study 4 weeks with a controlled diet (n=16)	hyper-cholesterolemic subjects with phenotype II and IV dyslipidemias	Two diets: (1) avocado rich diet (75% fat from avocado) diet; (2) low-saturated fat diet	In phenotype II both DRCA and DRSA significantly ↓ TC and LDL-C levels. On phenotype IV DRCA produced a mild ↓ on triglyceride levels while DRSA ↑ them. On HDL-C concentrations DRCA produced a significant ↑ in both phenotypes while DRSA did it only in phenotype IV.
Lopez-Ledesma *et al.* (1996)	randomized, controlled study for 7 days (n=67)	healthy normo-lipidemic subjects (<200 mg/dl) and mild hyper-cholesterolemia and type 2 diabetic patients (201-400 mg/dl)	Enriched avocado diet vs isocaloric non-avocado diets. 300 g Hass avocado substituted for other lipid sources (both diets contained about 50% kcal from fat	16% ↓ serum TC level followed the high MFA diet, while it ↑ after the control diet (P<0.001 between diets). In hypercholesterolemic subjects a significant (P<0.01) ↓ serum TC (17%), LDL-C (22%) and TG (22%), and increase of HDL-C (11%) levels occurred with the avocado diet, while no significant changes were noticed with the control diet.
Carranza-Madrigal *et al.* (1997)	randomized, prospective, transversal and comparative 4 week study and controlled diet (n=13)	dyslipidemic subjects with high BP	Three vegetarian diets: (1) 70% carbohydrate, 10% protein and 20% lipids; (2) 60% carbohydrates, 10% protein and 30% lipids (75% of the fat from Hass avocados); (3) diet 2 w/o avocado	AVD produced a significant ↓ LDL. ALVD did not change TC and LDL, while FDWA ↑ them slightly. The three diets ↓ TG levels, but only ALVD did so significantly. All three diets ↓ HDL levels, particularly ALVD, which produced the greatest ↓. Low-fat, carbohydrate-rich vegetarian diets may be harmful to hypercholesterolemic patients.

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Table 13.4. Continued.

Reference	Study design (n)	Study population	Dose	Results
Pieterse *et al.* (2005)	randomized, controlled, parallel study, free-living (n=61)	male (n=13) and female (n=48) adults with a age 40.8±8.9 years; BMI=32±3.9 free living population	Energy restricted diet for 6 weeks at the rate of 30% kcal from fat – 200 g avocado/day (30.6 g fat) substituted for 30 g of mixed fat (e.g. margarine and vegetable oil) compared to a control diet without avocado	The percentage of plasma oleic acid ↑ significantly with the consumption of avocado in the experimental group, whereas a decrease was seen in the percentage of myristic acid from baseline to the end of the intervention in both groups but was significant only in the experimental group. Anthropometric measurements (body mass, BMI, and percentage of body fat) ↓ significantly in both groups during the study ($P<0.001$), and the change was similar in both groups. Serum lipid concentrations (TC, LDL-C, HDL-C, and triacylglycerols), fibrinogen, BP, and arterial compliance did not change significantly within or between groups.

[1] ALVD = vegetarian diet composed of 70% carbohydrates, 10% proteins and 20% lipids; AVD = avocado; BMI = body mass index; BP = blood pressure; DRCA = diet rich in monounsaturated fatty acids; DRSA = low-saturated fat diet without avocado; FDWA = avocado-added free diet; FME = free monounsaturated-enriched; HDL-C = high-density-lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; LSF = low-saturated fat; RMF = rich-monounsaturated fat; TC = total cholesterol; TG = triglycerides.

13.5 Coenzyme Q10

CoQ10 or ubiquinone is a fat-soluble compound, an endogenous enzyme cofactor and provides antioxidant properties (Lee *et al.*, 2012). It is a potent antioxidant and is necessary for energy production in mitochondria. Soybean and canola oils, and nuts, fruits, vegetables, eggs, and dairy products are sources of CoQ10 (Mattila *et al.*, 2001). The main properties of CoQ10 are its role in biochemical process supplying cardiac cells with energy; its role as a cell membrane protecting antioxidant and its direct effect on genes involved in inflammation and lipid metabolism. CoQ10 may reduce blood pressure (Singh *et al.*, 1999). Hypertensives had low plasma CoQ10. CoQ10 significantly increased HDL and significantly reduced cardiac events and death when compared to vitamin B (Singh *et al.*, 1999). In a systematic review and meta-analysis of randomized controlled trials (seven clinical trials, n=356 patients, lasting at least 12 weeks) on CoQ 10 reported decrease in TG and no beneficial effects on glycemic control, lipid profile or blood pressure in patients with diabetes (Suksomboon *et al.*, 2015). CoQ10 is considered a part of adjunct therapy for congestive heart failure patients in Europe, Russia, and Japan (Tran *et al.*, 2001). Over all the evidence for CoQ10 in the treatment of heart failure is controversial and remains unclear.

Belardinelli *et al.* (2006) reported improved heart function. Daily supplementation of 100 mg CoQ10 (n=30, 12 week intervention) is effective in decreasing pro-inflammatory factors, such as IL-6 and high-sensitivity C-reactive protein, and increasing adiponectin in mild hypertensives (Bagheri *et al.*, 2015). Statins therapy may reduce the biosynthesis of CoQ10. Lee *et al.* (2012) reported CoQ10 supplements at a dose of 150 mg can decrease oxidative stress and increase antioxidant enzyme activity in patients with CAD. A higher dose of coenzyme Q10 supplements (>150 mg/d) might promote rapid and sustainable antioxidation in patients with CAD. CoQ10 supplementation (300 mg/d, n=42) significantly enhances antioxidant enzymes activities (superoxide dismutase, catalase, and glutathione peroxidase) and lowers inflammation (CRP, TNF-α, and IL-6) in a 12 week intervention trial (Lee *et al.*, 2013). Further studies are required to study the effects of CoQ10 on the quality of life, hospitalization and death rates of CVD.

13.6 Hawthorn berry fruit extract

The hawthorn berry (*Crataegus oxyacantha*) comes from a large genus of shrubs and trees in the family Rosaceae. Hawthorn (Crataegus spp.) may have some potential benefits in congestive heart failure, ability to increase the integrity of the blood vessel wall and improve coronary blood flow, and positive effects on oxygen utilization (Asher *et al.*, 2012; Rigelsky *et al.*, 2002; Zhang *et al.*, 2001). Hawthorn decreased serum TC, LDL-C and TG in hyperlipidemic subjects (Chen *et al.*, 1995; Von Eiff, 1994). The recommended daily dose of Hawthorn for heart patients is 160 to 900 mg of extract of the leaves of flowers administered in two or three doses.

In a meta analysis (eight trials, n=632) patients with chronic heart failure (New York Heart Association classes I to III) showed beneficial effects with hawthorn treatment (Pittler *et al.*, 2003). Symptoms such as dyspnea and fatigue improved significantly with hawthorn treatment

as compared with placebo. Hawthorn had a significantly stronger effect among the 70% of patients with ischemic disease (Lalukota *et al.*, 2004). Hawthorn may cause infrequent, mild, and transient adverse events such as mild rash, headache, sweating, nausea, dizziness, and cardiac and gastrointestinal symptoms. There were no reports of drug interactions but potential theoretic interactions exist with antiarrhythmics, antihypertensives, digoxin, and antihyperlipidemic agents.

13.7 Lycopene

Lycopene is a bright red carotenoid pigment, a phytochemical found in tomatoes and other red fruits. Lycopene is the most common carotenoid in the human body and is one of the most potent carotenoid antioxidants. In a recent met analysis (Cheng *et al.*, 2017; 1,189 publications identified, 21 fulfilled inclusion criteria) reported effects of tomato products and lycopene supplementation on CV risk factors among adult subjects >18 years of age. It was observed that interventions supplementing tomato were associated with significant reductions in LDL-cholesterol (P=0.006), IL-6 (P=0.03), and improvements in flow mediated vasodilation (2.53%; P=0.01); while lycopene supplementation reduced systolic-blood pressure (-5.66 mm Hg; P=0.002). No other outcome was significantly affected by these interventions. Overall, tomato products and lycopene supplementation decrease CV risk factors and improves endothelial function, blood lipids and blood pressure.

Tomato extract significantly reduced systolic and diastolic blood pressure in a hypertension trail (n=31, Grade I hypertension, 8 week trial). Systolic blood pressure decreased from 144 to 134 mm Hg (P<0.001), and diastolic blood pressure decreased from 87.4 to 83.4 mm Hg (P<0.05). In addition, Thiobarbituric acid-reactive substances, a lipid peroxidation products marker, decreased from 4.58 to 3.81 nmol/mg (P<0.05). These results suggest short-term treatment with antioxidant-rich tomato extract can reduce blood pressure (Engelhard *et al.*, 2006). The Kuopio Ischaemic Heart Disease Risk Factor Study (725 middle-aged men, Rissanen *et al.*, 2001) showed men in the lowest quartile of serum lycopene had more than a threefold risk of acute coronary event or stroke during a five-year follow-up relative to higher concentrations. These results suggest circulating levels of lycopene, a biomarker of tomato-rich food, may play a role in early stages of atherogenesis. Potential mechanism include possible mechanisms include enhanced LDL degradation, LDL particle size and composition, plaque rupture, and altered endothelial functions. Further studies are recommended to explore longer term lycopene supplementation and the incidence of cardiac events.

13.8 Curcumin/curcuminoids

Curcumin modulates multiple molecular targets (Figure 13.1), cell signaling proteins, cell cycle proteins, cytokines and chemokines, enzymes, receptors and cell surface adhesion molecules (Joe *et al.*, 2004; Shishodia *et al.*, 2005). Recent clinical studies have demonstrated curcumin

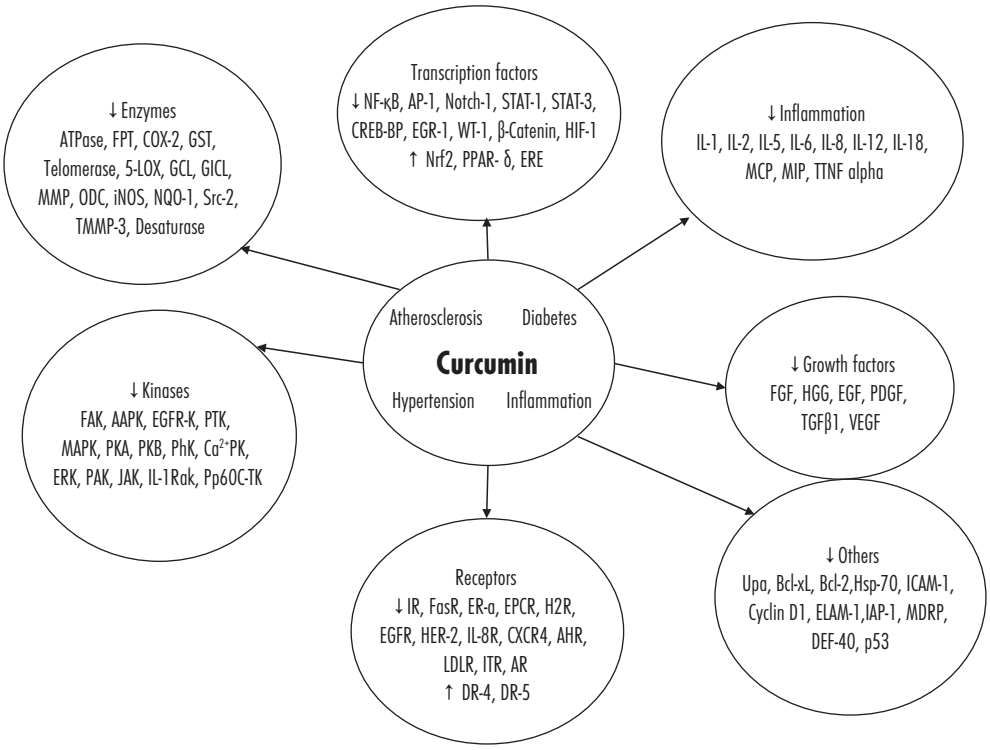

Figure 13.1. Curcumin potential role in cardio metabolic syndrome.

reduces blood cholesterol (Asai *et al.*, 2001; Keshavarz, 1976; Patil *et al.*, 1971; Rao, 1970; Soudamini, 1992); prevents low-density lipoprotein oxidation (Naidu *et al.*, 2002; Patro *et al.*, 2002; Ramírez-Tortosa *et al.*, 1999); inhibits platelet aggregation (Srivastava, 1986; Srivastava *et al.*, 1995); suppresses thrombosis and myocardial infarction (Dikshit *et al.*, 1995; Nirmala and Puvanakrishnan, 1996a,b; Venkatesan, 1998); suppresses symptoms associated with type II diabetes (Arun *et al.*, 2002; Babu *et al.*, 1995, 1997, Babu *et al.* 1998; Rahimi *et al.*, 2016), metabolic syndrome (Di Pierro *et al.*, 2015; Panahi *et al.*, 2014; Yang *et al.*, 2014) and 200 mg curcuminoids (healthy subjects, 8 weeks intervention) improved endothelial function by 3.0% increase (90% CI = 0.7 to 5.3%, P=0.032 (Oliver *et al.*, 2016). These results suggest curcumin/curcuminoids role in cardiovascular health. Further long term studies are required to explore its antioxidant activity in health and disease.

13.9 Gugulipid

Guggul is an extract from the resin of the mukul myrrh tree (*Commiphora mukul*), a tree that secretes a resinous substance called gum guggul. The medicinal uses of gugulipid are for obesity, atherosclerosis, and various inflammatory conditions (Dev, 1997). Gugulipid reduced levels of

TC by 11%, LDL-C by 12%, and TG by 15% (Nityanand *et al.*, 1989; Singh *et al.*, 1994) in Asian Indian population. Guggulsterones are the bioactive compounds of guggul may potentially involve in action for the hypolipidemic effects. In 2003, Szapary *et al.* studied the short-term safety and efficacy of oral doses, three times daily, of standard-dose gugulipid (1,000 mg), high-dose gugulipid (2,000 mg), (gugulipid, containing 2.5% guggulsterones) in 103 ambulatory, community-dwelling, healthy adults with hypercholesterolemia in U.S for 8 weeks. LDL-C was increased by 4% at eight weeks. No significant changes in levels of TC, HDL-C, TG, or very low-density lipoprotein cholesterol in response to any dose of gugulipid. Gugulipid was generally well tolerated. There were no significant changes in liver and kidney functions but six subjects developed a dermatologic hypersensitivity reaction rash (Szapary *et al.*, 2003). In another study (Norwegian general practice. 43 women and men, age 27-70, with moderately increased cholesterol) guggul at 2,160 mg (4 capsules) daily, or placebo for 12 weeks used in healthy adults with moderately increased cholesterol (Nohr *et al.*, 2009). After 12 weeks, mean levels of TC and HDL-C in the active group were significantly reduced compared with the placebo group. No change in LDL-C, TG, and TC/HDL-C ratio were observed. Mild gastrointestinal discomfort, possible thyroid problems, and generalized skin rash were reported. Gugulipid received regulatory approval in India in 1987 for use as a lipid-lowering drug, and is available in the USA as a dietary supplement. Further safety studies are required and clinical studies are required in different populations.

13.10 Gotu kola

AA is a triterpenoid isolated from gotu kola (*Centella asiatica*), which has been used as a medicinal herb in South East Asian countries. In animal studies, AA significantly improved insulin sensitivity, lipid profiles, hemodynamic parameters, oxidative stress markers, plasma TNF-α, NOx, and recovered abnormality of endothelial/inducible nitric oxide synthase expressions (Pakdeechote *et al.*, 2014) and decreased vascular $O_2(\bullet\text{-})$ production, consistent with downregulation of phox expression, was also observed after AA treatment (Bunbupha *et al.*, 2014). Gotu kola has a diuretic and blood purifying property (Brinkhaus *et al.*, 2000). Standardized extracts contain 29 to 30% AA and 20 mg for scleroderma and up to 180 mg for venous insufficiency was reported. Gotu kola may help reduce swelling and improves blood flow. In another study, total triterpenic fraction of *C. asiatica* significantly decreased of the abnormally increased capillary filtration rate, ankle circumference, and Ankle edema coin tester time in patients in four weeks (Belcaro *et al.*, 1990). Further safety studies and clinical studies are required in different populations with CVD.

13.11 Concluding remarks

A number of modifiable and non-modifiable risk factors are known to increase heart disease. Some risk factors are modifiable such as diet and lifestyle factors. Improvements in modifiable risk factors can help reduce the risk of CHD.

Overall, some of these bioactive nutrients may reduce risk factors of diabetes, hypertension, inflammation and CVD. However further long term safety and efficacy studies including manufacturing and quality control are required for these bioactive nutrients. Long term effects are needed to explore in health and disease conditions to reduce cardiac events and to improve healthy metabolism.

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14. Dietary considerations for reducing cardiometabolic risk in older adults

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Abstract

The aim of dietary guidance specifically targeted for older adults to minimize the risk of developing cardiometabolic disorders is, for the most part, consistent with that associated with optimal health outcomes throughout adulthood. Evidence suggests that there is a direct link between healthy dietary patterns, cardiometabolic disorders and total mortality. Due to diminished levels of physical activity, decreased metabolic rates, and increased proportions of fat to lean muscle mass, energy requirements decline with advancing years whereas nutrient requirements remain either unchanged or increase. To accommodate these changes there need to be increased efforts to educate 'younger' older adults to these changing and how to gradually accommodate them, such as paying increased attention to choosing nutrient dense foods within and among food categories. The use of nutrient supplements by older adults should be monitored to minimize the risk of overconsumption. This is particularly important because older adults who report using nutrient supplements tend to be those who have dietary and lifestyle patterns that are most closely associated with lower rather than higher risk for cardiometablic disorders and nutrient insufficiency. With advancing age it may be necessary to adapt living environments to promote the ability to acquire and prepare familiar foods. This is particularly important during times of change such as living environment or composition of the household. The data suggestion at any age individuals can benefit from improvements in dietary and physical activity patterns. The period of time older adults can expect to remain active, productive and independent continues to expand. Hence, no one is too old to benefit from improvements in lifestyle behaviors.

Keywords: diet, nutrition, food, nutrients, cardiovascular disease, healthy dietary pattern, older adults

Ronald Ross Watson and Sherma Zibadi (eds.) Handbook of nutrition and heart health
Human Health Handbooks no. 14 - DOI 10.3920/978-90-8686-853-7_14, © Wageningen Academic Publishers 2017

Key facts

- Approximately 15% of the USA population was 65 years or older in 2014; this is expected to grow to ~22% by 2040 and ~30% by 2060.
- Worldwide the number of people age 65 years and older will increase from ~8% of the population to ~16% by 2050.
- Approximately 33% of Americans have high low ('bad') density lipoprotein cholesterol concentrations, whereas approximately 20% of Americans have low high ('good') density lipoprotein cholesterol concentrations.
- For individuals age 65-74 years ~68% of women and ~62% of men have high blood pressure; for individuals age 75 years and older this increases to ~80% of women and ~77% of men.

Summary points

- Energy requirements decrease with increasing age; whereas nutrient requirements remain stable or increase
- To meet nutrients requirements older adults should choose nutrient dense foods within each food category.
- Dietary recommendations to minimize cardiovascular disease risk in older adults are similar to younger adults; choose a dietary pattern containing a variety of vegetables from all of the subgroups – dark green, red and orange, legumes (beans and peas), starchy, and other; fruits, especially whole fruits; grains, at least half of which are whole grains; fat-free or low-fat dairy, including milk, yogurt, cheese, and/or fortified soy beverages; variety of protein foods, including seafood, lean meats and poultry, eggs, legumes (beans and peas), and nuts, seeds, and soy products; and liquid vegetable oils.
- Emphasis should be put on replacing major sources of dietary saturated fat (meat and dairy) with unsaturated fat (liquid vegetable oils), not reducing the total amount of fat.
- A healthy dietary pattern should be tailored to an individual's personal, cultural and traditional preferences.
- A healthy dietary pattern should result in blood lipid and glucose concentrations within optimal ranges.
- All individuals, particularly those with high blood pressure should reduce sodium (salt) intake.
- With advancing years, particularly if there is a change in living situations, accommodations should be made to facilitate adherence to a healthy dietary pattern.

Abbreviations

HDL-C	High-density lipoprotein cholesterol
LDL-C	Low-density lipoprotein cholesterol

14.1 Introduction

There is a consistent demographic trend towards a shift in the age distribution within populations of both developed and developing countries towards older age groups. In 2014 approximately 14.5% of the USA population was 65 years or older. That translates to 46.2 million people or about one in every seven. This proportion of older adults is expected to grow to 21.7% by 2040, doubling current numbers to about 98 million by 2060 (https://www.acl.gov/news-and-events/announcements/subject-profile-older-americans-2016). Similar trends are seen globally. In 2010 it was estimated that 524 million people were aged 65 or older, representing about 8% of the world's population. This number is expected to grow to 1.5 billion by 2050, representing 16% of the world's population. Although currently developed compared to developing countries tend to have older population profiles, the majority of this rapidly expending age group is occurring in latter group of countries. It has been estimated that between 2010 and 2050 the number of older adults will increase by about 71% in developed countries and 250% in developing countries (http://tinyurl.com/jxcn74c). As a larger proportion of the population enters the older age categories, more emphasis needs to be placed on dietary guidance early in life with the aim of enabling older adults to stay healthy and active longer, and delay the onset of chronic diseases.

14.2 Nutrient recommendations for older adults

In the late 1990's Food and Nutrition Board of the Institute of Medicine recognized the changing age demographics worldwide and altered its rubric with which recommended dietary allowances was established. With regard to this issue the major change was an expansion of the adult age categories. Prior iterations of the recommended dietary allowances only provided guidance for individuals above the age of 50 years (IOM, 1994). This was revised in the late 1990's and early 2000 to separate this category into those aged 51 to 70 years and greater than 70 years (Table 14.1) (IOM, 1997, 1998, 2000, 2001, 2004, 2005a,b). As yet to be determined is whether upcoming versions of the recommended dietary allowances will further distinguish among individuals within the 70 years and older category as new data emerges.

Older adults who report consuming higher quality diets (Anderson *et al.,* 2010; De Groot *et al.*, 1996; Houston *et al.*, 1997; Reedy *et al.*, 2014; Sahyoun *et al.*, 1996; Schwingshackl and Hoffmann, 2015) and engaging in the highest level of physical activity (Blain *et al.*, 2010; Studenski *et al.,* 2010) have the most favorable survival rates. Since lifestyle behaviors do not change significantly once established these data suggest dietary practices and physical activity paterns in younger years may be important in determining health outcomes in later life. Nevertheless, there is no

Table 14.1. Recommended dietary allowances for older adults age 31-50 years, 51-70 years and greater than 70 years (Lichtenstein, 2013; with permission of Springer).[1]

Nutrient	Females (years)			Males (years)		
	31-50	51-70	>70	31-50	51-70	>70
vitamin A (µg/d) [a]	700	700	700	900	900	900
vitamin C (mg/d)	75	75	75	90	90	90
vitamin D (µg/d)	15	15	20	15	15	20
vitamin E (mg/d)	15	15	15	15	15	15
vitamin K (µg/d) [b]	90	90	90	120	120	120
thiamin (mg/d)	1.1	1.1	1.1	1.2	1.2	1.2
riboflavin (mg/d)	1.1	1.1	1.1	1.3	1.3	1.3
niacin (mg/d)	14	14	14	16	16	16
vitamin B6 (mg/d)	1.3	1.5	1.5	1.3	1.7	1.7
folate (µg/d)	400	400	400	400	400	400
vitamin B12 (µg/d)	2.4	2.4	2.4	2.4	2.4	2.4
pantothenic acid (mg/d)	5	5	5	5	5	5
biotin (µg/d)	30	30	30	30	30	30
choline (mg/d)	425	425	425	550	550	550
calcium (mg/d)	1000	1,200	1,200	1000	1000	1,200
chromium (µg/d)	25	20	20	35	30	30
copper (µg/d)	900	900	900	900	900	900
fluoride (mg/d)	3	3	3	4	4	4
iodine (µg/d)	150	150	150	150	150	150
iron (mg/d)	18	8	8	8	8	8
magnesium (mg/d)	320	320	320	420	420	420
manganese (mg/d)	1.8	1.8	1.8	2.3	2.3	2.3
molybdenum (µg/d)	45	45	45	45	45	45
phosphorus (mg/d)	700	700	700	700	700	700
selenium (µg/d)	55	55	55	55	55	55
zinc (mg/d)	8	8	8	11	11	11
potassium (g/d)	4.7	4.7	4.7	4.7	4.7	4.7
sodium (g/d)	1.5	1.3	1.2	1.5	1.3	1.2
chloride (g/d)	2.3	2.0	1.8	2.3	2.0	1.8

[1] White cells: recommended dietary allowance values; grey cells: adequate intake values.

evidence to suggest it is never too late to reap benefits from a high quality diet. Assessing diet quality is challenging. A number of systems have been developed to score food and beverage intake. Of note, although small differences among these systems have been reported current data

suggestions common elements unite them (McCullough, 2014). These include a predominance of fruits, vegetables, whole grains, nuts/legumes, low fat dairy, fish, and monounsaturated and polyunsaturated fats, and less meat, sugar-sweetened beverages and sodium.

One critical component of maintaining optimal dietary patterns with advancing years is to recognize changes that occur as a result of the aging process and make real-time small accommodations. With regard to energy requirements they decline with advancing years. This can make it challenging to maintain a healthy body weight and achieve adequate nutrient intakes. The decline in the total energy requirements is attributed to a gradual shift in body composition from lean to fat even in the absence of a change in body weight, and a decline in levels of physical activity. This shift in body composition results in a reduced basal metabolic rate which in turn results in a lower energy requirement to maintain a constant body weight (Williamson, 1993). Because nutrient needs either remain the same or increase, this means it is essential to choose nutrient dense foods to ensure all essential nutrient requirements are met. This is of particular concern for vitamin D (Table 14.1) (IOM, 2010). Likewise, the Recommended Dietary Allowances for vitamin B6 and calcium are higher for individuals >70 years than those below 70 years (IOM, 1998). In contrast, the Recommended Dietary Allowances for sodium is lower for older adults, making it somewhat more challenging to comply within our current food environment.

14.3 Approaches to minimized cardiometabolic risk in older adults

The rate of cardiovascular disease increases with age, particularly after menopause in women (http://tinyurl.com/m2dpgyg). For individuals age 60-79 years approximately 10% of women and 20% of men have been diagnosed with coronary heart disease. For individuals aged 80 years and older this increases to approximately 19% of women and 32% of men. The average age of first heart attack is 70 years for women and 65 years for men. For individuals age 60-79 years approximately 5% of women and 6% of men have had a stroke. For individuals age 80 years and older this increases to approximately 14% of women and 16% of men.

Aging may be accompanied by slow changes in an individual's physical and psychosocial characteristics. When these changes occur the rate of onset is highly variable. Aging may also be accompanied by the development of chronic diseases such as dyslipidemia, hypertension and insulin resistance. In all cases, dietary modification is the cornerstone of treatment. Complying with recommended dietary modifications may make it necessary to curtain the consumption of familiar foods. In an attempt to circumvent these food and beverage restrictions older adults may turn to what appear to be more acceptable food fads and nutrient supplement claims, particularly those that promise to slow the aging process (http://tinyurl.com/ma3f8v6; Kantor *et al.*, 2016). Succumbing to these unsubstantiated claims can divert resources from food budgets and potentially result in unbalanced eating patterns or nutrient intakes. Of particular concern is nutrient overconsumption because they can interfere with prescription and non-prescription drug actions, and utilization of essential nutrients.

In formulating dietary guidance to minimize cardiometabolic disease risk the 2006 American Heart Association (Lichtenstein *et al.*, 2006), 2013 American Heart Association/American College of Cardiology Lifestyle Guidelines (Eckel *et al.*, 2014), and 2015 Dietary Guidelines for Americans (http://tinyurl.com/24w3y46) consistently focuses on dietary patterns rather than individual foods or nutrients. As summarized in the latter document, similar to the former two documents, the recommend dietary pattern to minimize cardiovascular disease risk includes a variety of vegetables from all of the subgroups – dark green, red and orange, legumes (beans and peas), starchy, and other; fruits, especially whole fruits; grains, at least half of which are whole grains; fat-free or low-fat dairy, including milk, yogurt, cheese, and/or fortified soy beverages; variety of protein foods, including seafood, lean meats and poultry, eggs, legumes (beans and peas), and nuts, seeds, and soy products; and liquid vegetable oils. An integral part of the guidance is that these patterns should be tailored to an individual's personal, cultural and traditional preferences. No specific modifications were recommended for older adults. In general, the response to dietary modifications aimed at optimizing cardiometabolic risk factors appears similar across different age groups and both sexes (Lapointe *et al.*, 2006).

14.4 Optimizing cardiometabolic risk factors

14.4.1 Meeting nutrient requirements within energy needs

A challenge with advancing years is to meet nutrient requirements within the context of reduced energy needs. In order to ensure nutrient requirements are met while not overconsuming energy it is essential to make individual foods choices within each food group relatively high in nutrient density (nutrients per calorie). For some individuals this may be easy to accomplish, for example, switching from pale to deeply colored fruits and vegetables, such as romaine lettuce and peaches. For others it may mean increasing the relative proportion of vegetables and fruits compared to other meal components or substituting nuts, seeds and fruit for traditional snack foods. Additional approaches include boasting fiber intake by shifting from refined to whole grain breads and cereals, or from juice to whole fruit and vegetables. The wide range of food preferences among individuals suggest these changes need to be customized to an individual's habitual food choices. Particularly for older adults, such changes should be customized within the context of established eating patterns and food availability so that the enjoyment of food is maintained and enhanced.

14.4.2 Achieving lipid concentrations within acceptable ranges

Approximately 33% of Americans have high ('bad') LDL-C concentrations, whereas approximately 20% of Americans have low ('good') HDL-C concentrations (http://tinyurl.com/m2dpgyg). For all age groups, particularly for older adults, ischemic heart disease death rates are positively correlated with LDL-C concentrations and inversely correlated with HDL-C concentrations. For individuals with elevated LDL-C concentrations the American Heart Association recommends to consume a dietary pattern that emphasizes intake of vegetables, fruits, and whole grains;

includes low-fat dairy products, poultry, fish, legumes, non-tropical vegetable oils, and nuts; and limits intake of sweets, sugar-sweetened beverages, and red meats (Eckel *et al.*, 2014). These recommendations are consistent with those of the 2015 Dietary Guidelines for Americans (2015). No specific modifications are recommended for older adults. Within these dietary patterns emphasis is no longer focused on amount of dietary fat, rather the type of dietary fat. Sources of saturated fat, primarily meat and dairy fat, should be replaced with liquid vegetable oils (Wang *et al.*, 2016).

14.4.3 Maintaining blood pressure within recommended values

The incidence of hypertension, particularly isolated systolic hypertension, increases with age (http://tinyurl.com/m2dpgyg). For individuals age 65-74 years approximately 68% of women and 62% of men have high blood pressure. For individuals age 75 years and older this increases to approximately 80% of women and 77% of men. Awareness and treatment of high blood pressure is highest in older age groups. Accumulation of excess body weight in adult years is partially responsible for increased rates of hypertension in later years. This is of particular concern because hypertension can result in damage to the vasculature and kidneys. Dietary modification has been demonstrated to reduce blood pressure in older adults who present with hypertension. For individuals with elevated blood pressure the American Heart Association recommends the same dietary pattern as for elevated LDL-C concentrations (see prior section). The additional recommendation is to lower sodium intake to no more than 2,400 mg per day and if possible, further reduce to 1,500 mg per day. Emphasized is that even without achieving these goals, reducing sodium intake by at least 1,000 mg day will result in lower blood pressure. The Dietary Approaches to Stop Hypertension, frequently referred to as DASH, is a dietary pattern rich in vegetables, fruits and fat-free and low-fat dairy products. It has been demonstrated to reduce blood pressure in wide range of individuals, including older adults (Appel *et al.*, 1997). Further coupling the DASH dietary pattern with sodium restriction further decreases blood pressure (Sacks *et al.*, 2001).

14.4.4 Maintaining blood glucose concentrations within acceptable concentrations

The prevalence of type 2 diabetes for adults worldwide was estimated to be approximately 6% in 2010 and was projected to rise to 8% in 2030. About 9% of people living in the USA have been diagnosed diabetes (http://tinyurl.com/m2dpgyg). About 35% of the adult population has pre-diabetes (insulin resistance). There is a higher incidence of type 2 diabetes mellitus and insulin resistance in older age groups (Ford *et al.*, 2007, 2008). As with hypertension, excess body weight in adulthood contributes to this trend. Modifications in diet and physical activity patterns can prevent the onset or play a key role in the treatment of type 2 diabetes mellitus (Knowler *et al.*, 2002; Sakane *et al.*, 2011; Tuomilehto *et al.*, 2001). These lifestyle interventions include consuming a dietary pattern consistent with the recommendations for LDL-C lowering, engaging in regular physical activity and achieving and maintaining a healthy body weight. Low fat diets should be avoided. Foods high in unsaturated fat should replace foods high in saturated fat.

14.5 Special considerations for maintaining diet quality in older adults

Gradual physical and biological changes occur with advancing years that can influence food intake. Simple modifications can minimize potential negative impacts associated with these changes.

14.5.1 Dexterity and strength

Frequently advancing years is accompanied by diminished strength or arthritis which in turn can lead to difficulties with food preparation. If not addressed, these changes can negatively impact on dietary variety, quality and overall food enjoyment (Table 14.2). Food preparation tasks that may become more difficult include opening jars, cans or other food and beverage containers, and chopping and peeling vegetables and fruits. Some of these challenges can be addressed by the introduction of small accommodations such as ergonomically designed kitchen

Table 14.2. Factors that may contribute to compromised nutrient intake in older adults.

Factor	Change
energy intake	↓ requirement
	↑ geriatric cachexia
dexterity and strength	↑ sarcopenia
	↑ arthritis of the fingers and hand joints
	↑ tremor
	↓ manual dexterity
	↓ strength
senses	↓ taste acuity
	↓ smell acuity
mobility	↓ respiratory capacity
	↑ arthritis
	↑ osteoporosis
	↓ gait
	↓ balance
vision	↓ visual acuity
	↑ macular degeneration
oral cavity	↓ salivary secretions
	↑ peritoneal disease
	↑ ill-fitting dentures
	↓ salivary gland function
cognitive function	↑ dementia
	↑ cognitive impairment

utensils such as can openers and scissors, and kitchen modifications such as reducing clutter and reorganizing draws and cabinets to increase accessibility. Suggestions related towards using newer forms of familiar foods, such as partially processed or prepared items, may be helpful. Examples include bags of frozen vegetables and fruits that are pre-cleaned, trimmed and chopped; boneless chicken breasts; and pre-washed salad greens or grated carrots. Selection of these items may not be intuitive to older adults because they have not used them in the past and may not be aware of their availability.

14.5.2 Taste and smell

Taste and smell are critical factors associated with food enjoyment and subsequent nutritional status. Older adults frequently exhibit a decrease in taste acuity. This change appears to be related to a reduction in the number of taste buds, particularly those sensing salt and sweet. The negative effect of these changes on food intake can be amplified by the necessity to restrict dietary sodium coupled with an enhanced sensitivity to acid and bitter (Lipson and Bray, 1986). Taste acuity can also be diminished by alterations in sense of smell. Data suggests that in older adults' diminished odor perception is associated with poorer diet quality (Griep *et al.*, 1996a,b; 2000). Experimenting with herbs and spices may overcome some of these changes.

14.5.3 Mobility and vision

Living independently and the ability to go for trips outside the home can be challenged by limitations in mobility and vision associated with advancing years. This is of particular concern in certain regions with excessively hot or cold weather occurring for a large part of the year. Not only is the ability to acquire food and beverages compromised but also the ability to engage in social interaction and exposure to sunlight. The extent to which mobility and vision are of concern can depend on the availability of transportation options and stores within a short distance of home. Potential limitations can be addressed by actively identifying alternative forms of transportation, such as taxi-pooling to food stores and determining the availability of senior shuttles or senior taxi discounts.

In addition to addressing transportation related issues, other approaches to minimize the effect of diminished mobility and vision on diet quality include the introduction of food items that can be stored for longer periods than fresh; for example, re-sealable bags of frozen vegetables and fruits. Not only do they have a long shelf life, they are amenable to the removal of small quantities at any one time, they minimize the need to peel and cut, reduce waste due to spoilage and provide for increased food variety during periods of inclement weather. Although there is concern about the introduction of processed foods due to higher sodium and sugar levels than home prepared foods newer varieties with reduced sodium and added sugar are now available. Another example of a product particularly helpful to older adults that they might not automatically consider is shelf-stable milk. It is critical to find solutions to limitations imposed by changes in mobility and vision to avoid the onset of negative impacts on psychological well-being and nutritional status.

14.5.4 Oral health and dentition

Advancing years are accompanied by a decrease in salivary secretions. Likewise, changes in bite pattern may occur due to partial or complete tooth extraction/loss (Papas *et al.*, 1998a,b). If these changes result in poorly fitting dentures the consequence may cause an avoidance of certain types of foods and/or variety of foods. Fibrous foods such as whole fruits and vegetables may become painful to chew and difficult to swallow, leading to a shift towards highly processed foods or juices that are low in fiber (NIDDK, 2007). When evaluating the diet of older adults in terms of cardiovascular disease risk it is important not to overlook potential changes in oral health and dentition. If textural issues are identified that appear to be related to oral health a discussion of food preparation methods may help address some of the issues. These include longer cooking times and changes in preparation techniques, such as grating rather than slicing vegetables, can remedy some of the problems.

14.6 Psychosocial factors

With advancing years not only are there declines in physical capacity and physiological processes there may also be changes in the social environment that can have a major impact on diet quality (Table 14.3). Such psychosocial factors include emptying of the nest and loss of a spouse or other family members with whom an individual shared and prepared meals. These changes can be accompanied by less attention paid to mealtime and interest in preparing and consuming a balanced and varied diet. If these changes are also accompanied by deterioration in mental health

Table 14.3. Psycho-social factors that may contribute to compromised nutrient intake in older adults (Lichtenstein, 2013; with permission of Springer).

Factor	Change
companionship	↑ loss of spouse and contemporaries
	↑ social isolation
cognitive state	↑ depression
	↑ mental deterioration (dementia)
	↑ alcoholism
economic status	↑ fixed income
	↓ choice, variety and availability of foods
nutrition knowledge	↑ susceptibility to food fads
housing	↑ loss of home
	↓ control over food choices
	↓ access to preferred foods
	↓ ability to prepare favored foods

or economic status, leading to a change in living environment, older adults may be at increased risk for nutrient inadequacies. This latter factor, a new living environment, can result in changes in meal times, dining companions, opportunities for food preparation and types of food available. If these changes lead to the onset of depression and alcohol abuse particular attention needs to be paid to directly addressing these issues to ensure healthy eating patterns are maintained. All of these factors have been associated with altered dietary patterns predisposing to a decline in nutrient status(James *et al.*, 1997).

14.7 Nutrient supplements and older adults

Older adults report a higher use of nutrient supplement than younger adults (Block *et al.*, 2007; Foote *et al.*, 2003; Gardiner *et al.*, 2007; Rock, 2007; Yoon and Schaffer, 2006). Between 1999-2000 and 2011-2012 this trend has become more pronounced relative to the younger age groups (Kantor *et al.*, 2016). The reason older adults cite for choosing to take nutrient supplements include the desire to improve health and delay the onset of chronic disease, primarily cardiovascular disease (Buhr and Bales, 2009, 2010). Contrary to what would be expected, the subset of older adults who choose to use nutrient supplements are least likely to have biomarkers of nutrient inadequacy or poor dietary patterns (Block *et al.*, 2007; Sebastian *et al.*, 2007). Of concern, due to the high proportion of fortified foods in supermarkets this subgroup of older adults may be vulnerable to excess nutrient intakes and adverse drug-nutrient interactions (Kishiyama *et al.*, 2006; Murphy *et al.*, 2007; Radimer *et al.*, 2004; Rock, 2007; Yoon and Schaffer, 2006). There is limited amount of information available on the latter topic, making it difficult to determine the level of contemporary importance (Yetley, 2007). In general, those who report regularly taking nutrient supplements tend to be older (Block *et al.*, 2007; Foote *et al.*, 2003; Gardiner *et al.*, 2007; Rock, 2007; Yoon and Schaffer, 2006), and more likely to be female (Block *et al.*, 2007; Gardiner *et al.*, 2007; Rock, 2007), non-Hispanic white (Block *et al.*, 2007; Rock, 2007), college educated or beyond (Block *et al.*, 2007; Foote *et al.*, 2003; Gardiner *et al.*, 2007; Rock, 2007), and affluent (Block *et al.*, 2007).They also are more likely to have body mass indices within the normal range (Foote *et al.*, 2003; Rock, 2007), engage in regular physical activity (Foote *et al.*, 2003; Rock, 2007), have optimal chronic disease biomarkers (Block *et al.*, 2007), have low rates of smoking (Foote *et al.*, 2003), have better nutrient intakes, and hold strong attitudes about the importance of a good diet (Buhr and Bales, 2010; Murphy *et al.*, 2007; Sebastian *et al.*, 2007). Early work suggested low-dose anti-oxidant supplementation lowered all-cause mortality in middle-aged and older adult men but not women (Hercberg *et al.*, 2004). However, the effect was attributed to lower baseline status in men than women rather than the supplements themselves. A large study in women, Women's Health Initiative, concluded after approximately 8 years of follow-up that multivitamin use had little or no influence on the risk of common cancers, cardiovascular disease or total mortality in postmenopausal women (Neuhouser *et al.*, 2009).

14.8 Dietary guidance for older adults

There has been considerable interest in the relationship of diet quality and healthy aging, particularly with respect to cardiometabolic disorders. Compared to younger adults, older adults have report a higher level of motivation to improve their diet. Reasons cited include the desire to feel fit (52%), current health concerns (50%), desire to achieve a healthy body weight (39%) and to prevent the onset of disease (29%) (Dijkstra *et al.*, 2014). Of lesser concern, although important, include taste preference (15%), current disease (9%) and appearance (3%). In efforts to identify successful approaches to change dietary patterns in older adults a web-based health promotion program was reported to show promise (Cook *et al.*, 2015). Of those older adults who used the program, on average, they showed significant improvements in the areas of eating practices, diet change self-efficacy and planning healthful eating. There is considerable interest in identifying objective approaches to assessing diet quality in older adults. Using a relatively new approach, telomere length, did not provide useful (Milte *et al.*, in press). Additional data on this topic is likely to emerge in the future. Within the context of population-wide dietary guidance and evidence that older adults respond to dietary improvements positively in terms of cardiometabolic health efforts in this realm are important to pursue.

14.8.1 MyPlate for older adults

MyPlate for older adults provides a graphic representation of food-based recommendations (Figure 14.1; http://hnrca.tufts.edu/myplate). Modifications made to the original MyPlate specifically for older adults include the addition of food icons to the different sectors of the plate to provide illustrative examples of nutrient dense choices; shift of the dairy sector, a good source of high quality protein, into the protein sector; fusion of the vegetable and fruit sectors; creation of a fluid sector on the top right of the plate to emphasize the importance of this category for older adults; construction of a physical activity panel in the top of the plate to likewise emphasize the

Figure 14.1. MyPlate for older adults (http://hnrca.tufts.edu/myplate).

importance of engaging in regular physical activity; insertion of a sector in the center of the plate containing vegetable oils to emphasize the benefit across the diet of using liquid vegetable oils for food preparation in place of animal fats; and depiction of different forms of foods particularly useful to older adults such as bags of frozen fruits, pre-cut and pre-washed vegetables, and canned low sodium and low added sugar foods. Important to note, food based recommendations intended to support cardiometabolic health in older adults are, for the most part, appropriate for younger adults as well.

14.9 Conclusions

The aim of dietary guidance specifically targeted for older adults to minimize the risk of developing cardiometabolic disorders is, for the most part, consistent with that associated with optimal health outcomes throughout adulthood. Evidence suggests that there is a direct link between healthy dietary patterns, cardiometabolic disorders and total mortality. Due to diminished levels of physical activity, decreased metabolic rates, and increased proportions of fat to lean muscle mass, energy requirements decline with advancing years whereas nutrient requirements remain either unchanged or increase. To accommodate these changes there needs to be increased efforts to educate 'younger' older adults to these changing and how to gradually accommodate them, such as paying increased attention to choosing nutrient dense foods within and among food categories. The use of nutrient supplements by older adults should be monitored to minimize the risk of overconsumption. This is particularly important because older adults who report using nutrient supplements tend to be those who have dietary and lifestyle patterns that are most closely associated with lower rather than higher risk for cardiometablic disorders and nutrient insufficiency. With advancing age it may be necessary to adapt living environments to promote the ability to acquire and prepare familiar foods. This is particularly important during times of changes such as living environment or composition of the household. At any age individuals can benefit from improvements in dietary and physical activity patterns. The period of time older adults can expect to remain active, productive and independent continues to expand. Hence, no one is too old to benefit from improvements in lifestyle behaviors.

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15. Phytosterol consumption and coronary artery disease

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Abstract

Plant sterols and plant stanols, collectively called phytosterols in the following, are normal components of plants. They are present in all vegetable foods, especially in vegetable oils, nuts, and cereals. In a regular diet their daily intake is about 300 mg, and this amount has no or at most a modest effect on low-density lipoprotein (LDL) cholesterol level. When the intake of phytosterols added to foods or supplements is increased up to 2 g/day, they inhibit cholesterol absorption and decrease LDL cholesterol concentration by 8-10% demonstrated in over 100 randomised, controlled clinical interventions. Thus, phytosterols added to diet are a dietary, non-pharmacologic means to lower LDL cholesterol concentration without side-effects. They lower also other pro-atherogenic serum lipids and lipoproteins. They improve vascular health assessed as endothelial function and arterial stiffness in some, but not in all studies, in which they neither improve nor impair vascular health. Even though in animal studies phytosterols reduce atherosclerosis, no large-scale outcome trials for coronary artery disease prevention are available simply because the task is practically unfeasible. However, the recent beneficial effects of genetic and pharmacologic cholesterol absorption inhibition on coronary and cardiovascular outcomes suggest that cholesterol absorption inhibition with added phytosterol consumption conceivably has also a beneficial effect on cardiovascular health.

Keywords: atherosclerosis, cholesterol absorption, LDL cholesterol, sitosterol, cholestanol

Key facts

- Phytosterols (plant sterols and plant stanols) are normal components of plants. In regular diet their daily intake is about 300 mg.
- Increased phytosterol intake to 2 g/day in foods and supplements inhibit cholesterol absorption. Consequently, low-density lipoprotein (LDL) cholesterol concentration decreases by 8-10%. LDL cholesterol lowering reduces the risk of coronary artery disease (CAD).
- There is evidence that phytosterols improve biomarkers of vascular health, i.e. endothelial function and arterial stiffness.
- No large-scale outcome trials for CAD prevention are available because they are practically unfeasible.
- Genetically or pharmacologically caused cholesterol absorption inhibition reduce cardiovascular events. Likewise, added phytosterol intake conceivably could reduce cardiovascular events as a dietary means.

Summary points

- LDL cholesterol concentration is an important risk factor the development of cardiovascular disease, especially CAD. However, LDL cholesterol reduction by any means prevents the progression of the disease.
- Phytosterols (plant sterols and plant stanols) are normal components of plants. When added to diet up to 2 g/day they inhibit cholesterol absorption and lower LDL cholesterol level about 8-10%.
- Since 1995 food products with added phytosterols, such as spreads, yoghurts, softdrinks, and supplements, have been on the market worldwide to safely lower LDL cholesterol as a dietary means.
- Phytosterols 2 g/day lower LDL cholesterol irrespective of gender, age, ethnic background, body weight, background diet, or the cause of hypercholesterolemia. They can be added to statin treatment.
- In addition to LDL cholesterol lowering, phytosterols added to diet reduce other pro-atherogenic lipid risk factors and improve vascular health.

Abbreviations

CAD	Coronary artery disease
CAVI	Cardio-ankle vascular index
CVD	Cardiovascular disease
CRP	C-reactive protein
FMD	Flow-mediated dilation
HDL	High-density lipoprotein
hs-CRP	High sensitivity C-reactive protein
IL	Interleukin
LDL	Low-density lipoprotein
NPC1L1	Niemann-Pick C1-like 1 protein
PCSK9	Proprotein convertase subtilisin/kexin 9
PWV	Pulse wave velocity
RCT	Randomized controlled trial
RHI	Reactive hyperemia index
VLDL	Very low-density lipoprotein

15.1 Introduction: overview of naturally occurring phytosterols

Plant sterols and plant stanols (collectively called phytosterols in the following) are normal components of plants. Plant sterols have similar functions in plants as cholesterol has in humans, e.g. being structural and functional components of cellular and intracellular membranes. They are present in all vegetable foods, especially in vegetable oils such as corn oil, rapeseed (canola) oil, soybean oil, and sunflower oil, and in nuts, seeds, grains, legumes, vegetables, and fruit in decreasing order of quantity (Racette *et al.*, 2015). Corn oil is the richest source of these compounds containing plant sterols 686-952 mg/100 g of oil, rapeseed oil contains 250-767 mg/100 g of oil, sunflower oil 263-376 mg/100 g of oil, soybean oil 221-328 mg/100 g of oil, and olive oil less than 200 mg/100 g of oil, respectively (Piironen and Lampi, 2004). Sesame seeds and wheat germ have the highest plant sterol content of seeds and nuts, about 400 mg/100 g, and peanuts, pistachio, and sunflower kernel from 200 to 289 mg/100 g (Phillips *et al.,* 2005). Rye contains plant sterols about 100 mg/100 g and wheat about 70 mg/100 g, respectively (Piironen and Lampi, 2004). In vegetables, fruits and berries the amounts of plant sterols are low varying from 40 mg/100 g in cauliflower to 16 mg/100 g in carrots and 13 mg/100 g in apples (Normén *et al.*, 1999; Piironen and Lampi, 2004). Plant stanols are present in vegetable foods in much lower amounts than the plant sterols. The richest sources of plant stanols are corn oil (about 30 mg/100 g of oil), rapeseed oil (about 20 mg/100 g of oil), and rye and wheat (about 15-20 mg/100 g) (Piironen and Lampi, 2004).

In a recent large European cohort the most important dietary sources of phytosterols were fruits and vegetables (25.5%), bread and grain products (25.1%), and fats, oils, and sauces (19%) (Ras *et al.*, 2015b). The intake of naturally occurring phytosterols in different populations seems to

be rather consistent all over the world and around 300 mg/d, on average (Andersson *et al.*, 2004; Racette *et al.*, 2015; Ras *et al.*, 2015b). In vegan diets, the amount of phytosterols varies from 300 to 500 mg/d (Abdulla *et al.*, 1981; Vuoristo and Miettinen, 1994). Most of the dietary phytosterols are plant sterols, so that the amount of naturally occurring dietary plant stanols is less than 30 mg/d (Klingberg *et al.*, 2008a; Valsta *et al.*, 2004). For comparison, the average cholesterol intake varied in these studies from 218 to 260 mg/day (Andersson *et al.,* 2004; Ras *et al.*, 2015b; Valsta *et al.*, 2004). In the vegan population, the daily intake of cholesterol was only ~30 mg/d (Abdulla *et al.*, 1981).

The most abundant plant sterols in human diet are sitosterol, campesterol, and stigmasterol, and the most abundant plant stanols are sitostanol and campestanol. In the EPIC-Norfolk cohort of 25,000 subjects, the proportion of sitosterol was 66% of the total phytosterol intake, that of campesterol 22%, stigmasterol 8%, sitostanol 3%, and campestanol 2%, respectively (Klingberg *et al.*, 2008a).

Plant sterols differ from cholesterol in the structure of their side chain (Figure 15.1). Plant stanols are 5α-saturated derivatives of plant sterols (Figure 15.1). Sitosterol and campesterol, similarly as sitostanol and campestanol, differ from each other by having an ethyl or methyl group in the side chain, and stigmasterol differ from sitosterol by having a double bond in the side chain. Even though these structural differences between cholesterol and phytosterols, and between different plant sterols and plant stanols, are small, they have profound effects on the biological functions of the sterols and stanols, so that they have to be considered as metabolically different molecules.

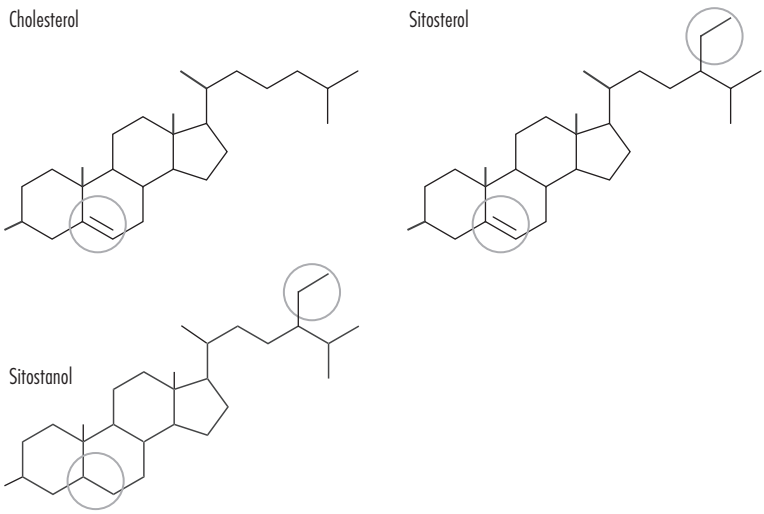

Figure 15.1. The molecular structures of cholesterol, sitosterol (plant sterol) and sitostanol (plant stanol). The differences between cholesterol and phytosterols are in the side chain, and the differences between sterols and stanols are in the ring structure, all marked with a blue circle.

The aim of this chapter is to deal with phytosterols added to foods and supplements as a dietary means to lower serum and LDL cholesterol concentrations and as a consequence reduce the risk of atherosclerosis and its clinical manifestations, especially CAD. We discuss shortly the metabolism and the cholesterol-lowering mechanisms of phytosterols in humans, and whether consuming phytosterols added to foods and supplements is cardioprotective.

15.2 The metabolism of phytosterols

The most marked differences between cholesterol and phytosterols in human metabolism are related to their *de novo* synthesis, intestinal uptake, and biliary secretion. Phytosterols are not synthesized in humans so that they are completely diet-derived. After entering the upper small intestine, both cholesterol and phytosterol esters are hydrolysed into free sterols and stanols by pancreatic cholesteryl esterase and are mixed to oil phase and micellar phase. From the micellar phase, cholesterol and phytosterols are actively transported by a membrane protein, NPC1L1, through the enterocyte membrane inside the cell (Davis *et al.*, 2004). In the enterocyte, cholesterol and phytosterols are similarly esterified with acyl-CoA cholesterol acyltransferase-2 to sterol esters (Gylling *et al.*, 2006), incorporated with triglycerides and apoprotein B-48 to form chylomicrons, which are then released to lymph to be transported to the liver.

However, in the enterocyte the processing of phytosterols diverges from that of cholesterol. Most of the phytosterols are pumped back to the intestinal lumen from the enterocyte by intestinal ATP-binding cassette transporters ABCG5 and ABCG8 (Berge *et al.*, 2000; Lee *et al.*, 2001). Thus, the intestinal absorption of phytosterols is very low compared to that of cholesterol. The absorption efficiency of plant sterols is less than 2% and that of plant stanols less than 0.2% (Ostlund *et al.*, 2002). For comparison, the absorption efficiency of cholesterol is 50%, on average. In the liver, the hepatic ABCG5 and ABCG8 transporters operate similarly as in the enterocyte and remove phytosterols effectively to bile to be excreted from the body. As a result, the circulating concentrations of total plant sterols are <24 μmol/l (<1.0 mg/dl), and those of total plant stanols <0.3 μmol/l (<0.012 mg/dl), respectively (reviewed in Gylling *et al.*, 2014). Therefore, in comparison with the recommended serum cholesterol level, 5.0 mmol/l (190 mg/dl), serum plant sterol and stanol levels are ~200-~16,000 times lower than the serum cholesterol level.

In circulation, about 60% of phytosterols are transported by LDL similarly to cholesterol. Proportionally the phytosterol ratio to cholesterol is highest in intermediate density lipoprotein and HDL (Simonen *et al.*, 2007). The circulating phytosterols are equilibrated with tissue phytosterol contents. This equilibration has been demonstrated between serum/plasma and aortic valve or arterial wall tissue (Helske *et al.*, 2008; Miettinen *et al.*, 2005, 2011; Schött *et al.*, 2014; Simonen *et al.*, 2015a; Weingärtner *et al.*, 2008), and between serum and liver cells (Hukkinen *et al.*, in press). Even during phytosterol supplementation up to 2 g/day, the serum/plasma phytosterol levels correlate with the respective levels in aortic valve and arterial tissue (Miettinen *et al.*, 2011; Simonen *et al.*, 2015a; Weingärtner *et al.,* 2008). Likewise, in extreme situations such as in phytosterolemia, a rare inherited disease with markedly elevated plasma and

tissue phytosterol levels resulting from genetic defects in the ABCG5/ABCG8 transporters, the proportion of phytosterols/total sterols in different tissues is similar to that in plasma (Salen *et al.*, 1985). These results suggest that high dietary intake of phytosterols or genetic defects in sterol metabolism increase their serum and tissue contents in the same proportion, so that there is no indication of uncontrolled phytosterol accumulation or retention into tissues.

15.3 Phytosterols and cholesterol lowering

The intake of naturally occurring phytosterols has no or at most a modest effect on LDL cholesterol concentration. At the population level, though, subjects with high natural phytosterol intake (~300-400 mg/day) had lower serum total and LDL cholesterol concentrations compared with subjects with low phytosterol intake (<200 mg/day) (Andersson *et al.*, 2004; Klingberg *et al.*, 2008b). However, the natural intake of phytosterols had no effect on the risk of CVD in women (Klingberg *et al.*, 2013) or in both genders (Ras *et al.*, 2015b), even though in the first study high intake of naturally occurring phytosterols seemed to have a cardioprotective effect in men (Klingberg *et al.*, 2013). This is the reason why phytosterols have been added to foods and food supplements since 1995 to obtain significant non-pharmacologic reduction in serum total and LDL cholesterol concentrations as part of a heart healthy diet.

The breakthrough landmark study of the efficacy and safety of added dietary phytosterols in order to lower LDL cholesterol concentration was the one-year clinical RCT by Miettinen *et al.* in 1995. In this double-blind study, 152 subjects with mild hypercholesterolemia consumed margarine without (control group, n=51) or with (n=102) plant stanol ester (1.8 or 2.6 g plant stanol/day) for 12 months. Plant stanol ester consumption significantly decreased serum total and LDL cholesterol concentrations. LDL cholesterol concentration was reduced by 14% in the plant stanol ester group and 1% in the control group, so that the difference between the groups was -21 mg/dl (95% confidence interval -14 to -29 mg/dl, $P<0.001$). HDL cholesterol and serum triglyceride concentrations remained unchanged, and no side effects were reported.

The concept of phytosterols added in foods and supplements as part of heart healthy diet in order to effectively and safely lower serum total and LDL cholesterol concentrations without drugs as a dietary means received enormous attention, and numerous amounts of food products with added phytosterols have been developed and launched to the market. The interest in these products also brought about intensive research activity. Recently three large meta-analyses have been published including 84-124 RCTs and altogether about 7,000 study subjects (Demonty *et al.*, 2009; Musa-Veloso *et al.*, 2011; Ras *et al.*, 2014). The meta-analyses included women and men from 22 to over 60 years of age, body mass index varied from normal to obesity, and the study populations were from different ethnic groups all over the world. The cholesterol-lowering effect of phytosterol added food products has been evaluated in subjects with primary hypercholesterolemia, familial hypercholesterolemia, combined hyperlipidemia, type 1 and type 2 diabetes, metabolic syndrome, CAD, and renal insufficiency. Most of these studies have been performed with esterified phytosterols added to low-fat spread/margarine, yoghurt, or minidrinks. The efficacy

and safety of phytosterol supplementation has been confirmed also in children and adolescents with normolipidemia, primary hypercholesterolemia or familial hypercholesterolemia (reviewed in Gylling *et al.*, 2014).

Accordingly, it is carefully documented that phytosterols 2 g/day added to food products or supplements significantly lower LDL cholesterol concentration by 8%-10% irrespective of gender, age, ethnic background, body weight, background diet, or the cause of hypercholesterolemia. When added to statin treatment, they have an additive cholesterol-lowering effect, which is larger than doubling the statin dose. There is a dose-response effect between the amount of added phytosterols and LDL cholesterol lowering, so that increasing the phytosterol intake from 0.6 g/day to 3.3 g/day LDL cholesterol is lowered from 6% to 12% (Ras *et al.*, 2014). Phytosterols do not affect HDL cholesterol concentration, and there is some indication of serum triglyceride lowering especially if the baseline level is slightly elevated. They are well tolerated, and there is a large body of information regarding them safe in long-term use. Plant sterols and plant stanols lower LDL cholesterol similarly. However, the better absorption efficiency of plant sterols over plant stanols increases the serum concentration of plant sterols by 40%, on average, which stabilizes within four weeks of the intake, and remains at very low levels compared to that of LDL cholesterol (~144 times lower) (Ras *et al.,* 2016). It is important to note that plant stanol supplementation, in addition to inhibiting cholesterol absorption, inhibits also the absorption of plant sterols and reduces serum plant sterol levels.

15.3.1 Mechanism of cholesterol lowering

Phytosterols lower LDL cholesterol concentration by interfering with cholesterol absorption. Of the different theories of the mechanisms of cholesterol absorption inhibition, e.g. by displacing cholesterol from mixed micelles (the micellar theory), by modifying the expression of genes encoding the sterol transporters, or by increasing cholesterol removal from the body via the transintestinal cholesterol excretion pathway, only the micellar theory has gained experimental support both in animal and human studies (e.g. Hassan and Rampone, 1980; Ikeda *et al.*, 1988; Nissinen *et al.*, 2002). The activation of the genes of the intestinal transporters has not been demonstrated either in animal or human studies (De Smet *et al.*, 2015; Field *et al.*, 2004; Plösch *et al.*, 2006), and the transintestinal cholesterol excretion pathway has intensely been studied in experimental animals only. Regarding the micellar theory, mixed micelles serve cholesterol and phytosterols to NPC1L1. When the phytosterol concentration in proximal small intestine is high, cholesterol loses its micellar solubility, and cholesterol absorption is decreased (Nissinen *et al.*, 2002). Consequently, less cholesterol will be transported to the liver.

Phytosterols reduce cholesterol absorption efficiency in a dose-dependent manner. In a well-controlled dietary intervention, the intake of phytosterols 459 mg/day and 2,059 mg/day compared to 59 mg/day (control) reduced cholesterol absorption efficiency by 10 and 25%, increased fecal cholesterol excretion by 36 and 74%, and increased cholesterol synthesis evaluated by the serum biomarker lathosterol/cholesterol ratio (Miettinen *et al.*, 1990) by 31 and 50% (Racette *et al.*,

2010). Control-related LDL cholesterol lowering was non-significant (5%) with the 459 mg/day phytosterol dose, but significant (9%) with the 2,059 mg/day dose.

15.4 Phytosterol consumption and coronary artery disease

15.4.1 Effects on risk factors

The elevated LDL cholesterol concentration is an essential risk factor for atherosclerosis and CAD, and lowering LDL cholesterol prevents the progression of atherosclerosis and CAD and reduces CAD outcomes. It has been estimated that for every 1% reduction in LDL cholesterol concentration there is a corresponding 1% decrease in the risk of CAD (LaRosa, 2007). The extensive body of information obtained from RCTs reveals that dietary phytosterols 2-3 g/day decrease LDL cholesterol level by 8-10%, so that according to the above estimation, phytosterols 2-3 g/day added to foods and supplements should reduce the risk of CAD by 8-10%.

In addition to serum total and LDL cholesterol reduction, phytosterols added to foods and supplements reduce non-HDL cholesterol concentration by about the same amount as LDL cholesterol (e.g. Lau *et al.*, 2005; McKenney *et al.*, 2014; Plat *et al.*, 2009). Serum apoprotein B-100 concentration is also reduced (Garoufi *et al.*, 2014; Gylling and Simonen, 2015; Theuwissen *et al.,* 2009).

Phytosterols added to foods do not change LDL particle size (Gylling and Miettinen, 1994; Padro *et al.*, 2015). They reduce cholesterol content in the pro-atherogenic small dense LDL particles (Garoufi *et al.*, 2014; Gylling and Miettinen, 1994; Sialvera *et al.*, 2012). They also reduce large and medium sized VLDL concentrations in subjects with moderate hypertriglyceridemia and metabolic syndrome (Plat and Mensink, 2009) resulting in serum triglyceride lowering in these subjects. Phytosterols also reduce postprandial lipoproteins without affecting postprandial cholesterol and triglyceride values (reviewed in Gylling and Simonen, 2015). Phytosterol consumption does not affect the serum concentrations of the pro-atherogenic lipoprotein (a) or PCSK9 (Garoufi *et al.*, 2014; Plat and Mensink, 2000; Simonen *et al.*, 2015b).

Elevated levels of circulating inflammatory biomarkers are present in several chronic diseases such as obesity, dyslipidemia, and atherosclerosis. The effect of added phytosterol consumption has been evaluated especially on CRP or hs-CRP concentrations in numerous studies (Clifton *et al.*, 2008; De Jong *et al.*, 2008; Devaraj *et al.*, 2006; Gylling *et al.*, 2009; Hallikainen *et al.*, 2006; Houweling *et al.*, 2009; Micallef and Garg, 2009; Sialvera *et al.*, 2012; Theuwissen *et al.*, 2009). These interventions included altogether 908 subjects, both men and women with mild to moderate primary hypercholesterolemia, familial combined hyperlipidemia, or metabolic syndrome. Age was ranging from 18 to 72 years and body mass index from normal to obese. The added phytosterols were in most studies in esterified form added to spread or margarine, yoghurt, or orange juice. The daily phýtosterol dose varied from 1.6 to 4 g. In all but one of the studies, added phytosterols had no effect on CRP or hs-CRP values in spite of significant LDL cholesterol

lowering in all studies. In one study, 2 g/day of phytosterols added to orange juice reduced CRP by 12% compared with controls in mildly hypercholesterolemic normal weight subjects (Devaraj *et al.*, 2006).

Of the pro-atherogenic cytokines IL-1beta and IL-6 were reduced by phytosterol consumption (Devaraj *et al.*, 2011; Micallef and Garg, 2009). No changes have been observed in IL-8, IL-10, tumor necrosis factor α, monocyte chemotactic protein-1, soluble vascular cell or intercellular adhesion molecules, or plasminogen activator inhibitor-1 concentrations (De Jong *et al.*, 2008; Devaraj *et al.*, 2011; Hallikainen *et al.*, 2006; Micallef and Garg, 2009). On the other hand, incubation of cultured myofibroblasts derived from stenotic aortic valves with sitostanol or sitosterol significantly decreased mRNA expression of the monocyte chemotactic protein-1 and IL-1 beta (Simonen *et al.*, 2015a).

Thus, phytosterols added to foods and supplements reduce the pro-atherogenic lipid and lipoprotein risk factors. Their effect on inflammatory biomarkers is mainly neutral even though some indications of reducing pro-atherogenic cytokines have been documented. Accordingly, they have a favorable effect on the most essential risk factors on atherosclerosis suggesting that the burden on atherosclerosis is conceivably reduced.

15.4.2 Effects on atherosclerosis and coronary artery disease

The effects of added phytosterols on atherosclerosis have been evaluated in clinical trials by assessing the surrogate biomarkers of cardiovascular health, i.e. endothelial function and arterial stiffness. Eight RCTs have been performed including two interventions in children with familial hypercholesterolemia and age ranging from 5 to 12 years, five interventions in adults with primary hypercholesterolemia (872 subjects, males and females, mean age from 40 to 54 years), and one intervention in adult type 1 diabetes subjects (Table 15.1). All studies were double-blind, randomized, placebo-controlled clinical plant sterol or plant stanol ester interventions, in which the duration varied from 4 to 52 weeks. The phytosterol doses varied from 1.93 g phytosterol/day to 3 g phytosterol/day, which are the doses recommended in international expert guidelines. In most studies the food matrix was low-fat spread. Endothelial function was measured as FMD in the brachial artery (De Jongh *et al.*, 2003; Gylling *et al.*, 2009; Hallikainen *et al.*, 2006, 2008; Jakulj *et al.*, 2006, Raitakari *et al.*, 2008; Ras *et al.*, 2015a) and in one study as RHI using peripheral arterial tonometry (Gylling *et al.*, 2013). Arterial stiffness was evaluated in three studies. In one study it was assessed as carotid artery compliance (Raitakari *et al.*, 2008), and in two studies as AI in small arteries (Gylling *et al.*, 2013; Ras *et al.*, 2015a), and in large arteries using PWV measuring CAVI or PWV using carotid-femoral distance (Gylling *et al.*, 2013; Ras *et al.*, 2015a).

In all studies, LDL cholesterol concentration was significantly reduced from 7 to 16% compared to controls (Table 15.1). In seven studies, in spite of the effective cholesterol lowering, valid phytosterol doses, taking phytosterol products with a meal, and food matrices proven to release phytosterols properly in the intestinal tract, phytosterol consumption had no significant effect on the vascular biomarkers. In one study, however, phytosterol consumption reduced arterial

Table 15.1. Randomized, controlled trials of phytosterols added in food products on vascular health assessed as endothelial function and arterial stiffness.[1,2]

Reference	Patients (n)	Study design	Phytosterol dose (g/day), food matrix	Duration	Assessment	Change in assessments vs controls	Average change in LDL-C vs controls (%)
De Jongh *et al.* (2003)	FH children (41)	C-O	sterol 2.3, spread	4 wks	FMD	NS	-14*
Jakulj *et al.* (2006)	FH children (42)	C-O	stanol 2.0, yoghurt	4 wks	FMD	NS	-9*
Hallikainen *et al.* (2006)	hypercholesterolemia (76)	C-O, parallel	sterol 1.93, spread; stanol 1.98, spread	10 wks	FMD	NS	-9-12*
Hallikainen *et al.* (2008)	type 1 diabetes (19)	parallel	stanol 2.15, spread	12 wks	FMD	NS	-16*
Raitakari *et al.* (2008)	hypercholesterolemia (190)	parallel	stanol 2.0, spread	12 wks	FMD; CA compliance	NS	-9*
Gylling *et al.* (2009)	hypercholesterolemia (282)	parallel	sterol 2.15, spread; stanol 2.13, spread	52 wks	FMD; IMT	NS; NS	-4* (TC)
Gylling *et al.* (2013)	hypercholesterolemia (92)	parallel	stanol 3.0, spread	26 wks	AI; CAVI; RHI	*P*=0.046; *P*=0.023, men; NS	-10*
Ras *et al.* (2015a)	hypercholesterolemia (232)	parallel	sterol 3.0, spread	12 wks	FMD; AI; PWV	NS; NS; NS	-7*

[1] AI = augmentation index; CA = carotid artery; CAVI = cardio-ankle vascular index; C-O = crossover; FH = familial hypercholesterolemic; FMD = flow-mediated dilation; IMT = intima-media thickness; LDL-C = low-density lipoprotein cholesterol; RHI = reactive hyperemia index; PWV = pulse wave velocity; TC = serum total cholesterol. FMD and RHI reflect endothelial function. CA compliance, CAVI, and PWV reflect arterial stiffness in large and AI in small arteries.

[2] *$P<0.05$; NS= non-significant.

stiffness in small arteries compared to controls (Gylling *et al.*, 2013). In a subgroup analysis of the same study, phytosterol consumption had a beneficial effect on arterial stiffness in large arteries in men, but not in women Gylling *et al.* (2013). Also in a subgroup analysis in another study, phytosterols improved arterial stiffness and FMD in men and women, in whom the respective values were initially reduced (Raitakari *et al.*, 2008). In none of the studies, phytosterols impaired vascular function.

Later on a combination analysis of the FMD results from the first six studies in Table 15.1 was performed (Plat *et al.*, 2012). The compiled results from the individual studies suggested an improvement in FMD by added phytosterol consumption. Thus, the lack of beneficial effects of phytosterol consumption in most of the individual studies may be caused by underrepresentativeness. Since initial normality of the vascular health was not an exclusion criteria, subjects with normal vascular health were included in the trials resulting in possible bias and underpowering of the individual studies.

In animal models, the protective role of added phytosterol intake on atherosclerosis has conclusively been documented (reviewed in Gylling *et al.*, 2014). In humans, however, no hard CAD endpoint studies are available. A possibility to conduct a phytosterol consumption study with hard CAD endpoints was considered in detail in the European Atherosclerosis Society Consensus Panel on Phytosterols. The result was that large-scale outcome trials of food products with added phytosterols for CAD/CVD prevention are not practically feasible requiring enrollment of over 50,000 subjects in the setting of low to intermediate cardiovascular risk, and about 30,000 subjects in the setting of high cardiovascular risk (reviewed in Gylling *et al.*, 2014).

However, there is evidence that cholesterol absorption inhibition per se may reduce atherosclerosis and CAD. Ezetimibe by pharmacologically inhibiting cholesterol absorption reduced not only plasma cholesterol level but also the development of atherosclerosis in mice (Davis *et al.*, 2001). In addition to LDL cholesterol lowering, another possible mechanism for atheroprotection following cholesterol absorption inhibition has been suggested. In a mouse model with genetically reduced cholesterol absorption, reverse cholesterol transport from peripheral tissue macrophages was markedly increased (Greenberg *et al.*, 2009). In this model, a 41% decrease in cholesterol absorption resulted in about 30% decrease in plasma cholesterol level, 70% decrease in aortic root atherosclerosis, and 60% increase in reverse cholesterol transport from peripheral tissue macrophages. Theoretically, these macrophages could be located in arterial wall and reduce cholesterol load from the intima.

These experimental studies open an interesting scenario between cholesterol absorption inhibition and prevention of atherosclerosis and CAD. There is some evidence that high cholesterol absorption efficiency may increase the risk of CAD. The original observation demonstrated that the higher the cholesterol absorption assessed with serum cholestanol/cholesterol ratio, a biomarker of cholesterol absorption efficiency (Miettinen *et al.*, 1989), the greater was the risk of major coronary events in the Finnish subgroup (n=868 CAD subjects) of the Scandinavian simvastatin survival study (4S) (Miettinen *et al.*, 1998). In large population cohorts high

cholesterol absorption was associated with the prevalence of CVD and with the risk alleles in ABCG8, the sterol transporter gene, and ABO, the blood group gene, and (Silbernagel *et al*, 2013). At the moment there is recent evidence that by reducing intestinal cholesterol absorption cardiovascular outcomes are improved.

First, the inactivating (loss-of function) mutations in the NPC1L1 sterol transporter are associated with reduced sterol absorption and reduced LDL cholesterol level (Cohen *et al.*, 2006). LDL cholesterol concentration was about 8% lower in the subjects carrying the rare inactivating NPC1L1 variants, and the serum biomarker level of cholesterol absorption efficiency was 23% lower in the mutation carriers. Second, in the heterozygous carriers of the inactivating NPC1L1 mutations mean LDL cholesterol concentration was 0.31 mmol/l (12 mg/dl) lower than in noncarriers, and their risk for CAD was reduced by 53% compared to the noncarriers (The Myocardial Infarction Genetics Consortium Investigators, 2014). Finally, the lessons from the IMPROVE-IT study demonstrated that reducing cholesterol absorption with ezetimibe added to statin treatment diminished LDL cholesterol concentration by 24% and decreased the CVD event rate by 6.4% compared to the statin-only group (Cannon *et al.*, 2015). Accordingly, reduced cholesterol absorption either by genetic or pharmacologic means resulted in reduced LDL cholesterol concentration and decreased cardiovascular risk.

Are these results applicable to phytosterols? Ezetimibe 10 mg/d reduces control-related mean cholesterol absorption efficiency by 54%, LDL cholesterol concentration by 14%, and serum biomarker of cholesterol absorption efficiency by 66% in subjects with mild to moderate hypercholesterolemia (Sudhop *et al.*, 2002). For comparison, phytosterol consumption 0.8-3 g/day reduces mean cholesterol absorption efficiency by 45%, LDL cholesterol concentration by 10%, and serum biomarker of cholesterol absorption efficiency by 37% (Miettinen and Gylling, 2003). The absorption inhibition and LDL cholesterol lowering are not markedly different between inactivating NPC1L1 mutations, ezetimibe treatment, and phytosterol consumption. Accordingly, it could be assumed that the lessons from hard CAD endpoint studies with inactivating NPC1L1 mutations and ezetimibe treatment could be applicable to phytosterol consumption added in foods and supplements.

15.5 Conclusions

Phytosterols added to foods and supplements diminish cholesterol absorption so that less cholesterol is carried from intestine to liver. This is followed by reduction of serum total-LDL, and small dense LDL cholesterol, non-HDL cholesterol, and serum apoprotein B-100 concentrations in quantities large enough to be expected to have an impact on vascular health. There is some indication that pro-atherogenic cytokines are reduced as well. Thus, phytosterol consumption has a favorable effect on the most essential risk factors of atherosclerosis and CAD. On the other side, they do not affect HDL cholesterol or apoprotein A-I, lipoprotein (a), or PCSK9 concentrations, and they are well tolerated. Phytosterol consumption is either neutral or improves arterial stiffness and endothelial function suggesting beneficial effects on vascular health. No hard CAD endpoint

studies are available in humans. It has been discussed in detail that large-scale outcome trials for CAD/CVD prevention are not practically feasible because of the enrollment would require 30,000-50,000 subjects. In addition, there is no indication available that added phytosterol consumption has any harmful effect on vascular wall or is pro-atherogenic or otherwise harmful. Lessons from genetic and pharmacologic inhibition of cholesterol absorption demonstrate reduced hard CAD/CVD event rates. The inhibition of cholesterol absorption and LDL cholesterol reduction are of similar magnitude between inactivating mutations of NPC1L1, ezetimibe treatment, and phytosterols added to food products suggesting that reduced cardiovascular risk conceivably is applicable also to added dietary phytosterol consumption.

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16. The role of dietary saturated fatty acids in cardiovascular disease

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Abstract

The relationship between saturated fat intake and risk of cardiovascular disease (CVD) has long been a subject of interest. When the effect of saturated fat on lipid profile measurements such as cholesterol and low-density lipoprotein particles became apparent, dietary guidelines promoted a saturated fatty acids (SFA) intake of less than 10 energy%. The interpretation of available studies that have investigated SFA intake and risk of CVD (risk factors) is heavily debated. There is limited evidence for a clinically relevant individual effect of SFA intake on CVD risk factors other than cholesterol, such as type 2 diabetes, obesity and inflammation. Higher saturated fat intake might increase risk of cardiovascular events, but may lower stroke risk in specific populations. Studies that have considered macronutrients that are consumed instead of SFA suggest that replacing SFA by polyunsaturated fatty acids (PUFA) (or possibly monounsaturated fatty acids) decreases risk of CVD, but evidence is less convincing for replacement by carbohydrates. When investigating SFA, it is important to consider the replacing macronutrients, food sources and interactions within the food matrix. Biomarkers for SFA intake can be used, but the endogenous production of circulating SFA subtypes needs to be considered. Previous studies are limited either through their design or their statistical approach and this has led to a polarised discussion between researchers. Advancements in genetic research might be able to clarify the biological mechanism for the previously found associations between SFA and CVD. There is fear of sending mixed signals to the general public, thereby causing confusion. It is therefore important that new studies address the limitations of previous research when investigating the relationship between SFA intake and CVD.

Keywords: saturated fat, CVD, diet, nutrition

Key facts

- Dietary guidelines promote a saturated fatty acids (SFA) intake <10 energy% and replacement of SFA by polyunsaturated fatty acids (PUFA).
- Saturated fat intake has a detrimental effect on lipid profile measurements such as low-density lipoprotein-cholesterol, but its relationship with manifest cardiovascular disease (CVD) is debated.
- Reduction of saturated fats has limited effect on CVD, but replacement of saturated fat by *cis*-PUFA seems effective in lowering CVD risk.
- However, a relatively high intake of saturated fat has been related to lower risk of stroke, especially in Asian populations that generally have a lower saturated fat intake.
- Subtypes SFA or SFA derived from meat or dairy products can have different effects on disease outcomes

Summary points

- Dietary guidelines suggest a SFA intake <10 energy% and replacement by *cis*-PUFA, but SFA intake in Western countries remains higher. SFA reduction usually does not lead to higher *cis*-PUFA intake.
- Intake of *cis*-PUFA or *cis*-monounsaturated fatty acids instead of SFA leads to a better lipid profile, which is an important intermediate for CVD.
- Evidence for an independent effect of SFA intake on other risk factors for CVD, such as hypertension, diabetes mellitus type 2 or body weight is limited, partially due to study limitations.
- Inflammation might be influenced by SFA intake but the magnitude of the observed associations makes clinical relevance unlikely.
- A reduction or modification of SFA intake does not lead to less cardiovascular death, but might lead to fewer cardiovascular events.
- There is no convincing evidence for a substantial effect of SFA reduction on risk of coronary heart disease (CHD), but replacement of SFA by *cis*-PUFA seems to lower risk of CHD.
- Intake of SFA generally does not seem to affect stroke risk. There are suggestions that stroke risk is actually lowered with higher SFA intake in specific populations.
- Biomarkers for SFA intake from specific food sources, for example C16:0 from meat and C15:0 from dairy, can have different relationships with CVD outcomes, though endogenous production of these biomarkers needs consideration.
- The effect of SFA can vary depending on food sources, possibly through interactions within the food or correlations with other nutrients.
- Studies that can clarify causality and give further insight into the biological mechanisms of observed associations between SFA intake and risk of CVD are needed.

Abbreviations

ABI	Ankle-brachial index
BP	Blood pressure
CAC	Coronary artery calcification
CHD	Coronary heart disease
CHO	Carbohydrates
CI	Confidence interval
CIMT	Carotid intima media thickness
CRP	C-reactive protein
CVD	Cardiovascular disease
DASH	Dietary Approaches to Stop Hypertension
DM2	Diabetes mellitus type 2
EPIC	European Prospective Investigation into Cancer and Nutrition
FA	Fatty acids
GI	Glycemic index
HDL	High-density lipoprotein
HR	Hazard ratio
IL	Interleukin
LDL	Low-density lipoprotein
MI	Myocardial infarction
MR	Mendelian randomization
MUFA	Monounsaturated fatty acid
NHS	Nurses' Health study
PAD	Peripheral artery disease
PUFA	Polyunsaturated fatty acid
RCT	Randomized controlled trial
RR	Relative risk
SDHS	Sydney Diet Heart Study
SFA	Saturated fatty acid
WHI	Women's Health Initiative

16.1 Introduction

Dietary fats have long been hypothesized to influence risk of CVD, for instance through mediators such as lipid profile (Keys, 1965), body weight (Lissner and Heitmann, 1995) or more recently through induction of a low inflammatory state leading to vascular endothelial dysfunction (Santos *et al.*, 2013).

There is a great variety in FA with regard to degree of saturation, carbon atom chain length and *cis* or *trans* configuration of double bonds in unsaturated fats (Calder, 2015; Suburu *et al.*, 2013). Historically, unsaturated fats are considered to be healthier than SFA (Keys, 1965) and

SFA are considered to be healthier than *trans* unsaturated fats (Zock *et al.*, 1995). The influence of SFA on lipid profile has been investigated since the early sixties (Keys, 1965) and the observed relationship provides the basis for the hypothesis that high SFA intake increases risk of CVD.

The relationship between SFA intake and disease outcomes has first been investigated in the Seven Countries Study. A total of 12,763 middle-aged men from 7 countries were included between 1958 and 1964. In these men, higher coronary death rates were observed in men with higher SFA intake. Researchers concluded that this was consistent with the hypothesis that SFA could increase risk of coronary diseases (Anonymous, 1981). The Seven Countries Study was the starting sign for a large number of studies investigating dietary SFA intake and risk of CVD.

According to current dietary guidelines, our SFA intake should be limited to <10% of our total energy intake. In a diet of 2,500 kcal per day, this translates to a maximum of about 28 grams of saturated fat. Replacement of SFA should preferably be done by PUFA.

However, adherence to the SFA intake recommendation is generally low in North America and Europe (2003), and if SFA intake is reduced, this is usually not related to a higher PUFA intake (Eilander *et al.*, 2015). In the Netherlands, less than 10% of people achieve a saturated fat intake <10% (Van Rossum *et al.*, 2011).

Though dietary recommendations are clear regarding SFA intake, there has been heavy debate regarding the interpretation of available research that has investigated the effect of SFA intake on CVD outcomes. This chapter will provide a review of the available literature for SFA intake in relation to CVD risk factors, intermediates and endpoints. Also, difficulties and considerations when investigating SFA will be described, such as correcting for confounders, interactions, use of biomarkers and nutrigenetics.

16.2 Risk factors for CVD

Risk factors are important to consider when investigating CVD outcomes. Predictive models for development of CVD include characteristics that have been related to increased CVD risk, such as smoking, blood pressure, diabetes and cholesterol measurements (D'Agostino *et al.*, 2008). Dietary habits in general are known to affect cardiovascular risk through cholesterol, blood pressure, body weight and diabetes (Verschuren, 2012). To explore the relationship between intake of SFA and risk of CVD, it is important to first examine the relationship between SFA intake and CVD risk factors.

16.2.1 Lipid profile

The blood lipid profile consists of several lipoproteins such as very low-density lipoprotein, LDL, intermediate-density lipoprotein and HDL. Also triglycerides and cholesterol, which can be transported to peripheral tissues by lipoproteins, are a part of the lipid profile. LDL is the major

cholesterol transporting particle in plasma, delivering sterol to cells for growth and hormone production (Mu and Hoy, 2004). LDL-cholesterol levels have been related to increased CVD risk and can be lowered through lifestyle and medication (Ray *et al.*, 2014). In contrast to LDL, HDL particles are hypothesized to have a beneficial effect on CVD through their role in cholesterol transport back to the liver and anti-oxidant and anti-inflammatory effects (Rosenson, 2010). The ratio of total/HDL-cholesterol seems to be the most informative cholesterol measurement CVD risk (Lewington *et al.*, 2007), but non-HDL cholesterol and triglycerides measurements seem to be very similar risk factors (Colantonio *et al.*, 2016; Di Angelantonio *et al.*, 2009). Apolipoproteins are the proteins that bind lipids to form lipoproteins and seem to have an effect on CHD risk that is independent from triglycerides and HDL-cholesterol (Di Angelantonio *et al.*, 2009). For all lipid profile measurements, the proportional risk reduction of CVD decreases with higher age (Lewington *et al.*, 2007).

The relationship between dietary intake of SFA and lipid profile has been extensively investigated in both observational studies and trials. A meta-analysis of 84 controlled trials was recently published by the WHO investigating the effect of FA and CHO on lipid profile (Mensink, 2016), updating a previous meta-analysis from 2003 (Mensink *et al.*, 2003). Replacement of SFA by *cis*-PUFAs and *cis*-MUFAs in trials had a small effect on total/HDL ratio (normal value <5 mmol/l), lowering it by 3.4×10^{-2} (95% CI: -4.0×10^{-2} to -2.8×10^{-2}), and 2.7×10^{-2} (95% CI: -3.3×10^{-2} to -2.2×10^{-2}), respectively. The reduction of total/HDL ratio for replacement of SFA by CHO was very small and not statistically significant (2.0×10^{-3}, 95% CI: -9.0×10^{-3} to 5.0×10^{-3}). Triglyceride levels increased with higher CHO intake instead of SFA, but other lipid profile measurements such as apolipoproteins suggested a beneficial effect of this replacement (Mensink, 2016). Specifying SFA into subtypes and using these subtypes as substituting nutrient for CHO has shown that lauric acid (C12:0), mystiric acid (C14:0) and palmitic acid (C16:0) increase HDL-cholesterol. Replacement of CHO by lauric acid even leads to a significant reduction in total/HDL cholesterol (-0.035, 95% CI: -0.048 to -0.033). There was not enough high-quality evidence available regarding replacement of SFA by CHO subtypes (eg sugars versus starch or low versus high GI) to meta-analyze these studies (Mensink, 2016). However, there is a suggestion that CHO rich diets low in GI lead to a more beneficial lipid spectrum when compared to CHO rich diets with high GI foods (Sloth *et al.*, 2004). Studies that provide further insight into the replacement of SFA by CHO subtypes would therefore be very useful in determining the value of replacing SFA by CHO in general.

Discussion remains as to what lipid profile measurement should be used for CVD risk estimation. Based on current evidence, SFA replacement by *cis*-PUFAs and *cis*-MUFAs leads to a more favorable lipid profile with regard to total/HDL cholesterol ratio, triglycerides and apolipoproteins. CHO might be beneficial as a replacing nutrient, but more studies are warranted to understand the effects of different subtypes of CHO.

16.2.2 Hypertension

Hypertension is related to a decrease in vessel compliance through endothelial dysfunction and this has also been related to atherosclerosis, suggesting an overlap in pathophysiological mechanisms (Cines *et al.*, 1998). Decreased vessel compliance requires a higher cardiac output to supply organs with enough oxygen, which would explain a rise in blood pressure as result of endothelial dysfunction, but it is also possible that high blood pressure is actually the cause of the endothelial dysfunction (Cines *et al.*, 1998). Hypertension has strong associations with CVD outcomes (D'Agostino *et al.*, 2008) and is considered the main modifiable risk factor for stroke (Sacco *et al.*, 1997). A 5 mm Hg lower diastolic BP or a 10 mm Hg lower systolic BP has been associated with approximately 30-40% lower stroke risk in a linear fashion (Lawes *et al.*, 2004). The relationship between blood pressure and total CVD mortality is characterized by a J-shaped curve, especially for diastolic BP (Messerli and Panjrath, 2009).

There is some evidence from animal experiments that suggests that SFA intake could increase risk of hypertension, since higher intake of SFA has shown to increase blood pressure in rats (Gerber *et al.*, 1999; Valensi, 2005). In humans, intake of high fat-meals have shown to have a detrimental effect on vascular function, which could lead to hypertension, but it remains unclear if a high SFA meal leads to a clinically relevant stronger effect on vascular function (Vafeiadou *et al.*, 2012).

It is important to realize that blood pressure is a continuous trait and that this differs from hypertension, which is commonly defined as a systolic BP >140 mm Hg and/or a diastolic BP >90 mm Hg. RCTs are generally more appropriate to investigate an effect on blood pressure than hypertension. Contrarily, in observational prospective studies, hypertension is often used as outcome.

In 1962, a large RCT started investigating non-isocaloric diets with different PUFA/SFA ratio's, but found no relationship with blood pressure (Anonymous, 1968). This lack of association was confirmed in several other RCTs, though some studies did suggest an effect (Morris, 1994; Schwab *et al.*, 2014). Observational studies found a similar lack of association between SFA intake and hypertension. Three large prospective studies among American cohorts did not find evidence for associations of intake of SFA and hypertension after adjustment for obesity related factors and dietary intake of fiber and micronutrients such as calcium, potassium and sodium (Ascherio *et al.*, 1992; Wang *et al.*, 2010; Witteman *et al.*, 1989).

Dietary patterns that are generally low in SFA have also been associated with hypertension. With respect to low SFA dietary patterns, the DASH diet (Appel *et al.*, 1997) is often highlighted. It is characterized by high intake of vegetables, fruit and low-fat dairy foods. It prescribes a relatively high intake of potassium, magnesium and calcium. Furthermore, it is low in saturated and total fat. The dietary intakes of the DASH trial participants were fully controlled. After three weeks, systolic BP was 5.5 and diastolic BP 3.0 mm Hg lower with the DASH diet when compared to the control diet. The blood pressure reduction was stronger for hypertensive participants. It is theoretically possible that the effect of these diets is caused by the low saturated fat intake.

However, these diets are also high in fruit, vegetables and other potential beneficial compounds, which make it impossible to disentangle this from the effect of SFA intake.

Although there seems to be a beneficial effect of dietary patterns low in SFA on blood pressure, there is no convincing evidence of an independent effect of dietary SFA intake on blood pressure or development of hypertension.

16.2.3 Type 2 diabetes

Diabetes is a risk factor for CVD. Diabetics are more prone to developing atherosclerosis, and complications such as PAD and CHD are therefore relatively common. Risk of stroke is higher for a patient with diabetes and a diabetic's prognosis is worse – mortality from stroke is three times higher for diabetics compared to non-diabetics. Renal disease is a serious complication that may develop after a long period of diabetes and further increases risk of CVD, especially for patients on renal dialysis (Grundy *et al.*, 1999). Type 1 diabetes is caused by destruction of islet cells in the pancreas and its incidence is not directly influenced by lifestyle (Atkinson and Maclaren, 1994). Type 2 diabetes, however, can be influenced by nutrition, among other factors (Grundy *et al.*, 1999).

Saturated fats have been hypothesized to influence risk of diabetes, based on results from animal studies. A high intake of SFA has shown to increase insulin resistance in rodents (Guldstrand and Simberg, 2007). According to several systematic reviews (Riserus *et al.*, 2009; Schwab *et al.*, 2014), there seems to be a beneficial effect of replacing intake of SFA with MUFA or PUFA (in particular omega-6 PUFA) on insulin resistance (Riserus *et al.*, 2009; Schwab *et al.*, 2014), but there is no evidence of an effect on fasting glucose (Schwab *et al.*, 2014). A meta-analysis of observational studies did not find a relationship between saturated fat intake and DM2 incidence after multivariable adjustment ($RR_{\text{high vs low intake}} = 0.95$, 95% CI: 0.88-1.03, 237,454 participants, 8,739 incident cases of DM2) (De Souza *et al.*, 2015).

These results from observational studies that investigate SFA intake and DM2 do not concur with previous RCTs investigating insulin resistance. It is possible that the association between SFA and DM2 has been attenuated by opposite effects of different SFA subtypes, since individual plasma SFAs have been differentially related to type 2 diabetes incidence (Forouhi *et al.*, 2009). Food sources of SFA also may play a role, as SFA derived from red meat are suggested to lead to higher DM2 incidence (Pan *et al.*, 2013), whereas dairy-derived SFA are suggested to have an inverse relationship or no relationship with DM2 risk in observational studies (Morio *et al.*, 2016).

Another aspect, is that the relationship between SFA intake and diabetes may be influenced by insulin resistance itself. Findings from the LIPGENE controlled dietary intervention study suggest that the metabolic phenotype of subjects determines their response to the quality of dietary fat on BMI, lipid spectrum measurements and insulin resistance (Yubero-Serrano *et al.*, 2015).

In summary, it is possible that higher SFA intake leads to increased insulin resistance based on results from RCTs. However, there is no convincing evidence that higher SFA intake leads to increased fasting glucose from RCTs and observational studies found no evidence of a higher diabetes incidence in participants with a high intake of SFA. A further complicating factor when investigating these associations is that having (pre)diabetes might alter the effect of dietary fat intake on the development of diabetes. We therefore conclude that no association of total SFA intake on diabetes incidence has been found, but that there are suggestions of an effect of SFA intake on intermediates for diabetes.

16.2.4 Obesity

Though recognized as a cardiovascular risk factor, obesity is not included in the calculation of the Framingham risk score (D'Agostino *et al.*, 2008), since a proportion of the effect of obesity is considered to be caused by the co-occurrence with glucose intolerance, dyslipidemia and hypertension ('the metabolic syndrome') (Meigs, 2000). These are factors that are included in the Framingham risk score (D'Agostino *et al.*, 2008). Co-occurrence of these different risk factors and the resulting higher risk of both DM2 and CVD, suggests that a part of the physiology of both diseases overlap, but causality is not yet clarified. A recent mediation analysis for the effect of BMI on CHD, investigated a total of 58,322 participants with 9,459 cases of CHD (Lu *et al.*, 2015). The increased CHD risk for participants with a higher than normal BMI seems mainly mediated by blood pressure, blood glucose and cholesterol, accounting for 47% to 52% of the effect of BMI (Lu *et al.*, 2015). Therefore, a large proportion of the effect of BMI does not seem mediated by other CVD risk factors and it is therefore important to consider BMI in modelling CVD outcomes after correction for other known risk factors.

The problem in investigating SFA intake and anthropometric measurements is that fat delivers energy to the body; if energy intake is higher than energy expenditure, the body will not be in energy balance and the extra energy will be stored, meaning that a person gains weight. However, this effect would be due to energy intake rather than SFA intake. A meta-analysis of RCTs that investigated low-fat diets and anthropometric measurements has shown that low-fat diets can lead to a reduction in weight (Hooper *et al.*, 2015a). However, sensitivity analyses where studies were categorized depending on the difference in total energy intake between intervention and control group showed that this effect was only statistically significant for studies where the control group had a higher total energy intake in comparison to the intervention group. The largest effect of a low-fat diet was observed if the reduced fat diet was >200 kcal lower in energy intake than the control diet (Hooper *et al.*, 2015a). This points to a limitation of investigating the relationship between fat intake and obesity measurements. Even in a controlled setting it remains difficult to disentangle the effect of energy intake and fat intake due to the necessity of *ad libitum* diets to make these long RCTs feasible.

Due to the high correlation between macronutrients and energy intake, observational studies often use iso-caloric statistical models to investigate the relationship between macronutrients and obesity measurements (Willet, 2013). In the NHS cohort, a 0.18 kg higher weight per 1%

increase in energy intake from SFA was recorded after eight years of follow-up after adjustment for age, BMI at start of study, leisure time physical activity, time spent sitting, percentage of calories from protein and change in percentage of calories from protein (Field *et al.*, 2007). Results from the European EPIC cohort were not consistent with results from the NHS, since a weak inverse association between SFA intake and weight was observed for women in the EPIC cohort (Forouhi *et al.*, 2009). In the total EPIC population, SFA intake was not related to obesity measurements (corrected for age, baseline weight and height, total energy intake, duration of follow-up, smoking, education and physical activity). Correction for energy was done using the residual, nutrient density and energy partition method (Forouhi *et al.*, 2009).

A reason for the discrepancy between observational studies could be an effect of residual confounding, since high SFA intake is related to unhealthy dietary habits (e.g. intake of sugar sweetened beverages) and lifestyle habits (e.g. lower physical activity). SFA subtype, determined by carbon chain length, might also play a role – longer-chain SFA are less likely to oxidize and it is hypothesized that they are therefore more likely to be stored in the body (DeLany *et al.*, 2000).

In short, there is no convincing evidence of a relationship between intake of saturated fats and obesity that is independent of energy intake. The necessity of *ad libitum* diets in RCTs and residual confounding in observational studies makes it very difficult to further clarify these associations.

16.2.5 Inflammation

Damage to the endothelial vessel wall due to subclinical inflammation has been hypothesized as part of the pathophysiology of CVD (Heinrich *et al.*, 1995; Pearson *et al.*, 2003). One possible mediator of this effect could be hypertension, since increased levels of inflammatory markers have been associated with hypertension several years in the future (Crowley, 2014). Other hypothesized mediators are BMI, hyperlipidemia and increased insulin resistance (Welty *et al.*, 2016). Inflammation might also have an atherothrombotic effect in itself (Libby *et al.*, 2011). Also childhood infections have been associated with CVD (risk factors) in adult life (Burgner *et al.*, 2015; Qanitha *et al.*, 2016), though it is difficult to infer causality based on these observational studies. A Mendelian randomization study investigating CRP levels and risk of CHD did not support a causal relationship (Elliott *et al.*, 2009), but studies investigating IL-6 and CHD did find evidence for causality of the relationship between inflammation and CVD (The Interleukin-6 Receptor Mendelian Randomisation Analysis (IL6R MR) Consortium, 2012; Sarwar *et al.*, 2012). IL-6 is found further upstream from CRP in the inflammatory cascade, suggesting that even though CRP might not be causally related to CVD, inflammation in general is. Medication aimed towards lowering the body's inflammatory response may even be used to improve cardiovascular health (Ridker, 2016).

Saturated fats have pro-inflammatory properties *in vitro* (Soto-Vaca *et al.*, 2013) and have shown to induce an inflammatory state in animal studies (Laugerette *et al.*, 2012; Shen *et al.*, 2013). A systematic review published in 2013 included 15 studies regarding SFA intake and inflammatory markers (i.e. adhesion molecules, cytokines, acute phase proteins and adipokines) in humans

(Santos *et al.*, 2013). Only high sensitivity CRP, an acute phase proteins, was convincingly associated with SFA intake (Guasch-Ferre *et al.*, 2015). More recently, a randomized controlled dietary intervention trial investigated the effect of SFA replacement by MUFA on E-selectin (an adhesion molecule) levels and found a 7.8% lower level of E-selectin levels in the intervention group (Vafeiadou *et al.*, 2015).

SFA intake has been linked to plasma inflammatory markers, which in turn have been associated with CVD outcomes. However, inflammatory markers are not broadly considered as CVD risk factors, since the underlying pathways are not yet fully elucidated. Some inflammatory markers may be causally related to CVD development, whereas others are not. Further studies are warranted to determine if and how the effect of SFA intake on inflammatory markers could be used to prevent CVD.

16.2.6 Subclinical atherosclerosis

Subclinical atherosclerosis is considered an intermediate factor or surrogate marker of CVD (Bots, 2006). The degree of atherosclerosis can be quantified with different non-invasive techniques. These techniques can assess the degree of atherosclerosis in the vessel bed of patients that do not have clinical signs and symptoms. One approach uses ultrasound measurements of the carotid artery to examine thickness of the two innermost layers of the vessel wall, called the *tunica intima* and *tunica media*. The resulting measurement is called CIMT and a thicker CIMT has been related to higher risk of CVD (Bots, 2006). Another approach measures CAC through CT scans. A higher degree of CAC has been related to CVD endpoints (Erbel and Budoff, 2012). The risk factors for subclinical atherosclerosis quantified with those techniques are the same as for CVD endpoints (Bots, 2006; Erbel and Budoff, 2012), underlining the usefulness of these measurements as surrogates for CVD. These measures are particularly useful for early detection of high risk patients. Moreover, using such endpoints in trials is very efficient, since effects of interventions can be observed in a relatively short time.

It seems logical to assume that SFA intake is related to measurements of subclinical atherosclerosis, since SFA intake has a strong relationship with lipid spectrum measurements (Mensink, 2016) and a detrimental lipid spectrum is related to subclinical atherosclerosis (Bots, 2006; Erbel and Budoff, 2012). Several cross-sectional studies have investigated SFA intake and CIMT and/or CAC (Masley *et al.*, 2015; Merchant *et al.*, 2008; Park *et al.*, 2006; Virtanen *et al.*, 2014). One study among a mixed ethnic population (Aboriginals, South Asian, Chinese, European) of 620 participants found that a 10 g higher SFA intake was related to a 0.03 higher CIMT with a *P*-value of 0.01, but the study did not mention a confidence interval for the estimate (Merchant *et al.*, 2008). The other studies did not find a relationship between SFA intake and subclinical atherosclerosis measurements (Masley *et al.*, 2015; Park *et al.*, 2006; Virtanen *et al.*, 2014). Another study investigated lifestyle habits and CIMT among 94 participants (Markus *et al.*, 1997) and found that a higher MUFA/SFA ratio was associated with a borderline significant slower progression of CIMT (-0.04, 95% CI: -0.07 to 0) after one year. Two RCTs have investigated SFA intake reduction as part of a broader set of interventions and both observed reduced progression

of CIMT with the intervention (Bemelmans *et al.*, 2002; Masquio *et al.*, 2015). No RCT has investigated SFA reduction as a separate intervention so it remains unclear if the observed effects are caused by SFA reduction or the other interventions regarding diet (Bemelmans *et al.*, 2002; Masquio *et al.*, 2015) or physical activity (Masquio *et al.*, 2015).

In summary, evidence regarding SFA intake and degree of subclinical atherosclerosis is scarce. Further studies are necessary to determine if there is an independent effect of SFA intake on carotid intima media thickness or CAC.

16.3 Cardiovascular disease endpoints

A large body of evidence has accumulated regarding dietary saturated fat intake and risk of CVDs. Of the CVD subtypes, most studies have focused on CHD or stroke. Providing a systematic review of all available evidence goes beyond the scope of this chapter. Therefore, recent and important systematic reviews and meta-analyses of observational and intervention studies regarding dietary intake of saturated fats and risk of CVD in humans are discussed (Table 16.1).

16.3.1 Saturated fat intake and cardiovascular disease

The meta-analysis by Hooper *et al.* (2015b) is most recent when it comes to the effect of SFA reduction or modification on risk of CVD, CHD and stroke. Studies with varying baseline risk of CVD were included, as were secondary prevention trials (i.e. after a CVD event). This meta-analysis updated results from a previous study in 2012 (Hooper *et al.*, 2012). From the 48 RCTs that were included in the 2012 systematic review, 15 were included for the updated review. A new search was performed to find studies that were published after the previous search, which led to the screening of 1,459 titles and abstracts, and yielded 5 potential RCTs, which were excluded after additional information from authors was requested.

In the most recent meta-analysis (Hooper *et al.*, 2015b), CVD mortality was defined as death from MI, stroke or sudden death. Combined CVD events were defined as cardiovascular death, cardiovascular morbidity (non-fatal MI, angina, stroke, heart failure, peripheral vascular events, atrial fibrillation) and emergency cardiovascular interventions (coronary artery bypass surgery or angioplasty). For the CVD outcomes, results from 12 RCTs were investigated after exclusion of 9 studies that were included in the previous meta-analysis (i.e. the Minnesota Coronary Men and Women study, the National Diet Heart study, the WHEL study, DO IT, MeDiet, PREMIER, and the studies by Ball and Søndergaard), due to a lack of adequate control group, no difference in SFA content of diets or short follow-up. In total, 314 trials were excluded from analysis. An explanation of reasons for exclusion is provided per study in the meta-analysis (Hooper *et al.*, 2015b).

For CVD mortality and CVD events as separate outcomes of interest there was a large overlap in studies included in the meta-analysis since CVD mortality was also considered to be a CVD event

Table 16.1. Description of recent meta-analyses that investigated the effect of saturated fatty acids (SFA) intake on cardiovascular disease (CVD) outcomes.[1,2]

Reference	End of data collection	Study designs	Investigated CVD outcome	SFA research question[3]
Cheng *et al.* (2016)	Feb. 2016	PCS	stroke	reduction
De Souza *et al.* (2015)	May 2015	PCS/RCS	CVD/CHD/stroke	reduction
Hooper *et al.* (2015b)	Mar. 2014	RCT	CVD/CHD/stroke	reduction and modification
Schwingshackl and Hoffmann (2014b)	Feb. 2014	RCT	secondary CHD	reduction and modification
Chowdhury *et al.* (2014)	July 2013	RCT	CHD	reduction and modification
		PCS	CHD	reduction
Siri-Tarino *et al.* (2010a)	Sept. 2009	PCS	CVD/CHD/stroke	reduction and substitution
Mozaffarian *et al.* (2010)	June 2009	RCT	CHD	modification
Jakobsen *et al.* (2009)[4]	2009	PCS	CHD	substitution
Skeaff and Miller (2009)	Sept. 2009	RCT	CHD	reduction and modification

[1] All studies are meta-analyses, except for the study by Jacobsen (2009), as this was a substitution analysis that used data from 11 cohorts. Studies may mention other outcomes or interventions as well.
[2] CHD = coronary heart disease; CVD = cardiovascular disease; PCS = prospective cohort study; RCS = retrospective cohort study; RCT = randomized controlled trial.
[3] SFA reduction in trials indicates that there was a difference in SFA intake between intervention and control. SFA modification indicates that the intervention aimed not only to reduce SFA, but also specified what nutrient should replace SFA. SFA reduction in observational studies indicates that high vs low SFA intake within a population was investigated. SFA substitution means that the analysis was modelled in SFA reduction with concomitant increase of a specified other nutrient.
[4] Publication year is noted as end of data collection was not mentioned.

(Hooper *et al.*, 2015b). Two RCTs noted only CVD events and were therefore not included in the mortality analysis (Houtsmuller *et al.*, 1980a,b,c; Moy *et al.*, 2001). Cardiovascular deaths in the SDHS (Ramsden *et al.*, 2013) were not included for the CVD events analysis without clarification.

Interventions were divided into a reduction of SFA intake and SFA modification. A reduction of SFA intake did not influence CVD death ($RR_{CVD\ death}$ = 0.95, 95% CI: 0.80-1.12, 53,421 participants, 1,096 CVD deaths, I^2=30%), but a lower risk of CVD events was found ($RR_{CVD\ events}$ = 0.83, 95% CI: 0.72-0.96, 53,400 participants, 4,377 CVD events, I^2=65%). Modification of diets high in SFA to diets lower in SFA and higher in PUFA did not change conclusions for CVD death. Diets comparably low in SFA and high in PUFA (but not MUFA or CHO) showed a stronger inversely correlation with CVD events ($RR_{CVD\ events}$ = 0.73, 95% CI: 0.58-0.92, I^2=69%, >3,000 participants, 884 CVD events). This is consistent with findings from the previous meta-analysis (Hooper *et al.*, 2012).

One meta-analysis limited study inclusion to only secondary prevention studies of CVD, and included 12 studies with a total of 7,150 participants (Schwingshackl and Hoffmann, 2014b). No association was found between high versus low SFA intake and cardiovascular mortality (RR=0.93, 95% CI: 0.66-1.31, I^2=0%), nor was there a relationship between SFA intake and CVD events (RR=0.93, 95% CI: 0.65-1.34, I^2=57%). Fat modification was not convincingly beneficial either ($RR_{CVD\ mortality}$ = 0.96, 95% CI: 0.65-1.42, I^2=69%; $RR_{CVD\ events}$ = 0.85, 95% CI: 0.63-1.15, I^2=75%). Funnel plot of the available studies showed little to moderate asymmetry, suggesting some degree of publication bias (Schwingshackl and Hoffmann, 2014b).

A meta-analysis of three observational studies did not find a relationship between SFA intake and CVD mortality with an RR of 0.97 (95% CI: 0.84-1.12) after multivariable adjustments, though investigated outcomes in original studies differed strongly (from only stroke mortality to mortality due to any circulatory diagnosis). CVD events were not investigated separately (De Souza *et al.*, 2015). A previous meta-analysis had a more limited definition of CVD (stroke plus CHD), but included both fatal and non-fatal CVD events and found a risk ratio of 1.00 (95% CI: 0.89-1.11,I^2=56%) (Siri-Tarino *et al.*, 2010a).

Recently, a prospective observational study was published that investigated the relationship between total and specific fat subtypes and (cause-specific) mortality in the American NHS and Health Professional's Follow-up Study cohorts (Wang *et al.*, 2016). Intake of SFA, when substituted for CHO, was not significantly associated with higher CVD mortality after multivariable adjustments ($HR_{high\ vs\ low}$ = 1.03, 95% CI: 0.93-1.13). However, substituting PUFA for CHO did show lower CVD mortality risk, which would suggest that there might also be a beneficial effect of substituting PUFA for SFA. Sensitivity analyses after exclusion of the first four years of follow-up suggested a possible detrimental effect of high SFA intake for CVD risk ($HR_{high\ vs\ low}$ = 1.13, 95% CI: 1.01-1.26), but the possibility of a chance finding cannot be excluded given the large number of analyses performed in this study (Wang *et al.*, 2016).

There is substantial heterogeneity between (outcome definition in) studies regarding CVD. Lowering (or replacing) intake of SFA does not seem to lead to lower CVD mortality in meta-analyses of trials and observational studies, although some individual studies do suggest a beneficial effect of substituting SFA by PUFA on CVD mortality. Meta-analyzing evidence from RCTs that investigated CVD events (combining mortality and morbidity), has shown that lowering SFA intake modestly decreases risk of CVD events and that this effect is stronger when PUFA replaces SFA. This association could not be confirmed in meta-analysis of secondary prevention trials for participants with established CVD.

16.3.2 Saturated fat reduction and coronary heart disease

The meta-analysis by Hooper *et al.* (2015b) showed that interventions with SFA intake reduction did not influence CHD mortality (RR=0.98, 95% CI: 0.84-1.15, I^2=22%, >53,000 participants with 886 CHD deaths CHD deaths). CHD events did not decrease after SFA reduction either ($RR_{CHD\ event}$ = 0.87, 95% CI: 0.74-1.04), I^2=66%, >53,000 participants, 3,307 cases). There was a

borderline significant reduced risk of MI events with SFA reduction (RR=0.90, 95% CI: 0.80-1.01, I^2=10%, >50,000 participants, 1,714 incident cases of MI), but this was not found for non-fatal MI (RR=0.95, 95% CI: 0.80-1.13, I^2=27%, >52,000 participants with 1,345 incident cases of MI) (Hooper *et al.*, 2015b).

In the most recent meta-analysis of observational studies (De Souza *et al.*, 2015), RR of CHD mortality for high versus low SFA intake was 1.15 (95% CI: 0.97-1.39, I^2=70%, 101,712 participants, 2,970 cases of CHD mortality), based on multivariable adjusted models. Including only studies that corrected for most relevant confounders (age, smoking, sex and total energy intake) did not change conclusions. SFA intake was not associated with CHD events either (RR=1.09, 95% CI: 0.95-1.17, I^2=47%, 267,416 participants, 6,383 cases of CHD) when comparing groups of highest versus lowest SFA intake. Substantial heterogeneity was observed between studies, for which no clear explanation was found in extensive subgroup analyses with meta-regression (De Souza *et al.*, 2015). One meta-analysis that examined CHD events included only prospective observational studies (Chowdhury *et al.*, 2014) and also found non-significant results; an RR of 1.03 (95% CI: 0.98-1.07, I^2=35.5%) of CHD events was observed when comparing the highest versus lowest third of SFA intake (Chowdhury *et al.*, 2014). This is in line with results from previous meta-analyses of prospective cohort studies (Siri-Tarino *et al.*, 2010a; Skeaff and Miller, 2009).

In summary, there is no convincing evidence that SFA reduction alone leads to a lower CHD risk.

16.3.3 Saturated fat replacement and coronary heart disease

Though there is limited evidence for a beneficial effect of reduction in SFA intake on CHD risk, it might be a bit premature to conclude that there is no relationship between SFA intake and CHD at all. When lowering intake of SFA, the effect of this intervention also depends on the substituting macronutrient. In a Western diet (with high SFA intake), replacing 1% of energy from SFA with PUFAs lowers LDL-cholesterol and is estimated (based on the LDL-cholesterol lowering effect) to reduce CHD incidence with 2-3% (Astrup *et al.*, 2011). Therefore, we also report results from studies that have investigated replacement of SFA rather than SFA reduction.

Mozaffarian (2010) found a statistically significant risk reduction of CHD events with fat modification of SFA to PUFA of 19% (RR=0.81, 95% CI: 0.70-0.95, 13,614 participants, 1,042 CHD events, I^2=37%) in the intervention groups versus control groups (Mozaffarian *et al.*, 2010). This translates to a 10% reduced CHD risk per 5 energy% replacement of SFA by PUFA, which is consistent with the hypothesized effect of this replacement based on lipid profile measurements (Astrup *et al.*, 2011). Another meta-analysis that was published around the same time also investigated SFA-PUFA replacement and included the same intervention trials, plus one additional trial (Skeaff and Miller, 2009). The meta-analysis by Skeaff (2009) yielded slightly different estimates for CHD events and results were only borderline significant (RR=0.83, 95% CI: 0.69-1.00, I^2=44.2%) (Skeaff and Miller, 2009).

In the meta-analysis by Chowdhury *et al.* (2014), one of the aims was to investigate omega-6 PUFA supplementation on CVD risk, but included studies and analyses were similar to previous meta-analyses. All RCTs from Mozaffarian (2010) were included, plus one other trial, the SDHS (Ramsden *et al.*, 2013). Without this latter trial, the effect estimate was identical between the two meta-analyses, showing a 19% risk reduction of CHD events (RR=0.81, 95% CI: 0.68-0.98) for PUFA intervention groups. However, after including the SDHS (Ramsden *et al.*, 2013), results were no longer statistically significant (RR=0.90, 95% CI: 0.79-1.02). Inclusion of this study in the meta-analyses by Chowdhury (2014) was criticized. The study was said to be extreme due to the very high PUFA content and the PUFA margarine was supposedly high in *trans*-fats (Liebman *et al.*, 2014; Te Morenga *et al.*, 2014; Willett *et al.*, 2014).

In secondary prevention of CHD, evidence regarding the effect of SFA reduction seems to be very limited. The meta-analysis by Schwingshackl and Hoffmann (2014b) found no significant risk reduction of MI for reduced SFA intake (RR=1.18, 95% CI: 0.88-1.59, I^2=19%, 2,584 participants, 240 cases of MI) or for modified SFA intake (RR=0.76, 95% CI: 0.54-1.09, I^2=55%, 3,523 participants, 292 cases of MI), though fat modification included both MUFA and PUFA interventions (Schwingshackl and Hoffmann, 2014b).

Jakobsen (2009) performed a pooled analysis of 11 prospective European and American cohorts that investigated substitution of SFA by PUFA, MUFA and CHO with regard to CHD incidence (Jakobsen *et al.*, 2009). In total, there were 344,696 participants with 5,249 coronary events and 2,155 coronary deaths. For a 5% lower energy intake from SFA and concomitant higher energy intake from PUFA, a lower risk of both CHD death (HR=0.74, 95% CI: 0.61-0.89) and CHD events (HR=0.87, 95% CI: 0.77-0.97) was observed. Substitution of SFA by MUFA and CHO was not associated with a lower risk of CHD (mortality) (Jakobsen *et al.*, 2009). GI of CHO might play a role, since replacing SFA by CHO with high-GI values has been related to higher risk of MI (Jakobsen *et al.*, 2010; Praagman *et al.*, 2016a). The null finding of replacing SFA by MUFA was somewhat surprising, since MUFA would be expected to have a beneficial effect based on their effect on lipid spectrum (Mozaffarian *et al.*, 2010) and intake of MUFA has indeed been suggested to lower risk of CVD (Schwingshackl and Hoffmann, 2014c). Authors noted that the source of MUFA in these cohorts with a Western diet might explain these unexpected findings, since most MUFA were derived from meat products (Jakobsen *et al.*, 2009).

Reduction of SFA intake in itself, without specifying the replacing macronutrient, has not convincingly shown to decrease CHD risk in observational studies or RCTs. Overall, many of the trials included in meta-analyses suffer from design limitations such as single-blinding or open enrolment. Duration of follow-up varied substantially and funnel plots suggested the possibility of publication bias. The effect of an 'SFA reduction' seems to be determined by the macronutrient that replaces saturated fat – both RCTs and observational studies suggest that eating PUFA instead of SFA reduces CHD risk. Replacement of SFA by CHO or MUFA has not shown to lower CHD risk, though it is still possible that certain CHO subtypes or MUFAs from specific food sources do lower CHD risk. In established CHD, there is no evidence of a beneficial effect of fat modification.

16.3.4 Saturated fat intake and stroke

Hooper *et al.* (2015b) did not observe an effect of altering dietary fat on stroke events (including both ischemic and hemorrhagic strokes) after meta-analyzing results of >50,000 participants with 1,125 incident strokes (RR=1.00, 95% CI: 0.89-1.12, I^2=0%) (Hooper *et al.*, 2015b). The number of studies investigating SFA intake and risk of stroke is limited and the WHI trial, with its sample size of 48,835 (1,076 stroke cases), has a large weight in the analysis. Without this trial, a possible protective effect of SFA intake is suggested (RR=0.63, 95% CI: 0.34-1.14, >2,000 participants, 46 cases of stroke, I^2=0%), but results are not statistically significant (Hooper *et al.*, 2015b). This meta-analysis is based on a previous meta-analysis (Hooper *et al.*, 2012), but does not include any trials that were performed after the previous search date. However, different studies were included in the two meta-analyses. Comparison of the two studies shows that confidence intervals for results after exclusion of WHI were slightly different in the previous meta-analysis (RR=0.61, 95% CI: 0.39-1.02, 11,018 participants, 64 cases of stroke, I^2=0%) (Hooper *et al.*, 2012).

Two recent meta-analyses of observational studies have looked into SFA intake and risk of stroke, yielding somewhat different results (Cheng *et al.*, 2016; De Souza *et al.*, 2015). De Souza (2015) meta-analyzed retrospective and prospective studies regarding SFA intake and ischemic stroke. Two of the studies that were included investigated plasma fatty acid measurements rather than a direct measure of SFA intake (Wiberg *et al.*, 2006; Yamagishi *et al.*, 2013). Among 339,090 participants, 6,226 cases of ischemic stroke were ascertained. In random-effect meta-analysis, RR of developing an ischemic stroke in the highest versus lowest SFA intake group was 1.02 (95% CI: 0.09-1.15, I^2=59%). Results were substantially different in Asian populations, where an 18% decrease in ischemic stroke risk (95% CI: 0.69-0.98) was observed (De Souza *et al.*, 2015).

Cheng *et al.* (2016) investigated only prospective studies examining intake of SFA and risk of stroke, but also included studies with hemorrhagic or unspecified strokes. This led to inclusion of five additional studies, two of which were performed in Japanese populations (Iso *et al.*, 2003; Yamagishi *et al.*, 2010). Relative risk of stroke in the highest versus lowest intake group of SFA was 0.89 (95% CI: 0.82-0.96, I^2=37.4%, 476,569 participants, 11,072 incident cases of stroke) in a fixed-effect meta-analysis (Cheng *et al.*, 2016). This association was stronger for hemorrhagic stroke (RR=0.76, 95% CI: 0.63-0.93, I^2=42.5%). Estimates for studies with an intake less than 25 gram of SFA per day (= 9 energy% based on a total energy intake of 2,500 kcal) showed substantially different RRs for the top versus bottom third of SFA intake, with an RR of 0.81 (95% CI: 0.71-0.92, I^2=45.3%) versus 1.02 (95% CI: 0.89-1.15, I^2=2.7%) in studies with SFA intake >25 gram/day. Studies with low SFA intake were mostly performed in Asian populations where background SFA intake was on average 9% of energy (range 5-14%) and this was lower than in populations from North-America (12% of energy, range 9-16%). Japanese populations had a particularly low SFA intake (Cheng *et al.*, 2016; De Souza *et al.*, 2015).

All in all, there are inconsistent results regarding a possible association between SFA intake and risk of stroke. In a meta-analysis of RCTs that were mainly performed in cohorts from North-America, no effect of SFA was observed. In contrast, an inverse association between SFA intake

and risk of stroke is suggested in observational studies that have also included Asian populations with low average SFA intake. It is possible that the relationship between SFA intake and stroke differs between populations, between background SFA intake levels, or between subtypes of stroke. Further studies in specific subgroups are warranted to elucidate these results.

16.3.5 Saturated fat intake and peripheral arterial disease

PAD is caused by peripheral vascular calcification of the lower extremities. ABI is the ratio between blood pressure at the ankle to blood pressure in the arm and is used determine degree of PAD. The ABI is considered to be normal when >0.9. The relationship between SFA and PAD or ABI has been investigated in several cohorts, but results have been inconsistent (Donnan *et al.*, 1993; Gimeno *et al.*, 2008; Lane *et al.*, 2008; Naqvi *et al.*, 2012). Therefore, no conclusions can be drawn with regard to SFA intake and PAD risk based on current evidence.

16.4 Shifting focus: SFA subtypes and biomarkers, food matrix and genetics

16.4.1 Saturated fatty acid subtypes

SFA subtypes can be differentiated by their carbon atom chain length. Palmitic acid, stearic acid (C18:0) and myristic acid are most commonly consumed, followed by lauric acid and other medium-sized FA (6-12 carbon atoms) (Calder, 2015). In meat, 30-40% of the total fat content consists of SFA. Most of this fat is palmitic acid (15-25%) and stearic acid (10-20%). Myristic and lauric acid are also present in animal fat, but in smaller quantities (<1%). Lauric acid is found in large quantities in palm and coconut oil. Myristic acid is also found in palm and coconut oil and represents about 10% of FA in milk fat (Rioux and Legrand, 2007). The odd-chain FA pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) are mainly found in dairy products (Hodson *et al.*, 2008).

As noted in the section on risk factors, the effect of SFA subtypes on the lipid spectrum differs (Mensink, 2016; Mensink *et al.*, 2003). Furthermore, there are suggestions that longer-chain FA might contribute more strongly to total energy availability in the body due to a lower oxidation rate (DeLany *et al.*, 2000). Variability in the effects of SFA subtypes on CVD (risk factors) is therefore conceivable. The relationship between intake of SFA subtypes and risk of CHD was first investigated in the NHS cohort (Hu *et al.*, 1999b). Stearic acid showed a strong association with increased risk of CHD in age adjusted analyses comparing quintiles of highest versus lowest intake (HR=1.97, 95% CI: 1.62-2.42). Additional correction for confounders, in particular correction for intake of MUFA, PUFA, *trans* fat, protein, dietary cholesterol, fiber and total energy intake, strongly attenuated the findings and results were no longer statistically significant (HR=1.16, 95% CI: 0.81-1.66). Results for the sum of C12:0 to C18:0 per 1 energy% were borderline significant (HR=1.29, 95% CI: 1.00-1.66, P=0.05) in the multivariable adjusted model. A strong correlation between stearic acid and several other SFA subtypes was observed (Pearson correlation coefficient of 0.92 for palmitic acid, 0.66 for mystiric acid) (Hu *et al.*, 1999b). Results from the more recent

EPIC-NL study suggest that a combined intake of the sum of short-chain SFA (C4:0-C10:0) relate to lower CHD risk, as do myristic acid, pentadecanoic acid and hexadecanoic acid. Higher total SFA intake was also associated with lower CHD risk in this study (Praagman *et al.*, 2016a). Subtypes of SFA were also investigated in another Dutch cohort, namely the Rotterdam Study (Praagman *et al.*, 2016b). In this cohort of mid-age to elderly participants, total SFA was not significantly related to CHD risk, but palmitic acid, the largest contributor (50%) to total SFA intake, was significantly related to a higher risk of CHD with an HR of 1.26 (95% CI: 1.05-1.52).

Comparison of these seemingly inconsistent results is difficult, since the interpretation of the effect of SFA subtypes should take effect estimates for total SFA intake and the background SFA intake in that cohort into account – think about different effects of SFA intake on risk of stroke in Asian versus Western cohorts (Cheng *et al.*, 2016; De Souza *et al.*, 2015). Food source of SFA subtypes is a second important factor that should be taken into account, which will be discussed later.

Studies regarding intake of SFA subtypes and risk of CVD outcomes are limited, but intake of long-chain SFA seems more detrimental than consumption of shorter chain SFA. More observational studies that investigate SFA subtype and food source are warranted. These studies need to take the background SFA intake, and previously established effect of total SFA intake on CVD risk within that population into consideration, in order to elucidate results from previous studies.

16.4.2 Biomarkers for saturated fat intake

There have been studies that attempted to clarify the relationship between SFA subtypes and risk of CVD by using different measurable indicators of SFA intake in biological material (e.g. blood, fat tissue). These so-called biomarkers have inherent limitations which need to be considered in order to correctly interpret study results. Biomarkers are not simply a tool to measure SFA intake and we will therefore discuss SFA biomarkers in this section separately.

There is a wide variety of biomarkers available for intake of SFA that can be chosen depending on research question. Long-term SFA exposure can be measured in adipose tissue, ascertained through a biopsy. Adipose tissue is usually not available in epidemiological studies, so plasma phospholipids, plasma cholesterol esters or erythrocyte measurements are often used (Hodson *et al.*, 2008). After a change in diet, plasma biomarker measurements start to change within days (Skeaff *et al.*, 2006) and generally reach a plateau phase within two weeks (Hodson *et al.*, 2014).

Biomarker measurements of even-chain FA, especially myristic, stearic and palmitic acid, have been adversely associated with risk factors for CVD (Ebbesson *et al.*, 2015; Forouhi *et al.*, 2014) and CVD outcomes (Chowdhury *et al.*, 2014). Circulating odd-chain FA have been inversely associated with diabetes (Forouhi *et al.*, 2014; Santaren *et al.*, 2014; Yakoob *et al.*, 2016), MI (De Oliveira Otto *et al.*, 2013; Warensjo *et al.*, 2010) and total CVD (De Oliveira Otto *et al.*, 2012), but no relationship was found with stroke (Yakoob *et al.*, 2014). However, the interpretation of these studies investigating circulating SFA subtypes is complicated. Since plasma biomarker

measurements can reflect endogenous production as well as dietary intake (Rioux and Legrand, 2007; Saadatian-Elahi *et al.*, 2009), results from these studies cannot be interpreted as if they represent an effect of dietary SFA subtype intake on CVD or CVD risk factors. The dairy derived odd-chain SFAs, pentadecanoic acid and heptadecanoic acid, are suggested to be mostly exogenously derived (Hodson *et al.*, 2008), though there seems to be some endogenous production of hexadecanoic acid as well (Jenkins *et al.*, 2015). Endogenous production of even-chain SFA is influenced by diet and increases with high intake of CHO (Hudgins *et al.*, 1996, 1998) and alcohol (Siler *et al.*, 1999).

Circulating even-chain SFA, especially long-chain SFA, have been related to higher risk of CVD, whereas odd-chain SFA might decrease CVD risk. However, these results must be interpreted with caution before they can be translated into advice on dietary intake, since there is insufficient knowledge regarding the influence of endogenous production on some SFA subtypes. Based on current evidence, it is not possible to make statements regarding causality of observed associations between biomarkers for SFA intake and CVD.

16.4.3 Saturated fatty acids within the food matrix

Even if we are able to tease out the effects of endogenous SFA production and SFA intake, we are still left with the interpretation of these associations. A specific SFA subtype can be abundantly present in a specific food, such as lauric acid in coconut oil, pentadecanoic acid in dairy products and palmitic (and stearic) acid in animal foods (e.g. dairy products, meat) (Rioux and Legrand, 2007). By studying these SFA subtypes, one might, in fact, be studying the intake of the food source as a whole, or the intake of other nutrients within the whole food. It is also possible that there is interaction of nutrients within the whole food or that the whole food is correlated with intake of other foods within a dietary pattern. Available evidence regarding SFA (subtype) intake from meats and dairy products will be used to illustrate some of these food matrix considerations.

First, we will explore meat derived SFA subtypes. Palmitic and stearic acid have been associated with higher CHD risk (Chowdhury *et al.*, 2014), and are mostly derived from animal fat, i.e. non-lean red meat. Red meat has been associated with higher risk of CHD (Abete *et al.*, 2014; Bovalino *et al.*, 2016; Kelemen *et al.*, 2005; Kontogianni *et al.*, 2008) and recent prospective studies and meta-analyses are pointing the accusing finger at processed red meat (Abete *et al.*, 2014; Bovalino *et al.*, 2016; Micha *et al.*, 2010). Heme-iron (Fang *et al.*, 2015) or preservatives in processed meat such as sodium (Micha *et al.*, 2013) are proposed as possible explanations for these associations. Furthermore, red meat has been found to be a part of a generally unhealthy 'Western' dietary pattern characterized by high intakes of red and processed meats, high-fat dairy products, refined foods and low intakes of vegetables and fruit (Hu *et al.*, 1999a). Determining causality of the observed relationship between palmitic or stearic acid and CVD in this example is very difficult.

The second example examines the effect of circulating odd-chain SFA on CVD. These SFA have been inversely associated with CHD (risk factors)(De Oliveira Otto *et al.*, 2013; Forouhi *et al.*, 2014; Warensjo *et al.*, 2010) and are mainly found in dairy products, a food group which has

been extensively investigated. High fat dairy products have not convincingly been related to CVD risk factors in meta-analyses of RCTs (Benatar *et al.*, 2013) and observational studies (Kratz *et al.*, 2013), though an inverse relationship has been observed between dairy product intake and obesity (Kratz *et al.*, 2013). Higher total dairy product intake has been associated with lower risk of total CVD (Qin *et al.*, 2015), as has higher milk intake (Soedamah-Muthu *et al.*, 2011). Also, milk and cheese intake have been related to lower risk of stroke (De Goede *et al.*, 2016). Even intake of butter, which is not recommended due to its high SFA content and hypothesized detrimental effect on CVD, has not shown to increase risk of CVD or diabetes (Pimpin *et al.*, 2016). Heterogeneity between studies and a possibility of publication bias (especially for stroke) is mentioned in most meta-analyses (Benatar *et al.*, 2013; De Goede *et al.*, 2016; Kratz *et al.*, 2013; Qin *et al.*, 2015; Soedamah-Muthu *et al.*, 2011). The beneficial effects of dairy products can hypothetically be contributed to calcium, dairy fats, vitamin D, magnesium, potassium, whey proteins (Rice *et al.*, 2011) and even probiotics (Thushara *et al.*, 2016). Furthermore, some of the components of dairy products, such as magnesium, potassium and calcium (Krishna and Kapoor, 1991; Lawton *et al.*, 1990; Lemann *et al.*, 1991) have been suggested to influence absorption or excretion of each other or other food factors and can work synergistically (Rice *et al.*, 2011). An example is the increased fecal fat excretion in the presence of calcium (Thorning *et al.*, 2015).

It is therefore important to consider the effect of whole foods, dietary patterns and interactions within the food matrix when investigating intake of SFA in order to interpret results correctly

16.4.4 Nutritional genetics and CVD

Cost reduction of genome sequencing has made it attainable to investigate genes that influence complex diseases such as CVD.

Large genome-wide association studies have led to the identification of a wide range of loci that are associated with CVD (risk factors) (Arking and Chakravarti, 2009) or that modulate the effect of risk factors for CVD (Polfus *et al.*, 2013). The use of genetic data can provide further clarification of biological pathways and differences in disease risk between populations, or help predict an individual's response to medication (Cambien and Tiret, 2007). Polymorphisms in the *apoE*, *ApoA1* and *PPARγ* genes have been related to differences in lipid profile response to dietary intake of fat (Lovegrove and Gitau, 2008), so even the effect of dietary interventions has a partial genetic explanation.

Furthermore, genetic information can be used as an instrumental variable in MR studies to estimate a causal relationship between a phenotype of interest and disease outcome (Burgess *et al.*, 2010). This technique has been used to assess the relationship between CRP (Burgess *et al.*, 2010) and alcohol intake (Holmes *et al.*, 2014) and CHD, among others.

In summary, genetic information allows us to investigate biological pathways between nutrition and disease. Hopefully, these genetic studies will help us understand why people respond differently to nutritional interventions and allow us to give more appropriate dietary advice.

Using genetic data as an instrumental variable in MR studies can even help us advocate causality of previously observed associations.

16.5 Methodological considerations

16.5.1 Comparability of study populations

The overwhelming amount of studies that have been performed on (saturated) fat intake and risk of CVD (with their sometimes contradictory results), has led to the necessity to combine results from studies to get a better overview of the evidence. The problem in combining these results is that it inevitably leads people to wonder if all these studies can, and should be combined.

There is heterogeneity in study populations that have been included in meta-analyses of CVD endpoints (Cheng *et al.*, 2016; Chowdhury *et al.*, 2014; De Souza *et al.*, 2015; Hooper *et al.*, 2015b; Mozaffarian *et al.*, 2010; Siri-Tarino *et al.*, 2010a). Some original studies have included participants with low CVD risk at baseline, while others have included participants with varying CVD risk profiles or participants with prevalent CVD. Average SFA intake varies between study populations, especially for stroke outcomes (Cheng *et al.*, 2016; De Souza *et al.*, 2015), with Asian populations consuming low amounts of SFA and Western populations consuming relatively high amounts of SFA. This could lead to population stratification when comparing high versus low SFA intake in meta-analyses. Furthermore, the strength of the association between cholesterol level and risk of CHD and stroke reduces with age, which would suggest that diet plays a stronger role in younger participants (Lewington *et al.*, 2007). Effect modification through age has indeed been suggested in a prospective study investigating SFA intake and risk of CHD (Jakobsen *et al.*, 2004). This would suggest that results from studies performed in different age categories might not be comparable.

There are concerns that meta-analyzing study results from the grossly different populations that have been investigated with regard to SFA intake and CVD might obscure relationships in subgroups (Davidoff and Rosenberg, 2014). It is also possible that an association that has been found in a meta-analysis, is not applicable to every subgroup. Though meta-analysis are sometimes required to achieve adequate statistical power, one should realize that this approach has its downsides as well.

16.5.2 Limitations of study designs

Studies investigating SFA intake and risk of CVD are mostly prospective observational studies or RCTs that are aimed at SFA reduction or SFA replacement by other macronutrients.

A long follow-up time in an RCT introduces the problem that people might not be able to stick to the intervention and this could attenuate its effect. An illustration of this is found in the WHI study – the largest single contributor to all RCT meta-analyses regarding CHD and stroke. The

WHI study is a long-term term dietary intervention trial, in which postmenopausal women with a diet >32% in total fat were randomized to either their regular diet, or a diet <20% in total fat. Participants received nutritional counselling to achieve this. After 6 years, there was a statistically significant difference between the intervention and control group with regard to energy% from fat (8.2%), but this difference was much smaller than initially expected. SFA intake was 2.3 energy% lower in the intervention group and there was a compensatory increase in energy% from CHO of 8.1%. As a result, LDL-cholesterol was only slightly lower in the intervention group after six years (Howard, 2007).

Observational studies have their own limitations. First of all, observational studies usually perform only one dietary measurement, which might not reflect dietary habits over time well. This effect might be strengthened by the fact that observational studies are generally performed in a population where the outcome is common enough to ensure adequate statistical power to find a difference in disease incidence. For CVD outcomes, this usually results in a population of middle-aged to older participants. Especially in that population, it is possible that people change their diet during the long follow-up, due to standard care dietary advice when they develop CVD risk factors.

A limitation in the data-analysis of some RCTs and observational studies is that authors have chosen to correct for measured cholesterol levels. Since the effect of SFA on CVD incidence is expected to be mainly mediated by lipid spectrum (Lewington *et al.*, 2007; Mensink *et al.*, 2003), this is a clear example of overcorrection that leads to attenuation of the observed effect, as described in a previous meta-analysis (De Souza *et al.*, 2015). The effect estimate in the meta-analysis by Siri-Tarino *et al.* (2010a) was said to be less extreme due to inclusion of studies that used fully adjusted models, including cholesterol measurements (Scarborough *et al.*, 2010; Stamler, 2010).

Both RCTs and prospective studies have limitations when it comes to investigating SFA intake and risk of CVD. Due to the aforementioned limitations, new studies and meta-analyses tend to lead to more discussion between researchers, rather than providing clarity, as illustrated by the high number of responses to a recent meta-analysis by Chowdhury (2014) (Davidoff and Rosenberg, 2014; Dawczynski *et al.*, 2014; Diekman *et al.*, 2014; Geleijnse *et al.*, 2014; Liebman *et al.*, 2014; McCaulley, 2014; O'Neil and Itsiopoulos, 2014; Schwingshackl and Hoffmann, 2014a; Te Morenga *et al.*, 2014).

16.5.3 Does SFA reduction as a single intervention actually exist?

Another aspect of the discussion regarding SFA intake and health and disease, is the question if it is even possible to reduce SFA intake. This question applies to participants of a trial, but also to people in real life that are trying to follow advice in dietary guidelines.

Short-term RCTs that investigate risk factors for CVD can choose to fully control study diets by providing all foods to participants. In these RCTs, the design of an intervention diet where the

only difference in macronutrient intake is a decrease in SFA is relatively easy. However, this leads to a reduction of total energy intake as well, which is related to CVD risk factors, resulting in uncertainty about the study results.

A fully controlled design is not feasible for RCTs with a longer duration in which CVD endpoints can be investigated. In long-term nutritional RCTs, *ad libitum* diets are commonly used. The change in dietary intake in these studies is achieved by providing dietary advice to participants. Given the long study duration, adherence to dietary advice that is different from the participant's habitual diets, will probably be lower than in studies of a short duration. Also, if the only intervention would be to explain how to decrease SFA intake, participants will either have a lower total energy intake (as in the previous example) or consume other macronutrients (other FA, CHO or protein) to remain in energy balance. The WHI study, an example of a long-term nutritional RCT with an *ad libitum* diet, reported an increased intake of CHO in women that received dietary advice to decrease their fat intake and differences between intervention and control arm were smaller than expected (Howard *et al.*, 2006). Replacement of SFA by CHO might attenuate effects of SFA reduction, since replacement of SFA by CHO has not convincingly shown to improve cardiovascular risk (Jakobsen *et al.*, 2009; Mensink, 2016; Mensink *et al.*, 2003; Wang *et al.*, 2016) and high-GI CHO might even increase risk of CHD (Jakobsen *et al.*, 2010; Praagman *et al.*, 2016a).

Given the uncertainties regarding 'reduction of SFA intake', there are doubts regarding the approach of attempting to look only at SFA intake (Astrup *et al.*, 2011; Mozaffarian, 2014; O'Neil and Itsiopoulos, 2014; Schwingshackl and Hoffmann, 2014a; Stanton, 2013).

Investigating a reduction in SFA, while taking the substituting macronutrient into account, is currently being considered as an appropriate way to investigate the effect of SFA on CVD (Geleijnse *et al.*, 2014; Liebman *et al.*, 2014; Siri-Tarino *et al.*, 2010b; Te Morenga *et al.*, 2014; Willett *et al.*, 2014). Observational studies that used to attempt to investigate intake of SFA and CVD by comparing groups of people with high and low SFA intake, are now starting to take substitution of the SFA by a specific macronutrient into account in their data-analysis.

Even more broadly, other lifestyle factors such as alcohol intake or smoking are suggested to have an interaction with SFA intake and lead to an even more detrimental effect on CVD risk (factors) (Corella *et al.*, 2011; Ivey *et al.*, 2014; Rumpler *et al.*, 1999; Sinha-Hikim *et al.*, 2014). This suggests that a focus on the generally unhealthy lifestyle that is often related to high SFA intake might be a more appropriate approach.

16.5.4 Dietary advice

Researchers still do not agree on the effect of saturated fat on cardiovascular health. Effect sizes are small and a part of the limitations of the research regarding SFA intake and risk of CVD is inherent to the nature of the research question.

Some researchers promote a low SFA intake to reduce CVD risk, pointing to study limitations or methodological errors as a cause for the lack of effects in meta-analyses (Hooper *et al.*, 2012; Skeaff and Miller, 2009). Others mainly focus on the lack of scientific support for current dietary guidelines that promote a SFA intake <10 energy% and consider if we should change them (Cheng *et al.*, 2016; Chowdhury *et al.*, 2014; De Souza *et al.*, 2015).

How can we translate results to feasible and understandable dietary advice, if even researchers cannot agree? Researchers have expressed their concerns regarding contradictory dietary advice that has reached the general public (Diekman *et al.*, 2014; Stein, 2006; Yngve *et al.*, 2006). Although most researchers do agree that we need new studies that also consider which macronutrients replace SFA or that investigate SFA subtypes or SFA food sources, this is not what reaches the public through media. The simplified discussion of 'is fat bad or not?' that is being held may lead to public distrust of dietary guidelines. Monitoring the effect of dietary guidelines is becoming more important (Astrup *et al.*, 2011) and so far, adherence to guidelines is not high (Van Rossum *et al.*, 2011; WHO, 2003).

16.6 Conclusions

Weighing and re-weighing available evidence with all its limitations has led to a very polarized discussion regarding the relationship between saturated fat intake and CVD. This makes it difficult to formulate new dietary advice, even though there is limited evidence that SFA reduction is beneficial if people choose to replace this with intake of CHO. The beneficial effect of SFA-PUFA substitution seems mainly based on the beneficial effect of PUFA. There are major concerns that spreading confusion regarding the SFA hypotheses by contradictive messages will lead to skepticism with the public. Since the goal of this type of research is to promote general population health, we should be aware of this effect. Given the limitations of study design in investigating the complexity of the SFA-CVD relationship, we must seek out new methodological approaches to clarify the previously found associations. Then, we need to translate these results into messages that the public can understand. Current evidence should be complemented with studies that investigate whole foods and dietary patterns, so we can better understand the sometimes contradictory results that have been found previously. Investigating subtypes of SFA can help to clarify possible counteracting effects and considering food sources of saturated fats allows us to interpret the effect of a macronutrient within the total diet. Genetic data could be used to elucidate the biological pathways that underlie observed associations.

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17. Bioactive attributes of traditional leafy vegetable *Talinum triangulare*

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Abstract

The leafy vegetable *Talinum triangulare* (Portulacaceae) commonly called waterleaf is traditionally well known for its nutritional and health benefits in southwestern India. It serves as a major leafy vegetable during the summer, hence a recent study which compared bioactive components and antioxidant potential of uncooked and cooked samples during wet and dry seasons. Irrespective of processing, total phenolics, vitamin C, phytic acid contents and trypsin inhibition were higher in dry season than in wet season. Tannins and flavonoids contents were higher in cooked samples from the wet season, and uncooked samples from the dry season. Total antioxidant activity and reducing power were higher in dry season than in wet season, while it was opposite for ferrous-chelating capacity. The 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity was higher in uncooked samples in dry season and cooked samples in wet season. The total phenolics, tannins, flavonoids and vitamin C were clustered with antioxidant potential in wet season, while in addition to these, phytic acid was also clustered in dry season. Selective differences in bioactive components and antioxidant potential of *T. triangulare* between seasons and processes help maneuvering in favor of nutrition as well as combating cardiovascular diseases. Being cosmopolitan in tropics, *T. triangulare* has become an inexpensive indigenous candidate with favorable nutraceutical attributes demands further insight on advantages in management of heart-related ailments.

Keywords: antioxidant activities, cardiovascular diseases, Ceylon/Florida spinach, nutraceutical value, wild herb

Key facts

- Leafy vegetables constitute part and parcel of human diet, known for nutrients and bioactive components capable to combat several age-related or lifestyle diseases.
- *Talinum triangulare* (water leaf or Ceylon/Florida spinach) is a widely grown terrestrial herbaceous perennial plant species.
- Potential nutritional and antioxidant properties of *T. triangulare* qualify as one of the health foods of nutraceutical significance.
- Being cosmopolitan throughout the tropics and adaptable to tropical habitats, *T. triangulare* is a potential indigenous cost-effective candidate which demands further research on its use in heart-related ailments.

Summary points

- Leafy vegetable *Talinum triangulare* is traditionally well known for its nutritional and therapeutic potential in Southwestern India.
- Bioactive components and antioxidant potential have been compared between uncooked and cooked samples collected in wet and dry seasons as its consumption is maximum during dry season.
- Total phenolics, vitamin C, phytic acid, total antioxidant activity and reducing power were higher in dry season than in wet season.
- Principal component analysis revealed a wide difference between seasons and process by clustering bioactive principles with antioxidant activities.
- Selective alteration of bioactive principles and antioxidant potential between seasons and processes facilitates to maneuver in favor of combating cardiovascular diseases.

Abbreviations

AAE	Ascorbic acid equivalent
DPPH	2,2-diphenyl-1-picrylhydrazyl
FCC	Ferrous ion-chelating capacity
FL	Flavonoids
L-DOPA	L-3,4-dihydroxyphenylalanine
PA	Phytic acid
PCA	Principal component analysis
RP	Reducing power
TAA	Total antioxidant activity
TA	Tannins
TI	Trypsin inhibition
TP	Total phenolics

17.1 Introduction

Scarcity of animal-derived proteins in developing countries forces a search for alternative sources to overcome protein-energy malnutrition and to fulfill basic health requirements (FAO, 2000; Pastor-Cavada *et al.*, 2009). Vegetables and fruits are most important in human diet as they are the potential source of many bioactive principles, which are of immense significance in nutrition as well as health. Leafy vegetables are popular dietary source especially in tropics owing to their diversity, adaptability and nutritional components (e.g. vitamin C, β-carotene, flavonoids, fiber and minerals) (Adefegha and Oboh, 2011; Fasuyi, 2007). Several wild or indigenous leafy vegetables serve as potential nutritional and health foods, which are commonly consumed by the ethnic groups in tropics (Acho *et al.*, 2014; Chibueze and Akubugwo, 2011).

Talinum triangulare (Jacq.) Willd. (family: Portulacaceae) is widely distributed in tropical regions and well known for its nutritional value especially proteins, minerals, vitamins and β-carotene (Ezekwe *et al.*, 2001; Fasuyi, 2007; Ogbonnaya and Chinedum, 2013). It is also endowed with several health-promoting qualities such as antioxidants, immunostimulation, prevention of hepatic problems, combat cancer, solve gastrointestinal problems, degenerative diseases and helps in management of cardiovascular problems (e.g. stroke and obesity) (Adefegha and Oboh, 2011; Afolabi and Oloyede, 2016; Agbonon *et al.*, 2010; Aja *et al.*, 2010; Andarwulan *et al.*, 2010; Laio *et al.*, 2015; Mensor *et al.*, 2001; Ogbonnaya and Chinedum, 2013; Oguntona, 1998; Olajire and Azeez, 2011). Considering the nutritional and medicinal properties of *T. triangulare* being used as nutritional source in Southwestern India, the present chapter addresses bioactive components and health-promoting potential. Emphasis has been laid to link bioactive principles of *T. triangulare* with antioxidant potential and possible prospects in management of cardiovascular diseases.

17.2 Leafy vegetable

Tender leaves and stem of *T. triangulare* (vernacular name in Kannada is 'Nela Basale') were collected during wet (July 2014) and dry (May 2015) seasons from five different locations (~50 m apart) in Payam, Kasaragod District, Kerala State (12°29'N, 75°7'E) (Figure 17.1). Samples were cleaned in the laboratory by removing inflorescence, roots and insect infested leaves. Samples were rinsed in running water to remove debris. Each sample was chopped into pieces

Figure 17.1. (A) *Talinum triangulare* grown on typical laterite soil in Southwestern region in India; (B) details of leaves and inflorescence; (C) harvested and cleaned tender leaves and stem; (D) uncooked dried flour; and (E) cooked dried flour.

and divided into two parts. The first part was dried in oven (50-55 °C) until moisture attains <10%, which served as uncooked sample. The second part was pressure-cooked in household cooker by addition of distilled water (3:1) followed by oven drying (50-55 °C), which served as cooked sample. Dried uncooked and cooked samples were pulverized in Wiley mill (mesh #30), refrigerated (4 °C) in airtight containers for analysis.

17.3 Bioactive components

17.3.1 Total phenolics and tannins

The content of TP of *T. triangulare* was determined by adapting the method outlined by Rosset *et al.* (1982). Tannic acid was used as standard and the results were presented in milligram equivalents of tannic acid per gram dry mass of vegetable (mg TAEs/g). Vanillin-Hydrochloric acid method outlined by Burns (1971) was employed to determine content of TA in vegetable. Catechin served as standard and the results were presented in milligram of catechin equivalents per gram dry mass of vegetable (mg catechin equivalent/g).

17.3.2 Flavonoids and vitamin C

The content of FL in vegetable was estimated based on the procedure by Chang *et al.* (2002). Quercetin has been used as standard and the results were presented in mg of quercetin equivalents per gram dry mass of vegetable (mg QEs/g).The vitamin C content in vegetable was evaluated based on Roe (1954) using ascorbic acid as standard. Content of vitamin C in vegetable was presented in milligram of ascorbic acid equivalents per gram dry mass of vegetable (mg AAEs/g).

17.3.3 Phytic acid and L-3,4-dihydroxyphenylalanine

The PA content in vegetable was evaluated based on the assay by Deshpande *et al.* (1982) and Sathe *et al.* (1983). The phytic acid content was expressed in percentage (mg/100 mg). The content of L-DOPA was estimated based on the protocol by Fujii *et al.* (1991) using high-performance liquid chromatography and liquid chromatography-electrospray ionization-tandem mass spectrometry.

17.3.4 Trypsin inhibition and hemagglutination

The enzymatic assay method proposed by Kakade *et al.* (1974) was followed to determine TI activity of vegetable. Release of p-nitroanilide (1 μM) per min by the enzyme per milligram dry sample has been considered as one trypsin inhibition unit (TIu/mg). The assay by Occenã *et al.* (2007) has been adapted to evaluate hemagglutinin activity in samples by serial dilution using human erythrocytes (A^+, B^+, AB^+ and O^+) in phosphate buffered saline. The activity has been expressed as hemagglutination unit per gram (HAu/g) dry mass of vegetable.

17.4 Antioxidant potential

Antioxidant potential of *T. triangulare* was assessed based on TAA, FCC, DPPH radical-scavenging activity and RP. Samples of vegetable flour (0.5 g) were extracted using methanol (30 ml) on a rotary shaker (150 rpm, 48 h). On centrifugation, the supernatant was collected on a pre-weighed Petri plate and dried at laboratory temperature. After determining the mass, it was dissolved in methanol at a concentration of one milligram per ml to assess antioxidant potential.

The method by Prieto *et al.* (1999) was employed to assess the TAA. Methanol served as blank, ascorbic acid served as standard and the TAA was presented in micromole equivalent of ascorbic acid per gram dry mass of vegetable (µm AAEs/g). The protocol by Hsu *et al.* (2003) was employed to determine the FCC. The sample without extract served as control to express FCC in percentage. Determination of reducing power of vegetable extract was carried out based on Oyaizu (1986) by considering absorbance at 700 nm. The DPPH free radical-scavenging activity was measured based on Singh *et al.* (2002) to express in percentage.

17.5 Data analysis

Bioactive principles and bioactivity between uncooked and cooked vegetable samples during wet and dry seasons were compared by *t*-test by Stastistica version # 8 (StatSoft Inc., 2008). To find out the relationship between bioactive components (total phenolics, tannins, flavonoids, vitamin C, phytic acid and trypin inhibition) and bioactive potential (total antioxidant activity, Fe^{2+} chelating capacity, reducing power and DPPH radical-scavenging activity) of vegetable collected during wet and dry seasons, the PCA was followed (SPSS version 16.0; SPSS Inc., Chicago, IL, USA). The plots of PCA score for wet and dry season samples were grouped among bioactive components with those of antioxidant potential.

17.6 Observations and discussion

Antioxidant properties of natural produce have been linked with various protective effects on human ailments like hepatic problems, gastrointestinal disorders, immune-deficiencies, diabetic complications, cancers, cardiac diseases and obesity. Indigenous leafy vegetables have upper hand in such protective effects due to their versatile bioactive principles. The waterleaf *T. triangulare* is a household vegetable used traditionally in Southwestern India as a nutraceutical source. This study showed major differences in antioxidant principles as well as antioxidant potential of *T. triangulare* in uncooked and cooked samples collected during wet and dry seasons. Besides leaves, mucilage extracted from leaves of *T. triangulare* also showed higher contents of phenolics and vitamin C, in turn higher antioxidant activity than leaves (Adetuyi and Dada, 2012).

17.6.1 Bioactive components

Phenolic compounds in vegetables are known to protect against oxidative stress, which is beneficial to control coronary heart diseases (Katalinic *et al.*, 2006; Kaur and Kapoor, 2002). TP content in *T. triangulare* was higher in uncooked than in cooked samples during both seasons (Figure 17.2A). Dry season possess higher quantity than wet season, however, cooked samples of wet season showed more tannins than dry season (Figure 17.2B). The quantity of tannin could be comparable with *T. triangulare* of Southwestern Nigeria (Fasuyi, 2007). In another study, the total phenolic content was higher during winter compared to summer as well as it was dependent on time of harvest (Brasileiro *et al.*, 2015).

Adequate quantity of flavonoids in diet leads to reduce the risks of stroke and cardiovascular diseases (Cutler *et al.*, 2008; Geleijnse *et al.*, 1999; Gross, 2004; Keli *et al.*, 1996). Intake of

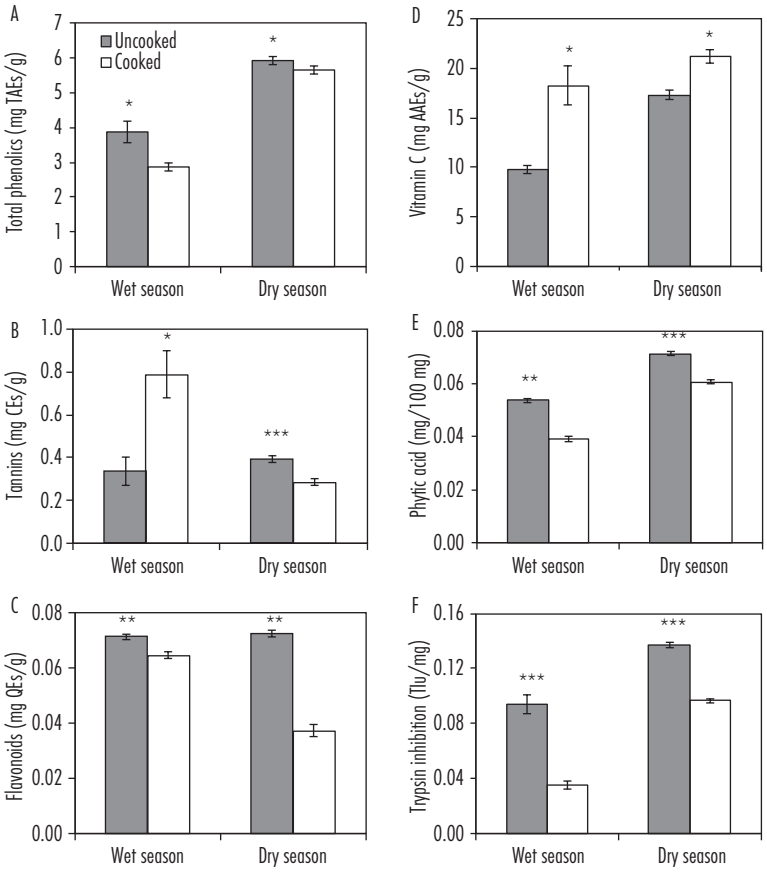

Figure 17.2. Bioactive principles of tender leaves and stem of *Talinum triangulare*: (A) total phenolics, (B) tannins, (C) flavonoids, (D) vitamin C, (E) phytic acid, and (F) trypsin inhibition (t-test: **P*<0.05; ***P*<0.01; ****P*<0.001).

flavonoids and carotenoids showed an inverse relationship with mortality from coronary heart diseases (Donald and Cristobal, 2006; Peterson *et al.*, 2012). Flavonoids content in *T. triangulare* was higher in uncooked samples than cooked samples of wet as well as dry seasons (Figure 17.2C). Depending on the requirements, *T. triangulare* could be sampled and processed to draw maximum benefit of flavonoids to overcome coronary heart diseases.

Vitamin C besides its antioxidant activity, helps in repairing the walls of arteries by hydroxylation reactions leading to synthesis of collagen fibers (Cobb, 2011). Vitamin C content in uncooked and cooked samples of *T. triangulare* was season-dependent and higher in dry season than wet season (Figure 17.2D). Extensive use of *T. triangulare* in diet during summer season will have a positive impact on coronary disease prevention. The vitamin C content is substantially higher in our study compared to another study carried out in Nigeria (Ogbonnaya and Chinedum, 2013). Leaves of *T. triangulare* also consist of a variety of vitamins and provitamins (e.g. thiamin, riboflavin, niacin, tocopherol and carotenoids), which are important in human nutrition as well as health (Ogbonnaya and Chinedum, 2013). In addition to vitamin C, dietary intake of phytic acid is known to protect against atherosclerosis and coronary heart diseases (Grases *et al.*, 2008; Jariwalla *et al.*, 1990; Konietzny *et al.*, 2006). PA is also responsible for the reduction of lipid content in the serum (Lee *et al.*, 2005). Similar to vitamin C, content of phytic acid was higher in uncooked and cooked samples of *T. triangulare* during dry season than wet season (Figure 17.2E). The quantity of phytic acid in cooked and uncooked samples is less than *T. triangulare* of Southwestern Nigeria (Fasuyi, 2007). The L-DOPA was below detectable level and trypsin inhibition activity in uncooked and cooked samples were higher in dry season than wet season (Figure 17.2F) (Table 17.1). Hemagglutinin activity was found only in uncooked sample during wet season against four blood groups (A^+, B^+, AB^+ and O^+) ranging from 100-200 HAu/g (Table 17.1).

Table 17.1. The L-3,4-dihydroxyphenylalanine (L-DOPA) and hemagglutinin activity of uncooked and cooked leafy vegetable *Talinum triangulare* against human blood groups.[1]

	Blood group	Wet season		Dry season	
		Uncooked	Cooked	Uncooked	Cooked
L-DOPA	–	BDL	BDL	BDL	BDL
Hemagglutinin activity (HAu/g)	A^+	200	ND	ND	ND
	B^+	100	ND	ND	ND
	O^+	100	ND	ND	ND
	AB^+	100	ND	ND	ND

[1] BDL = below detectable level; ND = not detectable.

17.6.2 Antioxidant potential

Uncontrolled production of free radicals causes several pathological problems including heart-related diseases (Barros *et al.*, 2007; Chanwitheesuk *et al.*, 2005; Jagadish *et al.*, 2009; Karadenz *et al.*, 2005; Marinova *et al.*, 2005). The antioxidant potential of biological materials is dependent on its bioactive components and demands to follow at least two methods for fair assessment (Wong *et al.*, 2006). TAA in uncooked and cooked samples of *T. triangulare* was higher in dry season than in wet season (Figure 17.3A). The ferrous-ion chelating capacity was higher in uncooked as well as cooked samples during wet season than dry season (Figure 17.3B). The DPPH radical-scavenging activity was higher in uncooked samples during dry season, it was the opposite for cooked samples (Figure 17.3C). The uncooked samples in dry season showed higher reducing power followed by cooked dry season, cooked wet season and uncooked wet season samples (Figure 17.3D). Similar to bioactive components, antioxidant activities also season- as well as process-dependent to select appropriate season and process for maximum benefit to manage diseases.

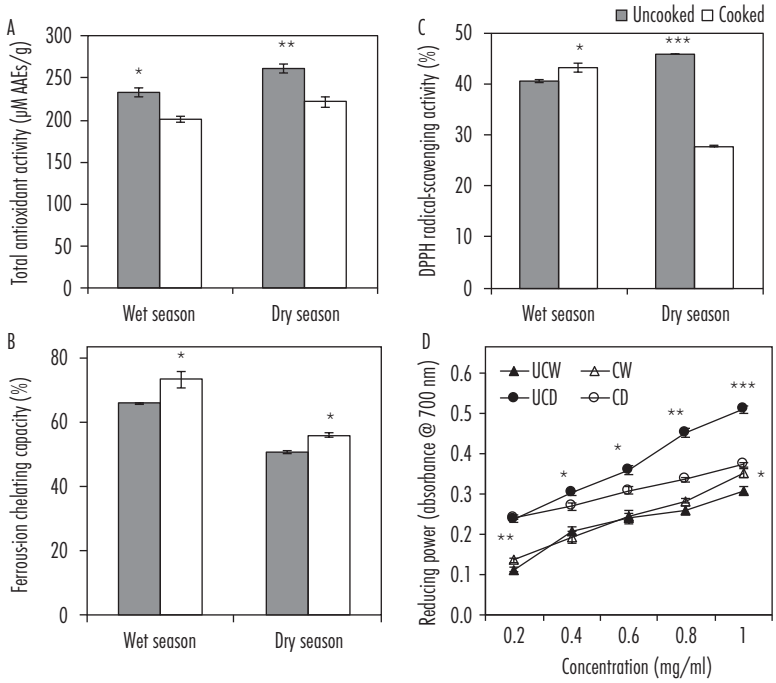

Figure 17.3. Antioxidant activities of tender leaves and stem of *Talinum triangulare*: (A) total antioxidant activity, (B) ferrous ion-chelating capacity, (C) DPPH radical-scavenging activity, and (D) reducing power (CD = cooked-dry season; CW = cooked-wet season; UCD = uncooked-dry season; UCW = uncooked-wet season) (t-test: **P*<0.05; ***P*<0.01; ****P*<0.001).

17.6.3 Principal component analysis

During wet season, the PCA of bioactive principles for uncooked *T. triangulare* against antioxidant potential resulted in two components with 100% variance (Eigen value <1; PC1: 64.48%; PC2: 35.52%), whereas for cooked samples respective figures were: Eigen value <1; variance, 100%; PC1: 73.48% and PC2: 26.52%. The total phenolics, tannins and vitamin C were clustered with antioxidant potential in uncooked samples (Figure 17.4A), while total phenolics, tannins, flavonoids and vitamin C in cooked samples (Figure 17.4B).

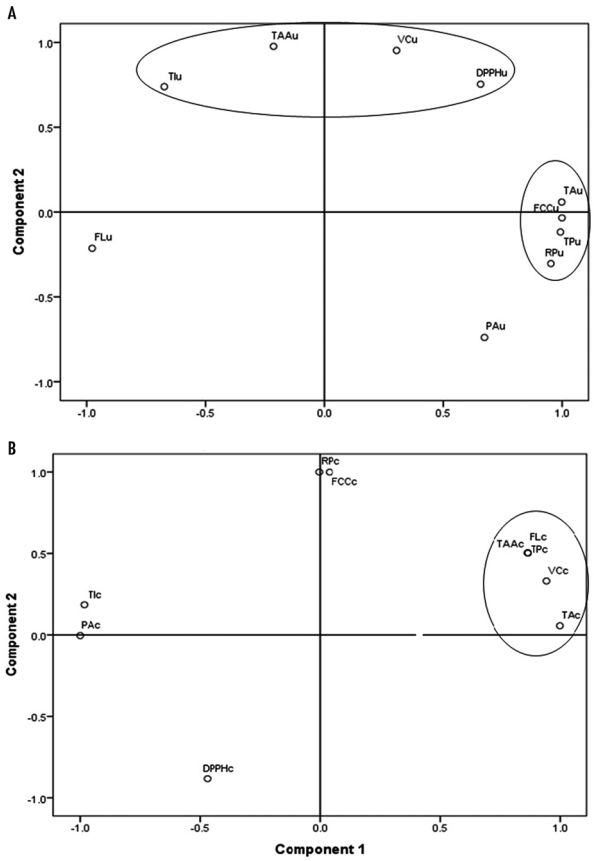

Figure 17.4. Principal component analysis of bioactive principles and antioxidant activities of (A) uncooked and (B) cooked tender leaves and stem of *Talinum triangulare* sampled during wet season. Notations followed by 'u' means uncooked and notations followed by 'c' means cooked. Bioactive principles: TP = total phenolics, TA = tannins, FL = flavonoids and VC = vitamin C, PA = phytic acid and TI = trypsin inhibition; antioxidant activities: TAA = total antioxidant activity, FCC = ferrous ion-chelating capacity, DPPH = DPPH radical-scavenging activity and RP = reducing power.

During dry season, the PCA of bioactive principles for uncooked *T. triangulare* against antioxidant potential resulted in two components with 100% variance (Eigen value <1; PC1: 74.30%; PC2: 25.71%), whereas for cooked samples respective figures were: Eigen value <1; variance: 100%; PC1: 58.06%; PC2: 41.94%. The total phenolics, tannins, vitamin C and phytic acid were clustered with antioxidant potential in uncooked samples (Figure 17.5A), while total phenolics, tannins, flavonoids and phytic acid in cooked samples (Figure 17.5B).

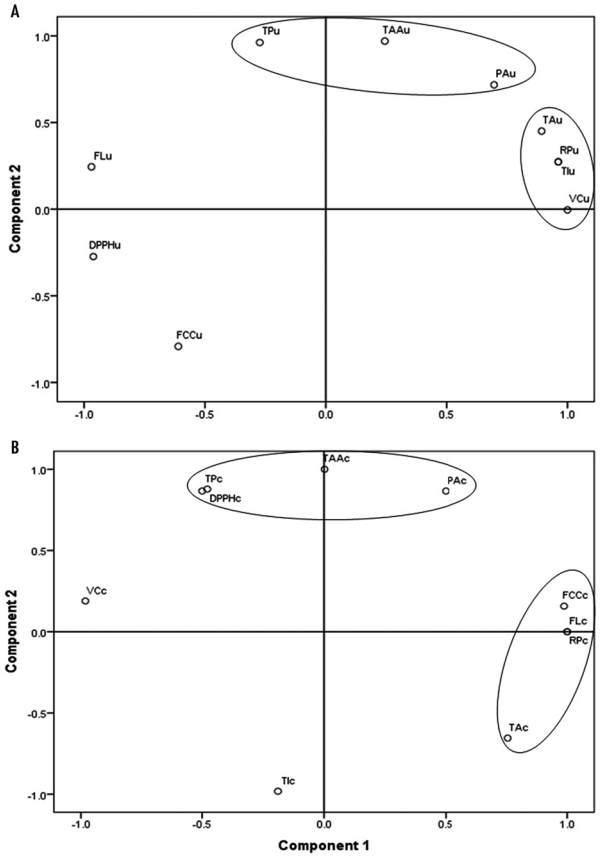

Figure 17.5. Principal component analysis of bioactive principles and antioxidant activities of (A) uncooked and (B) cooked tender leaves and stem of *Talinum triangulare* sampled during dry season. Notations followed by 'u' means uncooked and notations followed by 'c' means cooked; bioactive principles: TP = total phenolics, TA = tannins, FL = flavonoids and VC = vitamin C, PA = phytic acid and TI = trypsin inhibition; antioxidant activities: TAA = total antioxidant activity, FCC = ferrous ion-chelating capacity, DPPH = DPPH radical-scavenging activity and RP = reducing power.

Most of the bioactive principles evaluated in this study are closely associated with one or the other antioxidant activity of *T. triangulare* strengthens the assumption that they are indirectly responsible for disease prevention and valuable in disease management.

17.6.4 Role in disease management

Plant species in traditional medicine have been considered extensive in managing many diseases including stroke and obesity (Fontem and Schippers, 2004). The *T. triangulare* is thought to have medicinal potential useful for managing cardiovascular diseases like stroke and obesity (Aja *et al.*, 2010). For example, leaves of *T. triangulare* serve to treat anemia in pregnant women and children. Leaves are also known to reduce plasma cholesterol and boost plasma high-density lipoprotein, in turn to prevent coronary diseases (Ezekwe *et al.*, 2004). It has been predicted that cardioprotective as well as hypolipidemic effects were due to presence of substantial quantity of squalene in leaves (Biona *et al.*, 2015; Farvin *et al.*, 2006).

In addition to bioactive components, nutritional properties of *T. triangulare* are also aid in the management of heart-related diseases (M. Pavithra *et al.*, unpublished observations). For instance, digestion of starch and conversion of simple sugars will be delayed by fiber, which is very important in the management of diabetes mellitus (Monago and Uwakwe, 2009). Besides, absorption of cholesterol by dietary fiber, the gut provides protection against colorectal cancer, obesity and cardiovascular diseases (Ogbonnaya and Chinedum, 2013). Tender leaves and stem of *T. triangulare* possess sufficient fiber, which is significantly increased in cooking (14.1 vs 14.3% on dry mass basis) could be of immense value to those facing diabetes and obesity or cardiovascular problems. Magnesium is an essential mineral responsible to prevent ailments including cardiomyopathy, ischemic heart disease and bleeding disorders (Chaturvedi *et al.*, 2004; Gafar and Itodo, 2011). Magnesium content in uncooked and cooked *T. triangulare* surpassed NRC-NAS (1989) a recommended pattern for infants, children and adults, along with iron content in adequate quantity to combat anemia. The Na/K ratio of *T. triangulare* ranged between 0.003 (cooked) and 0.004 (uncooked) samples is an added advantage as the ratio <1 is preferable to combat high blood pressure (Yusuf *et al.*, 2007). As -3 and -6 fatty acids in food samples help controlling many heart diseases (Harris *et al.*, 2009; Katan, 2009; Roosha and Parloop, 2010), uncooked and cooked *T. triangulare* with linoleic and linolenic acids have additional advantages. The polyunsaturated/saturated fatty acid ratio in *T. triangulare* has increased in cooked samples which facilitates combating cardiovascular risks.

17.7 Conclusions

T. triangulare is a tropical cosmopolitan leafy vegetable well known for its nutritional and health benefits in Southwestern India. The leaves are endowed with a variety of bioactive compounds responsible for antioxidant activities. Several components in leaves such as phenolics, flavonoids, vitamin C and phytic acid are responsible for its versatility, and in turn helps control, treat and manage coronary heart diseases. The bioactive principles as well as antioxidant activities are

season- (wet and dry) and process- (uncooked and cooked) dependent. This leafy vegetable is also known for several nutritionally beneficial components (e.g. protein, fiber, minerals and essential fatty acids), thus there is a wide scope to utilize as nutraceutical source. Interestingly, oven drying of leaves at 60 °C has not resulted in substantial loss of nutritional and sensory properties (Oluwalana *et al.*, 2011). Future studies should focus to support the specific bioactive component and optimum dose necessary in relation to age, sex, status of health and other relevant parameters to prove its efficiency in control or treatment of coronary heart diseases. For instance, among flavonoid classes, only two are involved in lowering mortality by coronary heart disease (flavonol and flavone) (Peterson *et al.*, 2012). Similarly, among betalain pigment classes (produced during extreme environmental conditions in stem, leaf and flowers) are known for radical-scavenging activity (Swarna *et al.*, 2013), among them which one is beneficial for cardiovascular health? The pink-purple betalain pigments are a potential interest in production of foods and beverages because of their dual advantage as antioxidants and helpful in the management of cardiovascular diseases.

Acknowledgements

Authors are grateful to Mangalore University for permission to carry out this research in the Department of Biosciences. This work was supported by the University Grants Commission, New Delhi by awarding UGC-BSR Faculty Fellowship to KRS (Grant # F.18-1/64/2014/BSR].

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18. Bioactive foods and herbs in prevention and treatment of cardiovascular disease

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Abstract

Berberine, hated by horses and cows, has long been used as a suppressant drug for gastro-intestinal disorder. Surprisingly advantageous effects for the normalization of serum lipids were discovered recently. It may provide a new tool to fight against hyperlipidemea originated from excessive nutrients or diabetes, primary risk factors for the atheroscrelosis and coronary heart diseases. The medicinal effects of individual herbs on cardiovascular functions have been considered. The tenet 'One herb for one disease' is an oversimplification: each herb contains many different biologically active substances that in combination support the life of the plant as a whole. In the case of herbal medicines used by man the best mixtures of different herbs have been arrived at over long years. A recent example may be the remarkable hypolipidemic effects of a combination of the plant alkaloid, berberine, with a plant stanol where the two herbs act synergistically to reduce plasma hypercholesterol and triglycerides. Further advances in herbal medicine, that is the discovery of other useful herbs and new combinations, may be expected.

Keywords: hyperlipidemia, atherosclerosis, coronary heart diseases, herbal remedies, gastro-intestinal disorders, suppressant

Ronald Ross Watson and Sherma Zibadi (eds.) Handbook of nutrition and heart health
Human Health Handbooks no. 14 - DOI 10.3920/978-90-8686-853-7_18, © Wageningen Academic Publishers 2017

Key facts

- Instruments used these days in the biomolecular research fields are: molecular biological technique, electron beam computed tomography, electron microscopy, physico-chemical analytical techniques, laser speckle flowmeter.
- Berberine, hated by horses and cows, has long been used as a suppressant drug for gastro-intestinal disorder; surprisingly advantageous effects for the normalization of serum lipids were discovered recently.
- The tenet 'one herb for one disease' is an oversimplification: each herb contains many different biologically active substances that in combination support the life of the plant as a whole.
- In the case of herbal medicines used by man the best mixtures of different herbs have been arrived at over long years.

Summary points

- Medicinal effects of herbs are investigated carefully on the molecular level and better understood these days .
- Our medical care may depend more on the self-relied herbal medicine in the near future.
- Surprisingly advantageous effects for the normalization of serum lipids were discovered recently.
- A new tool may be provided to fight against hyperlipidemea originating from excessive nutrients or diabetes, primary risk factors for atheroscrelosis and coronary heart diseases.

Abbreviations

ACE	Angiotensin converting enzyme
BP	Blood pressure
CFR	Coronary flow reserve
EGb	*Ginkgo biloba* extract
GSH	Glutathione
HbA1c	Hemoglobine A1c
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
NF-kappa	Nuclear factor kappa
SHRSP	Stroke-prone spontaneously hypertensive rats
TC	Total cholesterol
TNF-α	Tumor necrosis factor alpha

18.1 Introduction

Herbal remedies were in use long before the development of modern medicine. For example, ginseng has been used in China from time immemorial (Cheng, 2007; Shen Nong, circa 2300 BC), while a third of all modern drugs are derived from herbs. Among these, digitalis and aspirin have been widely studied. Most medicinal herbs were first sampled empirically in rural areas and their value passed on from generation to generation. It is thought that one advantageous feature of the continuous long-term uptake of low concentrations of effective substances in the herbs is their slow, synergistic activity. Interestingly, there is evidence that chimpanzees, like man, also use medicinal herbs for certain conditions, including parasitic infestations (Fowler *et al.,* 2007; Huffman and Wrangham, 1994). The beneficial contribution of herbs to good health has long been recognized.

In Asia we have a saying 'healthy food, healthy life'. Herbs such as *Perilla* (a member of the mint family), sesame and capsicum, which are served at meals for their taste, are also health supplements as is garlic which is often included in herbs.

The legendary Chinese emperor, Shen Nong, examined more than 3,000 herbs. He identified 365 species as the best for use as herbal medicines with no side effects. His original record was lost but a copy prepared in the 2nd century AD shows us what the original records looked like (Shen Nong Beng CaoJing copied by Tao Hong Jing, 452-536). An older copy was found in 1971 in the grave of a lady, King Ma's Mound' (feudalistic lords, Mawanodai, Changsha City, 186 BC). A Japanese book published by Yoriyasu Tamba (Ishinhou, Book of Holy-mind, 918) listed over one thousand herbal medicines and more than 200 of these are still given in the current Japanese pharmacopeia.

Recently medicinal herbs have been analyzed to identify their effective components. The present chapter summarizes some of the interesting results obtained in investigations of the cardiovascular effects produced by herbs.

18.2 Chaga, cinder cork (*Fuscoporia obliqua*)

Fuscoporia obliqua, known as *Inonotos* in Russia and North Korea, has been used in some Russian rural areas instead of tea. Soljhenitsin, mentioned this in his Nobel Price-winning novel 'Cancer ward' (1970). In the countryside, farmers enjoyed good health with less illness than in other areas. It is a dark charcoal parasitic fungus on the birch. The Ainu people of northern Japan drank hot-water extracts of *Fuscoporia* for treating inflammation and stomach pains (Koyama *et al.,* 2008). Furthermore a pipe filled with the powdered fruiting body of *Fuscoporia* was lit during their religious ceremonies. The leader of the ceremony inhaled the smoke, then gave the pipe to his neighbor; circulation of the pipe continued until all participants had smoked it. Although the medicinal effects of the smoke are unknown, the tradition implies that *Fuscoporia* was highly regarded. Their general, rather than specific action, is at odds with the modern therapeutic principle of one drug for one syndrome.

18.2.1 Active compounds

Recent analyses shows that the major bioactive constituents are urusolic and oleanolic acids, lanosterol and inotodiol. Urusolic acid is known to down-regulate the anti-apoptotic factor, bcl-2. The hot water extract is highly antioxidative.

In alloxan-induced diabetic mice, an animal model for type-1 diabetes, hyperlipidemia was significantly reduced by *F. obliqua* (Sun *et al.,* 2008). When SHRSP rats were given a hot water-extract of *Fuscoporia* for 2 months, in place of drinking water (Koyama *et al.,* 2006), mean arterial blood pressure and cardiomyocyte cross-sectional area significantly decreased as did the number of alkaline phosphatase-stainable capillaries (which serve as an index of shear stress) The HbA1c level and capillary density showed a slight decrease but lipid constituents did not change significantly.

18.2.2 Human study

A double blind test on *Fuscoporia* extract was made in 60 adults. Volunteers were divided into three groups of 20 subjects (Yonei *et al.,* 2007). The first group received 15 ml of a water-diluted vinegar, the second group, 5 ml of *Fuscoporia* extract (3 times diluted with water) and the 3rd group 15 ml of the *Fuscoporia* extract prepared from cultured mycelia (obtained from Fujiwara Technoart, Japan). Aortic blood pressure, body weight, and blood glucose all showed slight but significant falls with a marked decrease in superoxidized blood lipids. Most subjects in both *Fuscoporia*-treated groups reported feeling warmer, sleeping better, and having improved volition. On the other hand, they complained of polyurea and increased sweating; in addition there was a

slight but significant increase in blood triglycerides. The researchers were therefore hesitant about making a general recommendation for the daily use of *Fuscoporia* extracts.

In another study, on 14 patients suffering from type 2 diabetes (Maenaka *et al.,* 2008), the effects of *Fuscoporia* on postprandial blood glucose levels and arterial endothelial cells (assessed by reactive hyperemia) were compared with those in 12 healthy subjects. Reactive hyperemia of the forearm was produced by a transient occlusion followed by reperfusion. In normal subjects, the peak forearm blood flow response and total reactive hyperemic flow, i.e. the flow debt repayment, were not affected by a meal. In diabetic patients, however, there was a remarkable change for both indices being significantly decreased at 120 and 240 min after the test meal. The prior administration of *Fuscoporia* decreased the postprandial glucose peak, accompanied by recovery of the peak forearm blood flow and flow repayment. *Fuscoporia* would thus appear to reduce the risk of endothelial injury and possible future arteriolar complications.

18.3 Danshen (*Salvia miltirrheria*)

Danshen is a red extract obtained from the roots of the plant, *Salvia miltirrheria* (salvia, means 'salvage'); many members of this genus have medicinal properties. Danshen has been used in China for hundreds of years for treating patients suffering from cardiovascular diseases including angina, coronary artery spasm, myocardial spasm, hyperlipidemia, and hypertension.

Danshen has therapeutic effects in cerebrovascular diseases. It has been mentioned that a related plant, *Salvia columbariae*, was used by Californian Indians for the treatment of strokes.

18.3.1 Active compounds

The many active compounds contained in danshen roots include tanshinones (diterpenoids), salvianolic acid, miltirone, and lithospermatic acid. The water-soluble compounds scavenging peroxides inhibit the expression of adhesion molecules in vascular endothelium and leukocytes. The lipophylic compounds prevent the development of vascular damage; NADPH oxidase and platelet aggregation are inhibited, as is mast cell degradation caused by ischemia followed by reperfusion.

Danshen inhibits ACE so it lowers blood pressure, dilates arteries, and decreases cardiac fibrillation and blood clotting. These actions are vital in the treatment of cardiovascular diseases. The inhibition of clot formation, and potential clot dissolution, has been demonstrated in many clinical trials with danshen; these showed it increased the proteolysis of fibrinogen (Chang *et al.,* 1991). Salvianolic acid A reduces thrombosis and platelet aggregation; it also protects neurons from ischemic damage (Wang *et al.,* 1991). Miltirone is a benzodiazepine receptor agonist (Kim *et al.,* 2002) while lithospermatic acid B inhibits ACE and reduces hypertension (Lam *et al.,* 2003). Tanshinone l protects against the damage that can result from myocardial ischemia and reperfusion (Kang *et al.,* 2000) and it reduces arachidonic acid metabolis. Tanshinone llA, in

contrast, decreases neuroprotection when cerebral ischemia is followed by reperfusion (Wang *et al.,* 2006). Dihydrotanshinone reduces expression of interleukin while rosmarinic acid and salvianolic acids inhibit thrombosis, thromboxane B2 formation and platelet aggregation (Wang *et al.,* 1991).

The use of danshen in cerebrovascular disease has been mentioned but no double blind tests using a placebo have been reported. The delay between the onset of ischemia and the start of treatment varied in different patients from a few hours to many days. Improvements were, however, still observed in several studies that started many days after the incident, emphasizing the value of danshen for ischemic episodes in heart and brain. The results also imply that danshen may stimulate brain repair mechanisms, perhaps by activating stem cell growth (Tang *et al.,* 2002).

18.3.2 Human study

To determine the clinical efficacy of Salvia a meta-analysis was undertaken in cases of chronic stable angina pectoris. The effects of the widely prescribed Salvia pellet (the main component is danshen mixed with sanqi (*Panax notoginseng*) and borneol (*Cinamon camphora*) were compared with those of nitrates. Results from twenty-seven randomized controlled trials (n=3,722) were collated. Statistical software was provided by the Cochrane Collaboration. It was concluded that Salvia pellet significantly improved both the angina and the electrocardiogram (Wang *et al.,* 2006).

18.4 Foxglove (*Digitalis purpura*)

A note on the use of topical digitalis therapy: the drug was applied to the skin by inunction, a kind of transdermal mediation. Groves and Bisset (1991) described that foxgloves which were beautiful with its pinky trumpet like blossoms, were poisonous to dogs, cats and human. The main component of digitalis, the glycoside ouabain, has been used for several centuries to treat cardiac patients by inuncuation of digitalis leaves on the patient skin. More than 200 years ago, it was known that leaves of foxglove reduced the edema caused by heart failure. It was later shown that ouabain obtained from digitalis leaves by Withering increased the contractile force in excised papillary muscle (Cattel and Gold, 1938). The main clinical effect of digitalis has been attributed to the increase in contraction. Closely related cardiotonic glycosides include, among others, digitoxin, gitoxin and glycosides A and B.

18.4.1 Active compounds

Structurally, digitoxin is a *cis*-type steroid with an unsaturated lactone ring (a cyclic ester containing 5 carbon atoms with a ketone group). It has an antibacterial action and is also a pheromone. Digitoxin forms a complex with the Na/K ATPase of the Na/K pumps on the cardiomyocyte membrane. Two amino acids, glutamine and asparagine, in the first extracellular domain of the pump protein are sensitive to ouabain. Replacement of these aminoacids with

arginine and asparagine reduced the sensitivity of the ATPase to ouabain (Dostanic *et al.,* 2004). The exact mechanism by which ouabain exerts its clinical effect is unclear but the following steps have been assumed. First ouabain forms a complex with the extracellular portion of the Na/K ATPase of the pump, resulting in a slight decrease in the pumping action of the Na, K exchanger. This will increase intracellular Na, thereby accelerating Na/Ca exchange: as the intracellular Na is pumped out, more Ca will be pumped in. The increase in intracellular Ca has an inotropic effect on the vascular and cardiac muscle, reducing contraction time, tachycardia, arrhythmia and hypertension and preventing congestive heart failure. Unfortunately the ready accumulation of ouabain causes side effects. A fuller account of the mechanism of digitalis action is given by Lingrel (2010).

18.4.2 Side effects

The therapeutic benefit was believed in the first century AD. More recent descriptions of the therapeutic use of foxglove, 200 years ago, included the toxicity (Bara, 2001). Breathing pollen of foxglove causes broncho-spasmic constriction. Eating 2 g of foxglove leaves (mistaking for confrey) may cause danger of life because of the suffocation.

Vincent van Gogh was an uncommon man. Automutilation, depression, insanity, and suicide are part of his medical history characterized by halos and the yellow color. Van Gogh may have been under the influence of digitalis intoxication and its side effects: xanthopsia and coronas. This hypothesis is based on having painted his physician holding a foxglove plant twice, that this medicine was used in the latter part of the 19th century in the treatment of epilepsy, and that the toxic effects of digitalis may have, in part, dictated the artist's technique (Lee, 1981).

18.5 Garlic (*Allium sativan*)

Garlic has been widely planted in Japan since its importation from China in the 8th century. As it is universally known, it is no longer listed in the current pharmacopeia. Although the medicinal effects of garlic were described on an Egyptian papyrus dating from 3200 BC, it was not included in early pharmacopeias from neither Japan nor China, probably because the pungent smell was disliked and its effective component, allicin, is unstable. Moreover, its invigorating effects were believed to disturb Buddhist training and discipline.

Garlic contains an enzyme, allinase that protects it against insects. Allinase is packed isolately in cells, but converts alliin to allicin when the garlic bulb is crushed. Allicin contains unstable sulfur atoms so is prone to change into other active sulfur-containing compounds.

18.5.1 Active compounds

The majority of many clinical trials on garlic have demonstrated its beneficial effects, with dried garlic proving superior to oily preparations. Among other actions, it improves arterial elasticity and prevents hypercholesterolemia and platelet aggregation (Fugh-Berman, 2000).

In terms of reducing cardiovascular risk factors, allicin and alliin are the most important constituents of garlic. The cheapest option for clinical use is commercial garlic powder intended for cooking; this contains high levels of allicin. 'Aged garlic' is also recommended. It is prepared by keeping sliced garlic roots in water for a year before drying them; this gives a relatively high concentration of alliin (Fugh-Berman, 2000).The allicin content in different commercial garlic products is variable since it is readily destroyed during processing. The contribution of alliyl and allicin in the protection against heart failure afforded by garlic has been evaluated in rats. Heart failure was induced by isoproterenol injection in three groups of rats previously fed for 2 weeks with normal rat chow alone, or chow with the addition of either aged garlic extract or S-allyl cysteine. The amount of the S-allyl cysteine in the chow was equal to that in the chow supplemented with aged garlic extract. Improvement of cardiac functions was more effective with aged garlic extract than with S-allyl cysteine alone, suggesting a synergistic effects of other ingredients (Asdaq and Inamdar, 2010).

18.5.2 Human study

One meta-analysis included 16 randomized controlled trials with a total of 952 subjects (Silagy and Neil, 1994a). In eight trials garlic powder (600-900 mg/day) reduced triglycerides by 13% (12 mg/dl). HDL cholesterol was not significantly affected. The effect increased progressively over three months. In 10 other trials 41 moderately hypercholesterolemic men TC between 220-290 mg/dl) were treated with 7.2 g per day aged garlic extract. Compared with values from those given a placebo, garlic administration caused a reduction of 6.1% in TC and of 4.6% in LDL (Steiner *et al.,* 1996). In a placebo-controlled study of 35 hypercholesterolemic renal transplant patients over six weeks, TC decreased from 290 to 276 mg/dl and LDL levels decreased from 193 to 181 mg/dl and these were maintained for 12 weeks (Budoff, 2006; Lash *et al.,* 1998).

The effects of garlic on arterial elasticity have been studied in healthy subjects. A hundred and one healthy, non-smoking adults aged 50-80 years old, with a history of regular garlic intake (300 mg/day for >2 years), demonstrated greater aortic elasticity, measured by a pulse wave velocity meter, than those who did not eat garlic habitually. (Breithaupt-Grögler *et al.,* 1997).

Forty-one normotensive hypercholesterolemic men were treated with aged garlic extract. They showed a mean reduction of 5.5% in systolic blood pressure, (Ried *et al.,* 2010). Two studies found that alliums/alliins inhibited platelet aggregation in human blood (Bordia, 1978; Kiesewetter *et al.,* 1993). Garlic also has an antioxidant action, decreasing the susceptibility of LDL to oxidation (Phelps and Harris, 1993).

18.5.3 Statistics

A double-blind parallel randomized placebo-controlled trial of aged garlic extract was carried out in 50 patients whose records routinely showed systolic BP >140 mm Hg. Oral administration of the extract (960 mg containing 2.4 mg S-allyl cysteine) for 12 weeks decreased systolic BP significantly by 10.2±4.3 mm Hg (Ried *et al.,* 2010).

A slight but significant decrease in blood pressure by garlic could be confirmed by a meta-analysis (Silagy and Neil, 1994b). The incremental benefits to statin were confirmed in a pilot study where garlic therapy was applied to patients who were on statin therapy (Budoff, 2006).

18.6 Ginkgo (*Ginkgo biloba*)

The ginkgo is venerated as a holy tree and is planted in shrines, temples and along main streets. However, its use as a food or as a culinary herb has a shorter history probably because of its strong smell and the skin irritation caused by the lactic acid in its juice. Simply touching the fruit causes a strong long-lasting allergic skin reaction over the whole body. Ginkgo was not listed in Shen Nong's herbal records. Probably the habit of eating ginkgo corns was not then wide-spread in Asia.

A recent oceanological study revealed ginkgo corns in the Mongol-Korean warships that were sunk off Japan in 1239. They were probably shipped for medicinal use by the overseas expedition. Definitive evidence of the consumption of ginkgo in 16th century Japan is found in a tea party menu listing baked ginkgo corns. Since then it has become the practice to include these in dishes served during prayers for health in the New Year.

18.6.1 Active compounds

The clinically effective compounds in gingko, gingkolide B, are terpenic lactones consisting of diterpenoids with 20-carbon skeletons. It improved the contraction of isolated cardiomyocytes from ischemic-reperfused rats. The DNA-protecting factor, Bcl-2, also increased in these ginkgolide B-treated cells (Fugh-Berman, 2000). Nowadays EGb is dispensed as tablets and used widely in the treatment of senile dementia. The efficacy of EGb treatment is similar to that of other cholinesterase inhibitors. In a trial comparing EGb 761 with donepezil, which inhibits acetycholinesterase, no statistically significant or clinically relevant differences were seen (Stein *et al.,* 2015).

Ginkgo extract has protective effects on cardiovascular functions in rats. Tachycardia produced in rats by injection of isoproterenol causes oxidative stress; it compromises cardiac function and several important endogenous antioxidants are depleted.

In EGb-treated rats there was a reduction in the deleterious effects on superoxide dismutase, catalase, glutathione peroxidesidase, glutathione reductase, and GSH, and on the serum marker enzymes aspartate aminotransferase, lactate dehydrogenase and creatine phosphokinase (Silagy and Neil, 1994a).

Following ischemia/reperfusion, the contractile power of hearts from rats that had been fed with EGb was greater than that of hearts from controls. Impaired cardiac function in spontaneously diabetic rats was largely restored by Ginkgo administration. EGb improved vasodilatation, prevented substance P accumulation and reduced the number of mast cells. It also protected the myocardial ultrastructure (Schneider *et al.,* 2009).

18.6.2 Human study

There is however little information from human patients about the beneficial effects of ginkgo on cardiac functions. *Ginkgo biloba* does not reduce blood pressure nor the incidence of cardiovascular accidents. The potassium-sparing diuretic use of *G. biloba* was associated in 192 aged persons with better verbal learning and memory measured by California verbal learning test as compared with no antihypertensive medication users (Yasar *et al.,* 2012).

18.7 Ginseng (*Panax ginseng*)

Panax ginseng (panax = almighty; gin = man; seng = to visit noble person) was classified by Shen Nong as an herb of the highest ranking. It is distributed in north-eastern China and Korea and was revered for its strength-giving property, its rejuvenating power and the human shape of its bifurcated root. The demand for ginseng outstripped the wild supply in the Middle Ages. Korea began commercial cultivation, with large exports of ginseng to Japan. In 1730 the 8th Shogun governor in Japan, Yoshimune Tokugawa, planned to increase the use of ginseng in Japanese medicine. He imported quantities of rough ginseng roots and distributed them all over Japan for cultivation by feudal lords. However, this was unsuccessful because the humid Japanese weather was unsuitable for these delicate plants. A closely related plant, *Panax notoginseng*, was found in Canada in the early 18th century and later in New England and New York. By the end of the 19th century farmers began to cultivate it, achieving success in both Canada and USA.

A related plant, *Acanthopanax* (spiky ginseng) is distributed in China, Siberia, Korea, and the northeastern part of Japan. Its leaves,root, and trunk bark contain medicinal substances. The extract of spiky ginseng was used in sports medicine. Its beneficial effects are experimentally studied in acute myocardial ischemic dogs (Sui *et al.,* 1994), in the aortic arch surgery in human patients (Di Eusanio *et al.,* 2015) and monocytic cell adhesion in spontaneously hypertensive rats (Park *et al.,* 2014).

18.7.1 Active compounds

Ginseng roots washed in boiled water and dried are called white ginseng. 6-year-old roots peered and boiled by steam become red and transparent, and are called red ginseng or radix ginseng. These are believed to be most effective medically. Ginseng contains ginsenosides, a class of steroid glycosides, and triterpene saponins and ursolic acid. The precise composition of the plants varies depending on where they grow, and their age.

In rats with ischemic myocardia following acute myocardial infarction eating radix ginseng and notoginseng increased capillary density and expression of vascular endothelial growth factor receptor-2 and hypoxia-inducible factor-alpha.

American Ginseng reduces oxidative stress and oxidative stress-induced cell death in cardiomyocytes through activating the nuclear factor erythroid 2-related factor 2 pathway, thereby providing cardioprotection against pathological cardiac remodeling.

The effects of total ginseng, and its components, panaxadiol and panaxatriol on ischemia/reperfusion injury were studied in isolated rat hearts. Rats received ginseng (20 mg/kg) or its components (5 mg/kg) every day for 7 days; on the 8th day hearts were excised. They were re-perfused after 30 minutes of ischemia. The myocardial damage was significantly reduced in hearts from rats whom also received ginseng constituents. This was probably attributable to their scavenging effects (Kim and Lee, 2010). The stalks of ginseng leaves contain bioactive constituents; their pharmacological functions have been confirmed in leaf extracts (Wang *et al.,* 2009). Ginsenoid-Rg1, one of the ingredients of ginseng induces angiogenesis via non-genomic cross-talk between the glucocorticoid receptor and fibroblast growth factor receptor-1 (Cheung *et al.,* 2011).

18.7.2 Human study

The effects of red ginseng extract were studied during an 8-month follow-up in patients with acute myocardial infarction. Mobilization of circulating angiogenic cells and microvascular integrity improved in those with an increased ST-elevation. After coronary stenting CFR was measured with an intracoronary Doppler wire. Circulating angiogenic cells had increased when assessed on days 1 and 5, and at 8 months when CFR was also significantly increased (Ahn *et al.,* 2011). Both radix ginseng and radix notoginseng were found to increase the secretion of vascular endothelial growth factor, and the expression of vascular endothelial growth factor receptor-2 in cultured human umbilical vein endothelial cells (Lei *et al.,* 2010). Ginsang extract was used for sport training also. Further details are given by Deyama *et al.* (2001) and Kwan *et al.* (2004)

18.8 Goldenseal (*Coptidis rhizome*) and *Hydrastis canadensis*

Goldenseal (*Coptidis rhizoma*) belongs to the buttercup family (*Ranunculacea*). Most species in this family contain compounds that are harmful to horses and cows when the plants are eaten raw; gazing stock usually avoid them. In contrast, the root of a closely related plant, *Hydrastis canadensis*, was used in Canada as a herbal medicine by the native Indians and, similarly, *C. rhizoma* has long been used in Asian medicine for psychological depression, bacterial infections, and gastro-intestinal disorders including gastritis colitis and liver disease. It is listed in the Japanese pharmacopeia. The main compound, the alkaloid berberine, is very bitter, effective against pains in digestive organs and its effects are long lasting. It has recently become clear that in man berberine is highly effective in reducing total plasma cholesterol and triglycerides (Kong *et al.,* 2004).

Raised TC and LDL-cholesterol, with a decrease in HDL-cholesterol, are primary risk factors in the development of atherosclerosis and coronary heart disease. Increased plasma triglycerides are a further, independent cause of cardiovascular disease in fetus (Jia *et al.,* 2008).

18.8.1 Active compounds

Berberine interacts with the micelles through their hydrophilic and hydrophobic binding sites to form alkaloid-bile salt agglomerates. The agglomerates decrease the capacity of micelles to solubilize cholesterol and thus affect cholesterol absorption. Berberine upregulates hepatic LDL receptor, so that to decompose LDLs (Kong *et al.,* 2004).

Furthermore, in rats, hamsters and man, berberine synergistically reduces the plasma lipids when given together with stanol, one of plant cholesterols (Wang *et al.,* 2010). Stanol is contained in fruits, nuts and vegetables, and forms 10% of grain sterols. It is recognized as a natural supplement that reduces cholesterol absorption and increases bile production. To study their effects three groups were prepared. Group 1 received only berberine, group 2 received stanol only, group 3 received a mixture of berberine and stanol. These supplementations significantly decreased plasma TC and non-HDL-C levels as compared with the control group. The supplementations lowered plasma TC by 22, 30 and 43% and non-HDL-C by 28, 45 and 63%, respectively. Substantial improvements in cholesterol-lowering efficacy were observed after berberine and phytostanol treatment. A synergistic action on plasma TC of berberine and phytostanol was marginally significant compared with either compound acting alone.

18.8.2 Human study

Oral administration in 32 hypercholesterolemic patients for 3 months reduced serum cholesterol by 29%, triglycerides by 35% and LDL-cholesterol by 25%. A reduction in LDL receptors was induced in human hepatic cells through a post-transcriptional mechanism that stabilizes the mRNA (Kong *et al.,* 2004).

18.9 Konjac (*Amorphophallus konjac*)

Glucomannan, or konjac, is a water-soluble straight-chain hydrocarbon polymer contained in roots of konjac, a plant originating in tropical Asia (Katsuraya *et al.,* 2003). It was transplanted to Japan during the Stone Age and became part of the diet. Cultivation is easy and it grows well in half-shaded areas, for instance among mulberry trees. Glucomannan is not digested in the human gut and blocks the adsorption of cholesterol but readily causes a sense of satiety. Konjac is boiled with other vegetables, fish, meat, and rice and can be served with soya sauce, as a side dish. It is also chewed. Konjac causes no side effects and is sold in different forms in markets. Unfortunately, a few years ago a type of konjac candy in the form of jelly balls came on the market. Several children were in danger of death when balls blocked the trachea. The tradition that konjac should be chewed must be retained.

18.9.1 Active compounds

Konjac can be applied in a powder or a polymerized rubbery jelly form. Both forms can absorb glucose and cholesterols contained in food. The rise speed in blood glucose level is decreased by konjac, and cholesterols exit in feces.

18.9.2 Human study

It has been found (Walsh *et al.,* 1984) that konjac produces statistically significant improvements in plasma cholesterol of obese patients. Martino *et al.* (2005) showed that in hypercholesterolemic children, the TC, LDL, triglycerides and systolic blood pressure decreased after 4 weeks of dietary supplementation with konjac. In type 2 diabetes, konjac improved the lipid status in blood serum (Chen *et al.,* 2003). Twenty two patients of type 2 diabetes took gelatin capsules each containing 0.5 g konjac powder three times daily for 28 days, one half hour before each meal with a glass water. TC decreased by 8.2%. LDL decreased significantly by 10.7%, leaving HDL unaffected.

18.10 Saji (China), sea-buckthorn (*Hippophae rhamnoides*)

Hippophae rhamnoides, a low spiky bush, grows in full sun in dry, sandy semi-desert areas, and also in cold areas. Its roots perform a nitrogen fixation role in the surrounding soils. During Gengis Khan's reign the fruits of *H. rhamnoides* were reserved for army horses, to enhance their speed; humans were forbidden to eat them. After the end of the Mongolian Empire the fruits were used to treat cardiovascular patients in northern China; the Ainu race in northern Japan also took them as a medicine. A recent field survey showed *Hippophae* fruits are still widely used therapeutically for man and domestic animals in India, near the Himalayas. (Dhyani *et al.,* 2010). They are taken to alleviate pain, and for pulmonary, and gastro-intestinal disorders.

18.10.1 Active compounds

The fruits, which are a shiny orange when ripe, contain superoxidase activity, quercetin, isorhamnetin, rutin, kaemferol, vitamin C, vitamin E, β-carotene and β-sitosterol.

The addition of total flavonoids from *H. rhamnoides* to smooth muscle cells in culture reduced intracellular free calcium; it seems likely that *Hippophae* fruits reduce peripheral vascular tone, and aortic blood pressure, by controlling intracellular calcium (Zhu *et al.,* 2005).

Repeated stretch of cultured cardiomyocytes activates NF-kappa B. It was found that administration of *H. rhamnoides* extract to culture fluid reduced NF-kappa B activity (Xiao *et al.,* 2003).

These effects seem to be advantageous for hypertensive patients. If hypertensive patients repeat isometric stretches when supine, blood pressure and heart rate increase via the sympathetic nervous system. The administration of total flavones of *H. rhamnoides* markedly/significantly reduced the cardiac response to the isometric movements (Zhang *et al.,* 2001).

It has been shown that aqueous extracts of *Hippophae* leaves also promote the healing of experimental burns in rats. In addition, the extract promotes angiogenesis in an *in vitro* chick chorioallantoic membrane model while *in vivo* it upregulates vascular endothelial growth factor (Upadhyay *et al.,* 2011).

The effects of *Hippophae* have been examined in male spontaneously hypertensive stroke prone rats. Dried *Hippophae* fruits from Northeastern China were powdered and added to the rat chow at a concentration of 0.7 g/kg. The chow was mixed into a dough with the addition of water, then dried to a solid block in a stream of cold air. The daily intake of chow, over 60 days, was about 40 g/day. Taking an average body weight, this gives a calculated daily dose of *Hippophae* of 136 mg/kg/day. Heart rate, mean blood pressure, HbA1c, TC, and plasma triglyceride were significantly lower in the *Hippophae*-treated group compared with the SHRSP group fed normal chow. In the ventricular wall, capillary portions stainable for alkaline phosphatase decreased, indicating a decrease in vascular shear stress. Capillary portions staining for dipeptidylpeptidase IV, a marker of the venular portion, increased (Koyama *et al.,* 2009).

18.10.2 Human study

The effects of *Hippophae* fruits on postprandial metabolism and insulin response were confirmed in ten healthy normal-weight male volunteers. Concomitant consumption of dried and crushed whole berries with high glucose meal reduced the difference between the postprandial hyperglycemia (at 30 min) in serum glucose concentration and subsequent hypoglycemia significantly (at 120 min) (Lehtonen *et al.,* 2010).

18.11 Sesame (*Sesamum indicum*)

Sesame originated in the African savanna and is distributed all over the world. In ancient Egypt and India it was used as a medicine and in divine ceremonies in Mesopotamia. In Japan sesame seeds were found near Tokyo in a mitten dating from the 12th century BC. Ancient records of Chinese fauna claim that eating sesame every day keeps men young. In Japan there is a word 'sesamize' which means powdering sesame seeds. Unappetizing food may become tasty only with a sprinkling of powdered sesame seeds.

18.11.1 Active compounds

More than 10 oily substances are confirmed in sesame seeds. The main substance, sesamin, is a stable lignan, comprising 0.5-1.0% of the whole seeds in weight. Sesamin is transformed in the liver to an active form, sesamin cathechol, a strong scavenger for active oxygens (Fukuda *et al.,* 1986). It may protect unsaturated fatty acids from oxidative destruction. Moreover, it inhibits cholesterol synthesis in the liver and suppresses cholesterol uptake in the intestine (Hirose *et al.,* 1991).

Sesame protects polyunsaturated fatty acids. The essential fatty acid docosahexaenoic acid is extremely susceptible to oxidation but sesamin, a lignan from sesame seeds, acting synergistically with alpha-tocopherol, can prevent this. (Yamashita *et al.,* 2000).

Sesamin has antihypertensive effects which have been studied in rats treated with deoxycorticosterone acetate. Unilaterally nephrectomized rats were separated into a control group, on a normal diet and a test group on a sesamin-containing diet. Systolic blood pressure, left ventricular weight and vascular wall thickness in the control group increased significantly in comparison with those on the sesamin diet (Matsumura *et al.,* 1995).

The antihypertensive effect of sesamin administration from 6 weeks of age, has also been studied in SHRSP which, from weaning, were either salt-loaded (1% NaCl in the drinking water) or served as controls. At 17 weeks of age, systolic blood pressure was significantly lower in the sesamin group (180±4 vs 215±4 mm Hg $P<0.01$). The left ventricular weight and aortic vascular wall were slightly but significantly lowered by sesamin feeding.

Sesamin is useful as a prophylactic in malignant hypertension reducing water and salt retention (Matsumura *et al.,* 1998). It also has chemoprotective effects through the suppression of NF-kappa B-regulation, thereby accelerating cell survival, proliferation, invasion, and angiogenic gene products (Harikumar *et al.,* 2010). Adhesion molecules are strongly expressed through the action of TNF-α on vascular endothelial cells. This can cause obstruction of blood vessel with fatal consequences. *in vitro*, sesamin reduces intercellular cell adhesion molecule-1 in TNF-α-treated human aortic endothelial cells, and exerts the same effect *in vivo* in apoprotein-E-deficient mice. (Wu *et al.,* 2010). Sesamin is a potent antioxidant and prevents endothelial dysfunction, followed by a cardiac protection for cardiovascular disease risk reduction. (Chung *et al.,* 2010).

18.11.2 Human study

Three studies using sesamin supplements have indicated a possible association between the lipid- and blood pressure-lowering properties. Furthermore, epidemiological studies in eleven human subjects examined dietary intakes of lignans in relation to cardiovascular disease risk (Peterson *et al.,* 2010).

The effects of sesame oil were studied in hypertensive patients who were on antihypertensive therapy either with diuretics (hydrochlorothiazide) or beta-blockers (atenolol). Fifty patients were given sesame oil and instructed to use it as the only edible oil for 45 days. Blood pressure, anthropometry, lipid profile, lipid peroxidation and enzymatic and non-enzymatic antioxidants were measured before, and immediately following the trial. Systolic and diastolic blood pressure were lowered to normal values. The same patients were then told to stop using sesame oil for another 45 days. Blood pressure values returned to the initial high level (Sankar *et al.,* 2006). A significant reduction in body weight and body mass index have been reported following sesame oil substitution but without any significant alterations in lipid profile except in triglycerides. Substitution resulted in a reduction in plasma sodium with a rise in potassium. Thiobarbituric acid reactive substance decreased while superoxide desmutase and catalase activities increased. Vitamin C, vitamin E, betacarotene, GSH (glutathione peroxidase).

The effect of 4-week administration of sesamin was studied on blood pressure in twenty-five mildly hypertensive patients by a double-blind, cross-over-controlled trial. 12 patients allocated to 4-week intake of capsules containing 60 mg sesamin per day, and 13 subjects placebo capsules. After a 4-week washout period the subjects received the alternative administration for 4 weeks. Systolic blood pressure decreased 137.6±2.2 to 134±1.7 mmHG, P=0.04, diastolic 87.7±1.3 to 85.8±1.0 mm Hg P=0.045. Sesamin caused small but significant effects in human patients (Miyawaki *et al.,* 2009).

18.12 Rosemary (*Rosemarius officinatis*)

Rosemary is a woody, perennial herb with evergreen, needle like leaves and purple or pink flowers. It has long been used as herb tea or herbal medicine for the activation of physical and psychological functions, as it is known to induce mental stimulation. For thousands of years rosemary has been used to improve skin, hair and memory. Ayurvadic medicine celebrates rosemary essential oil. In addition, recent study suggests cardio-protective effects of rosemary. It has mint like odor and contains calcium, vitamin B6 and iron.

It strengthens our memory and reduces muscle pain. It boosts the immune sensitivity, circulatory system and function, antioxidant capacity. It rejects free radicals and beta–amiroid. Rosemary shows potential effects against cardiovascular diseases, hypotensive, diabetes, anti-atheroscrelotic effects (Hassani *et al.,* 2016).

18.12.1 Bioactive substances

Rosemarinic acid, other anti-oxidants, phenolic diterpenes, flavonoids. Rosemary has been used in folk medicines to treat headaches, epilepsy, poor circulation, and many other ailments. Essential unsaturated fatty acids played important roles. Atherosclerosis is a chronic and progressive inflammatory disease. Novel anti-inflammatory therapies may be promising as treatment strategies also for cardiovascular risk reductor (Nabavi *et al.,* 2015).

Rosemary could act as a stimulant, mild analgesic, and inflammation reductant.

The effects of the rosemary components, carnosic acid (a kind of diterpenes, crosses blood-brain barrier and exerts neuroprotective effects) and carnosol, were studied on vascular smooth muscle cell migration (Chae *et al.,* 2012). Atherosclerosis is a chronic and progressive inflammatory disease of blood vessels. Novel anti-inflammatory therapies may be promising as treatment strategies for cardiovascular risk reduction. Rosemary is a rich source of phenolic phytochemicals having significant anti-oxidant, anti-inflammatory, hypoglycemic, hypolipidemic, hypotensive, anti-atherosclerotic, anti-thrombotic, hepatoprotective, and hypo-cholesterolemic effects. The activity of the inflammation-related substances, monocyte chemo-attractant protein-1 and matrix metalloproteinase-9 were suppressed.

18.12.2 Human study

Effectiveness of Rosmarinus officinalis essential oil as antihypotensive agent was shown in 32 primary hypotensive patients and its influence on health-related quality of life. The increase achieved in blood pressure values after 72 weeks administration of Rosemary essential oil was clinically significant. The results obtained from this prospective clinical trial prove the effectiveness of statistical methodology as a new approach to explain the antihypotensive effect of rosemary essential oil and its relationship with the improvement in patients' quality of life.

18.13 Egoma (*Perilla frutescens*)

Seeds and leaves of *Perilla* have widely been used in the life of ancient Japanese as food and medicinal material. Remains of baked cookies of *Perilla* seed were found in excavated remains of stone-age ovens near Tokyo. There are variations in *Perilla* leaf colors, green and red. Red leaves seem to contain more reducing capacity than the green (Banno *et al.,* 2004).

Since *Perilla* seeds contain much unsaturated essential fatty acids, they were important for the human life. It was used also for the oil lamp. The seed oil was used to make water-proof paper-suits and water-impermeable woven cloths. Once the oil dried, it could be used to make paper-made water-proof flexible clothes.

The multiply unsaturated oil is recognized as essential fatty acids and important to maintain our lives. Japanese monkeys recognize the importance of *Perilla* leaves and eat them but deer do not. It is said that Japanese deer recognize *Perilla* and dislike the smell of the unsaturated oil. (Asif *et al.,* 2011; Makino *et al.,* 2002).

Perilla frutescens seeds (4 mg/seed) contain much polyunsaturated fatty acids (35-45%). *Perilla* seed oil consistently contains omega-3 (α-linolenic acid) fatty acids, at 54-64%. The omega-6 (linoleic acid) component is usually around 14% and omega-9 (oleic acid) is also present in *Perilla* oil. These polyunsaturated fatty acids are beneficial to human health and in prevention of different diseases like cardiovascular disorders, cancer, inflammatory, rheumatoid arthritis, etc.

18.13.1 Bioactive substance

Polyunsaturated fatty acids obtained from *Perilla* (essential fatty acids) promote nitrogen monoxide production in vascular smooth muscle cells (Makino *et al.,* 2002). *Perilla* extract acts to restore the arterial blood flow reduced by vascular diseases.

In addition, *Perilla* extract shows suppressive effects on allergic inflammatory reactions (Oh *et al.,* 2011).

18.13.2 Human study

Perilla oil in addition to exercise was used with hyperlipidemic patients. Tumor necrosis factor-alpha, plasminogen activator inhibitor-1 and highly sensitive C-reactive protein ($P<0.05$) decreased in lipidemic patients with hyperlipidemia. *Perilla* is probably useful in the treatment against arteriosclerosis obiterans (Saita *et al.,* 2012; Wei *et al.,* 2013). In addition, *Perilla* extract shows suppressive effects on allergic inflammatory reactions.

18.14 Green tea (*Cammeille sinensis*)

The trees of *Cammeille* for green tea were distributed in nature on Tibetan and South-West Chinese mountains. The tea tree is now widely planted in Asia. Its medicinal effect was confirmed by the legendary emperor, Shen Nong about 2700 years BC for remedial use. Chinese people started to drink the water of boiled tea leaves in the 2nd century BC. The Japanese Buddhist, priest Saityu, brought seed corns of green tea tree back to Kyoto in the year 805 AD. They are now widely planted in Japan. Tea can be categorized into three types according to the different levels of fermentation: green (unfermented), oolong (partially fermented), and black (fermented).

In general, green tea extracts showed stronger antioxidant activity than the semifermented and black tea extracts, mainly because of the higher content of (−)-epigallocatechin gallate. The processes used in the manufacture of black tea are known to decrease levels of the monomeric catechins to a much greater extent than the less severe conditions applied to other teas.

18.14.1 Active compounds

The green tea from *Cammeille* leaves, richly contain bioactive compounds: 2% caffeine, and 13% tea cathekin (there are several cathekin compounds useful for tanning), 24% protein, 46% carbohydrates, 4-6% fat and many kinds of polyphenolic antioxidants. The taste of green tea depends mostly on the balance between catechins and proteins.

Green tea catechins prevented atherosclerotic changes in vascular cells *in vitro*. The effects were studied further in animal experiments. Rats were fed with cholesterol-enriched diet. Water or green tea-water were supplied to the rats. The beneficial effects were confirmed significant.

18.14.1 Human study

Three examples of human study are introduced here. Green tea catechins improved forearm vascular function measured in smokers by means of occlusion and releasing test. It seemed probable that catechins ameliorated endothelial dysfunction by scavenging free radicals with anti-inflammatory properties in healthy male smokers. (Sano *et al.,* 2004)

Sano *et al.* (2004) divided 203 heavy smokers in the area into two groups according to the daily green tea uptake amount. All patients received the coronary angiographic analysis and divided into two groups, drinker of much green tea (109 patients) and almost no green tea (94 patients). Those who drank much green tea daily showed almost no injury on the coronary artery. Those who drank no green tea showed stenotic shadows on coronary arteries.

Green tea catechins improved human forearm vascular function and have potent anti-inflammatory and anti-apoptotic effects as shown by means of the venous occlusion strain-gauge plethysmography (reactive hyperemia measurements). In 30-male healthy smokers who drank green tea cathekins daily for two weeks, the reactive hyperemia increased significantly in the forearm. It seemed probable that the uptake of tea catechins repaired the endothelial dysfunction caused by smoking (Maeda-Yamamoto, 2013; Oyama *et al.,* 2010).

18.15 Concluding remarks

Berberine, hated by horses and cows, has long been used as a suppressant drug for gastro-intestinal disorder. Surprisingly advantageous effects for the normalization of serum lipids were discovered recently. It may provide a new tool to fight against hyperlipidemea originated from excessive nutrients or diabetes, primary risk factors for the atheroscrelosis and coronary heart diseases.

In the present article the medicinal effects of individual herbs on cardiovascular functions have been considered (Table 18.1). The tenet 'one herb for one disease' is an over simplification: each herb contains many different biologically active substances that in combination support the

Table 18.1. Herbs and their medicinal effects.

Herb species	Syndrome	Expected merit	Main substance	Chemical units
chaga	lipidemea, arteriosclrosis	antitumor, antioxidation	ursolic acid	pentacyclic triterpene
danshen	angina pectoris	heart beat normalization	diterpenoids	comp. of two units of triterpnoids
foxglove	heart failure, Na+/K+-pump	heartbeat normalization	glycosides, digitoxin	Na+/K+-transporter
garlic	blood clotting	antiblood aggregation	alliine	thiosulfinate
ginkgo	senile dementia	partial recovery of dementia	gingkolide B	flavonol-glycoside
ginseng	diabetes infection	cardioprotection	ginsenoside, vascular endothelial growth factor	ginsenoside Rg
goldenseal, *Hydrastis*	high cholesterolemia	cholesterol normalization	berberine	hydrophilic,-phobic sites
Konjac	high cholesterolemia	cholesterol normalization	rejection of cholesterol in food	glucomannan
Saji	quercetin, rutin	angiogenesis	antioxydant, angiogenesis	quercetin, kaempherol, vitamin C
Sesame	hypertension	antihypertension	sesamine catechol	scavenger f. active oxygen
Rosemary	atherosclerosis	neuroprotection	carnosic acid	phenoric diterpene
Egoma	hyperlipidemia	antioxidation	polyunsaturated fatty acids	tumor necrosis factor alpha
Green tea	arteriosclerosis	antioxidation, anticancer	vascular endothelial growth factor, cathekin	vascular endothelial growth factor, pentacyclic triterpene

life of the plant as a whole. In the case of herbal medicines used by man the best mixtures of different herbs have been arrived at over long years. A recent example may be the remarkable hypolipidemic effects of a combination of the plant alkaloid, berberine, with a plant stanol where the two herbs act synergistically to reduce plasma hypercholesterol and triglycerides. Further advances in herbal medicine, that is the discovery of other useful herbs and new combinations, may be expected.

Herbs were historically often over harvested and were forced to be cultivated by human hands. However, we may not disturb the nature. An effort should be made to develop and mimic artificial

compounds or to introduce a hybrid mixture of herbs and artificial compounds to reduce the heavy load of harvest from the natural environment.

Acknowledgements

Publications on herbal medicines are often difficult to access. Much information was collected through PubMed. The author wishes to express his thanks to PubMed information. The author also wishes to express his cordial thanks to Professor Dr Watson for his kindness to give him the chance to prepare the present chapter. The author is much indebted to Dr Bethany Stevens for her kind reading of the manuscript, and also to Dr Ann Silver, Institute of Physiology, Cambridge for her valuable suggestions.

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19. Epidemiological aspects underlying the association between dietary salt intake and hypertension

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Abstract

The development of the human nutrition has walked side by side with the salt intake behavior. From the unpredictable conditions in the past when food was restricted to a high-fat diet rich in salt and red meat in Western countries, human health has been affected by certain components of the daily diet. Several epidemiological studies have shown that salt consumption in the general population is higher than the current recommendation. Also, people at high risk of developing cardiovascular and renal diseases have a high salt consumption. Several dietary factors cause changes in blood pressure, and high salt intake is one of the presumed causative factors. Based on that, reduction in salt intake is an obvious strategy that should be take place in all countries around the world. In fact, salt reduction reduces blood pressure and accounts for a reduction in morbidity and mortality. Also, reducing salt content in foods not only reduces the risk of cardiovascular diseases, but helps saving a big amount of money.

Keywords: sodium, cardiovascular diseases, hypertension, morbidity, sodium reduction

Key facts

- Louis Dahl showed a positive linear relationship between prevalence of hypertension and mean sodium intake.
- The International Study of Salt and Blood Pressure (INTERSALT) was conducted in 52 centers and showed the relationship between salt intake and blood pressure levels.
- The UK has effectively created a program of voluntary salt reduction in collaboration with the food industry Consensus Action on Salt and Health (CASH) that is one of the most successful initiatives to support salt reduction.
- The blood pressure response to a high-salt diet varies among individuals, a phenomenon that has been called salt sensitivity. Genetic factors have been reported to be involved in this pattern.
- In most of the cases, people are not aware of the deleterious effects of a high salt intake. Even when they know how much salt they are eating.

Summary points

- The average salt consumption is around 9-12 g/day, which is higher than stated in guidelines.
- The increased consumption of salt is not an exclusive pattern of the adult population. Salt intake by children and teenagers are also higher than recommended.
- Effective and progressive reduction in salt consumption reduces cardiovascular diseases (CVD) and is associated with lower blood pressure levels.
- Although a low-salt diet reduces the risk of CVD, a large part of the population is not aware of the deleterious effects of salt intake.
- People at higher risk of cardiovascular and kidney diseases eat more salt than those in the general population.

Abbreviations

CASH	Consensus Action on Salt and Health
CKD	Chronic kidney disease
CVD	Cardiovascular diseases
DASH	Dietary Approaches to Stop Hypertension
INTERSALT	International Study of Salt and Blood Pressure
T2D	Type-2 diabetes
WASH	World Action on Salt and Health

19.1 Introduction

During the evolution of human nutrition, salt intake appears as one of the most exciting stories. The rise and fall of this intriguing component of the human diet has walked together with the development of CVD (Baldo *et al.*, 2015; He *et al.*, 2012). Salt is found in natural form as a crystalline mineral present in abundance into seawater. Salt means sodium chloride, which is 40% sodium and 60% chloride. Indeed, very few people realize that in Latin, the words for health and healthy, *salus* and *salubris*, were derived from salt. Salt has been referred to as the fifth element, as vital as air, water, fire, and earth. Also, the Bible contains more than 50 references to salt, calling it 'the essence of life' (MacGregor and He, 1998; Roberts, 2001).

About five million years ago, wild fruits and vegetables were the main source of nutrients because at that time the agricultural methods or techniques of animal husbandry were not developed. Even after hunted animals were introduced into the diet and the meat consumption varied from 20-80% of the daily diet, the salt intake was less than 1.5 g per day (Baldo *et al.*, 2015; Jackson, 1991). Also, the potassium consumption coming from large quantities of vegetables and fruits was approximately 16 times greater than the salt intake (MacGregor and He, 1998; Roberts, 2001).

With the advent of agriculture as the dominant mode of subsistence 10,000 years ago, the initial stimulus for salt production and use may have been food preservation. Agriculture requires a settled population, which facilitates greater population densities. However, agriculture is a more precarious means of obtaining subsistence than is gathering and hunting. Under these conditions, food preservation became an important behavioral response to the new selective pressures of dietary uncertainty associated with agriculture (Jackson, 1991; MacGregor and He, 1998; Roberts, 2001). In the absence of opportunities for natural cold storage, or perhaps as an adjunct, the use of salt in food processing and the ingestion of nondiscretionary intakes assumed greater importance in the human diet, although supplementary (i.e. discretionary) salt use may still have remained minimal (Baldo *et al.*, 2015; Jackson, 1991; Roberts, 2001).

At present, the Yanomano Indians in Brazil consume less than 0.5 g of sodium per day (Baldo *et al.*, 2015). But the reality is critical, when the sodium intake is at least two times higher than the current recommendation. These different patterns of salt intake have aroused the interest of

several areas. The nomenclature 'salt appetite' refers to the state and behaviors associated with seeking and ingesting salty substances. It is noteworthy that saltiness is one of the five basic human tastes. Numidian nomads and certain Bedouins who used to eat fish and roasted meat had a strong dislike for salt (Roberts, 2001). On the other hand, Wilkins and Richter (1940) described a case of a child with abnormally low synthesis of aldosterone, a mineralocorticoid responsible for sodium retain. The child showed an impressive salt appetite eating salt directly from the shaker.

Thus, the main objective of this chapter is to describe the association between high salt intake and hypertension, and also to show the impact of initiatives to reduce salt intake worldwide.

19.2 Salt consumption around the world

The body functions are maintained through the balance of different elements. Sodium is a main element participating in cellular activities, being essential for nerves and muscles to work correctly. It is essential in the auto-regulation of the hidroelectrolitical balance of the body. Salt consumption reflects directly in the renal system activity. In fact, high dietary salt intake presents a major challenge to the kidneys to excrete large amounts of salt administered. Another system that is hugely vulnerable to the adverse effects of excessive salt in the diet is the cardiovascular system (Ha, 2014). In the 60's, Louis Dahl brought up to the attention of scientific community and also to the clinical practice around the world, a positive linear relationship between prevalence of hypertension and mean sodium intake across five different populations (Dahl, 1960). Louis' findings attracted interest of the scientific community to research concerning salt and CVD. After that, important advances in this field of research were made. Several small and large epidemiological studies have been completed in order to estimate salt consumption and to establish its association with CVD.

19.2.1 Sodium consumption by general population

Based on several studies, the salt intake in the general population is higher than the current recommendation. As part of the Global Burden of Diseases Nutritional and Chronic Diseases Expert Group, data on global sodium consumption was systematically identified and analyzed. The salt intake was estimated by 24-h urinary sodium excretion method or estimates of dietary intake. Data were arranged according to age and sex, from published reports or direct contacts for 205 surveys from 66 countries. In 2010, the estimated mean level of sodium intake worldwide was 3.95 g/day, which means an estimated salt intake of 9.9 g/day (Mozaffarian *et al.*, 2014; Powles *et al.*, 2013). Some other studies carried out around the world aimed to define the amount of salt intake in different populations. For instance, a study carried out in 418 Spanish adults aged 18-60 years aimed to determine the salt intake using a 24-hour urine collection. The estimated salt intake in the whole population was 9.8 g/day (11.5 g/day in men and 8.4 g/day in women), that means that 88.2% of the subjects had intakes of over the recommendation (Ortega *et al.*, 2011).

A cross-sectional survey conducted in Shandong-China showed a mean 24-h urinary sodium level of 5.46 g, which corresponds to 13.9 g of salt per day. In this study, the mean salt intake by rural residents was higher than that of urban residents and it was also higher in men than in women (Zhang *et al.*, 2014). However, salt intake can vary considerably even inside the same country, mostly due to cultural aspects. In a different plan, in the Jiangsu Province of China, salt intake was estimated in 2,345 adults aged from 18 to 69 years old using a 24-h urine sample collection. The mean 24-h urinary sodium was 4,310 mg, which represents an estimated salt intake of 11.0 g/day (Yongqing *et al.*, 2016). In Yantai, another region in China, salt intake was approximately 12 g/day, which is twice the recommendations (Xu *et al.*, 2014).

In order to identify a healthy range for sodium intake, Pubmed and other publicly available search mechanisms were used to identify published studies that contained 24-h urinary sodium collection datasets from free-living people. The analyses included a total of 69,011 individuals, 190 collection sites from 45 different countries, and a period more than 5 decades. The results showed an estimated salt intake of 9.3 g/day and range of 6.6-12.2 g/day (McCarron *et al.*, 2013).

This pattern of high sodium intake can be observed in different populations around the world, independent of being from develop or developing countries. Subjects from four countries of Latin America participated in a study to estimate their salt consumption. The overall mean salt excretion in those 17,033 adults included in the study protocols was 11.9 g/day (Lamelas *et al.*, 2016). The same behavior was observed in random samples of adult population from 12 Italian regions, including men and women aged 35-79 years. The salt consumption was estimated from 24-h urine collections, and it was higher than 5 g/day in 97% of men and 87% of women (Donfrancesco *et al.*, 2013).

The expressive findings from several epidemiological reports indicate that salt intake is not only a problem for adults. Indeed, high salt intake was also observed in children and is associated with CVD in early ages (He and MacGregor, 2006; Miersch *et al.*, 2013). In a cohort study, it was observed that 70% of 8-month-old infants had a sodium consumption of 400 mg over the current reference values (Cribb *et al.*, 2012). Similar results were observed for 1- to 6- (Mulder *et al.*, 2011) and 10- to 12-year-old (Cotter *et al.*, 2013) children, in which more than 90% of the whole sample were over the recommended amount for salt intake.

Sodium consumption was estimated in children and adolescents aged 8 to 18 years (n=6,235) who participated in National Health and Nutrition Examination Survey 2003 by using multiple 24-h dietary recalls. Salt intake was, on average, 8.6 g/day (Yang *et al.*, 2012). A study enrolled 1,424 subjects aged 6-18 years from 10 Italian regions representative of the whole Italian territory to estimate sodium intake using a 24-h urine collection. In this study, 90% of the participants had sodium intake over the recommendation (Campanozzi *et al.*, 2015). Another cross-sectional study to determine the salt intake of children observed the mean salt intake for the 5- to 6-year-olds was 3.75 g/day, which increased to 7.55 g/day for the 13- to 17-year-olds. The authors found that 70% of participants had salt intake above their respective maximum intake recommendation. (Marrero *et al.*, 2014)

19.2.2 Salt consumption by patients at risk

High salt consumption is not only observed in healthy people from general population. Indeed, people at high risk of cardiovascular and renal diseases also eat more salt than stated in guidelines. Several dietary factors cause changes in blood pressure, and high salt intake is one of the presumed causative factors, even in hypertensive patients.

The salt consumption was estimated by a brief self-administered diet-history questionnaire and a 24-h urine collection in a total of 136 hypertensive Japanese outpatients. The salt intake as estimated by the questionnaire was higher than the urinary salt excretion assessed by 24-h urinary collection. Furthermore, the urinary salt excretion estimated by 24-h urinary collection was 9.0 g/day, being higher in men than in women (Sakata *et al.*, 2015). A multistage-stratified (by age and sex) cluster random sampling method was used to select a nationally representative sample of the general Portuguese population ageing 18-90 years old. The average salt excretion was 10.7 g/day, which represents 4.2 g/day of sodium. However, sodium intake in hypertensive patients (4,246 mg/day) was significantly higher than in normotensive individuals (4,072 mg/day) (Polonia *et al.*, 2014).

Patients with T2D are at high risk of developing CVD, and CVD risks are even higher in those with a diagnostic of hypertension. This is of great importance because the abusive consumption of salt in dietary might contributes and/or aggravates previous chronic diseases, such as hypertension. The American Diabetes Association recommends that those at risk of heart disease, including those with T2D, should limit their dietary salt to up 6 g/day (Franz *et al.*, 2004). Besides the risk, people are not even close to this recommendation. In the Enhancing Adherence to Diabetes Self-Management study, a single center, randomized, controlled trial to test a 6-month behavioral intervention to improve lifestyle management of people with T2D, the estimated mean salt intake of 8.2 g/day was observed for diabetic patients (Provenzano *et al.*, 2014). Also, it was showed that 122 patients with T2D attending the Austin Health Diabetes Clinics from 2001 to 2008 had a urinary salt excretion of 9.9 g/day in men and 8.3 g/day in women. It is worthy emphasizing that only 3% of male patients and 14% of female patients with T2D met the Australian NHF guidelines for sodium consumption (Ekinci *et al.*, 2010).

Different reports observed that individuals with CKD are at an increased risk of death, and CVD is the leading cause of premature death in this population. Moreover, high salt consumption is related to an increased risk of progression to end-stage renal disease in CKD patients (Vegter *et al.*, 2012). In fact, current guidelines recommend that patients with CKD limit their daily dietary salt intake to less than 5 g (Verbeke *et al.*, 2014). Kutlugun *et al.* (2011) studied 373 consecutive outpatients with stable CKD for daily sodium excretion. The estimated mean 24-h urinary sodium levels of 2 consecutive urine samples were approximately 9.8 g/day (Kutlugun *et al.*, 2011). Also, it was demonstrated by Nerbass *et al.* (2015) in which excessive sodium intake was associated with CVD and CKD in people with CKD stage 3, and that estimated sodium intake above the recommended amount is an independent factor for increases in mean arterial pressure and albuminuria in this population (Nerbass *et al.*, 2015).

19.3 Salt and hypertension: data from large studies

CVD, mainly arterial hypertension, has quickly grown worldwide, and the consequences of this ailment affect a large portion of the world population (Baldo *et al.*, 2015). Regardless of the causes, the prevalence of hypertension depends on the region, but it varies between 20 and 40% (Cutler *et al.*, 2008; Egan *et al.*, 2010). Several experimental studies have showed the strong connection between high salt intake and hypertension (Baldo *et al.*, 2011, 2012, 2015; Forechi *et al.*, 2015). This link has also been evidenced by many large epidemiological investigations conducted worldwide.

In a pioneer large multicenter study addressing this issue, the INTERSALT was conducted in 52 centers from 32 countries, and each center recruited 200 men and women aged 20-59 years. A total of 10,079 participants were included in the study, and were asked to provide both a casual urine specimen and a 24-h urine collection (Anonymous, 1986). INTERSALT found a significant positive association between 24-h urinary sodium excretion and systolic and diastolic blood pressure, even after adjustment for confounders (Anonymous, 1988). These results were observed in a large range of salt intake among different population.

The European Prospective Investigation into Cancer in Norfolk was a prospective population study of 25,000 men and women aged 45-79 years unselectively recruited from general practice registers in Norfolk, UK, who attended to a baseline survey. In this study, sodium intake was estimated by a single casual urine sample. The significant association between systolic and diastolic blood pressure to urinary sodium: creatinine was independently associated to age, body mass index, cigarette smoking, and urinary potassium/creatinine. These findings were consistent between men and women (Khaw *et al.*, 2004).

Two large studies committed to analyze changes in dietary patterns were carried out. In both studies, sodium intake and its relation to hypertension was also evaluated. The DASH trial was a multicenter, randomized, controlled feeding study designed to compare 3 dietary patterns on blood pressure in persons with high-normal blood pressure and stage 1 hypertension. The DASH feeding trial demonstrated that combined effects on blood pressure of a low sodium intake and the DASH diet were greater than expected for either intervention alone (Sacks *et al.*, 2001). The other study, the International Collaborative Study of Macronutrients, Micronutrients and Blood Pressure was an international cross-sectional epidemiological study designed to clarify the role of multiple dietary factors in the etiology of blood pressure prevailing for most middle-aged and older individuals (Stamler *et al.*, 2003). The study enrolled 4,680 men and women aged 40-59 years from 17 diverse population samples from Japan, China, UK and USA. The highest mean values for urinary sodium excretion were found in China. In the USA, mean urinary sodium excretion were in the range of 4,140-4,370 mg/day for men and 2,990-3,450 mg/day for women (Zhou *et al.*, 2003).

More recently, a large study released consistent results regarding the association between salt intake and cardiovascular risk. The Prospective Urban Rural Epidemiology study is a large,

international, prospective cohort study design to ascertain the association between estimated sodium and potassium excretion and the composite of death and cardiovascular outcomes. The Prospective Urban Rural Epidemiology study enrolled and followed 156,424 participants, 35 to 70 years of age, residing in urban or rural communities of 17 countries. The 24-h sodium and potassium excretion was estimated by using the Kawasaki formula to a morning fasting midstream urine sample. O'Donnell et al (O'Donnell *et al.*, 2014) showed that an estimated sodium intake between 3-6 g/day was associated with lower risk of death and cardiovascular events than either a higher or lower estimated level of sodium intake. Moreover, the association between a high estimated sodium excretion and increased risk, which was significant only among participants with hypertension, was attenuated after adjustment for blood pressure, suggesting that the adverse effects of high sodium intake may be mediated to some degree by the effects of sodium intake on blood pressure (Mente *et al.*, 2014).

19.4 Reducing sodium intake

19.4.1 Evidences in support of salt reduction

Based on the data presented above, it is clear that there are compelling evidences supporting worldwide salt reduction. However, salt reduction programs are not an easy task to implement. The UK has effectively created a program of voluntary salt reduction in collaboration with the food industry. This initiative started after 22 renowned researchers on salt and blood pressure set up an action group, CASH. CASH waged a high-profile campaign to handle with food manufacturers and suppliers to reduce, universally and gradually, the salt content of processed foods. The initial goal was beyond reducion of salt in food products. It also developed and supported an education program to help the public in becoming more salt-aware in terms of understanding the impact of salt on their health, and explain the evidence into public health policy (Godlee, 1996; He *et al.*, 2014a; MacGregor and Sever, 1996).

The results from CASH interventions represented a great advance. The average salt intake in England was 9.5 g/day in 2003. Salt intake fell to 9.0 g/day in 2005/2006, 8.6 g/day in 2008 and further to 8.1 g/day by 2011. In the same period, there was a reduction in mortality by 42% and 40% due to stroke and ischemic heart disease, respectively (He *et al.*, 2014b).

Following the success of CASH group, a world action group – WASH – was established in 2005. The intent of WASH is to set up similar groups worldwide based on CASH. Appropriated strategies to reduce salt consumption of that particular country, and to stimulate actions from the government and/or department of health, the food industry, media, and public system (He *et al.*, 2010).

Some of these initiatives to reduce salt intake are supported by some small and large studies showing the benefits of salt reduction. For instance, adults aged 30-54 years with prehypertension were included into the Trials of Hypertension Prevention phase I and II, which was a comprehensive

education and counseling study to reduce sodium intake. Patients allocated in an interventional group experienced a 25% reduction on relative risk of CVD in the 10 to 15 years after the trial (Cook *et al.*, 2007).

The impact of reduction in salt consumptions is mandatory in order to keep healthier. The analyses of 4 salt reduction trials showed that a modest salt reduction (from ~10-12 g to 5-6 g) caused a significant reduction in systolic blood pressure of approximately 10 mm Hg in individuals with isolated systolic hypertension. Also, in those in patients with combined hypertension salt reduction caused a significant decrease of 7 and 4 mm Hg in systolic and diastolic blood pressure, respectively (He *et al.*, 2005). Swift *et al.* (2005) invited black hypertensives from African or African-Caribbean origin to participate in a salt reduction program. Reduction in salt intake to approximately 5 g/day lowered the systolic and diastolic blood pressure in 8 and 3 mm Hg, respectively. Additionally, urine protein excretion was reduced in those patients with reduced salt intake (Swift *et al.*, 2005).

The prevalence of hypertension in elderly people exceeds 50%, which means that a reduction on salt consumption would provide an important benefit for this population. The effects of sodium reduction on blood pressure and hypertension control were evaluated in 681 patients with hypertension, aged 60 to 80 years, randomly assigned to a reduced sodium intervention or the control group. The reduction in sodium intake resulted in a mean reduction in systolic blood pressure of 4.3 mm Hg and in diastolic blood pressure of 2.0 mm Hg after a mean follow-up of 27.8 months (Appel *et al.*, 2001).

A population health model including country-specific disease data was used to predict the changes in prevalence of ischemic heart disease and stroke for each country estimating the effect of salt reduction through its effect on blood pressure levels. Interventions with 30% of salt reduction would decrease the prevalence of stroke by 6.4% in Finland to 13.5% in Poland. Also, cases of ischemic heart disease would be decreased by 4.1% in Finland to 8.9% in Poland (Hendriksen *et al.*, 2015).

A computer-based modeling study to predict the impact of dietary salt reduction indicated a great reduction on future CVD. The study demonstrated that a population-wide reduction in dietary salt of 3 g/day is projected to reduce the annual number of new cases of coronary heart disease by 60,000 to 120,000, stroke by 32,000 to 66,000, and myocardial infarction by 54,000 to 99,000, and to reduce the annual number of deaths from any cause by 44,000 to 92,000. The computational model also showed that a national effort to decrease salt consumption by 3 g/day would result in an estimated saving of 10 billion to 24 billion in health care cost (Bibbins-Domingo *et al.*, 2010).

19.4.2 Excessive salt intake: do we know the risks?

Several reports from different professional associations support an expressive reduction in salt intake by the general population. Also, the Seventh Report of the Joint National Committee indicates a dietary sodium reduction to a level of no more than 2,300 mg/day for hypertensive

patients (Chobanian *et al.*, 2003). Also, it is highly recommended that physicians advise patients to reduce their salt intake. However, the efficacy of these recommendations is questionable.

A total of 664 patients with hypertension were interviewed and only 15% and 10.6% of all participants reported that they always look for sodium content in food and always buy low-sodium products, respectively (Westrick *et al.*, 2014). Also, a study investigated consumers' knowledge of health risks of high salt intake and the frequency of use and understanding of labeled salt information. A total of 474 valid surveys were obtained in shopping centers within metropolitan Melbourne. 65% of participants were unable to correctly identify the relationship between salt and sodium. Also, 88% knew of the risk of high blood pressure (Grimes *et al.*, 2009).

A study was set to investigate urinary salt excretion and the relationship between the awareness of salt restriction and the actual salt intake in hypertensive outpatients. The authors demonstrated that there was no relationship between the awareness of salt restriction and the actual salt intake estimated by 24-h urinary collection (Ohta *et al.*, 2004). Another similar study showed that although 62% of the patients indicated they had high or very high awareness of the necessity to restrict dietary salt. This awareness, however, did not correlate with salt intake (Takahashi *et al.*, 2015).

19.4.3 Salt reduction: why not?

There is compelling evidence showing that reduction in salt intake lowers blood pressure and reduces the risk for CVD (Appel *et al.*, 2001; Cook *et al.*, 2007; He *et al.*, 2014a,b; Hendriksen *et al.*, 2015; Sacks *et al.*, 2001). However, there are also some reports against the reduction in salt intake. Increased plasma renin activity and ANG II have been reported as the main argument against salt reduction recommendation (Alderman and Cohen, 2012). In fact, increased plasma renin activity (He *et al.*, 2005; Swift *et al.*, 2005) and aldosterone (He *et al.*, 2005) have been reported in people facing a moderate salt reduction. However, a recent meta-analysis of randomized controlled trials did not detect an association between low sodium intake and increase in plasma renin activity, supporting the speculation that renin activity returns to a baseline level after long-term sodium intake reduction, although renin activity is increased in the early phase of sodium intake restriction (Rhee *et al.*, 2016).

There are several other reports raising questions about the reliability of salt reduction strategies. Non-obese, non-treated hypertensive adults were fed strictly controlled diets. An initial week on a control diet (sodium = 3,680 mg/day) was followed by 3 weeks on low-salt intake (sodium = 1,380 mg/day). In hypertensive patients, low-salt intake reduced the 24-h arterial blood pressure, and increased the plasma triglyceride concentration, in the fasting and postprandial periods, consequently to the accumulation of chylomicron-cholesterol content and particles (represented by the apoB concentration), and very low-density lipoprotein lipid components. Fasting C reactive protein, interleukin-6 and tumor necrosis factor-concentrations increased after a low-salt intake (Nakandakare *et al.*, 2008).

19.5 Conclusion

Salt consumption varies around the world. However, the consumption is higher than the recommendations no matter where. It highlights for the needing of strategies to reduce salt form diet. Indeed, some strategies already in course in a few countries show the beneficial of reducing salt intake. But it is important to focus on the high risk patients, in which the consumption is higher than healthy people.

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20. Resveratrol and metabolic syndrome in obese men – a review

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Abstract

There is currently an unprecedented need for the development of safe and effective methods to treat obesity and its comorbidities. One such promising method is the use of dietary supplements that have the potential of diminishing the incidence of hypertension, excess fat accumulation, insulin resistance and the other components of what researchers refer to as 'metabolic syndrome'. Among these potential dietary supplements is the compound resveratrol (RESV), a stilbene first identified in 1978 that has been examined as a potential therapeutic agent in the treatment of hypertension and some forms of cancer. This short review will attempt to highlight studies that have examined how RESV might be implicated in not only extending lifespan, but also how it may have significant and beneficial impact on excessive fat deposition, insulin sensitivity, dyslipidemia and hypertension.

Keywords: stilbene, insulin resistance, dyslipidemia, hypertension

Ronald Ross Watson and Sherma Zibadi (eds.) Handbook of nutrition and heart health
Human Health Handbooks no. 14 - DOI 10.3920/978-90-8686-853-7_20, © Wageningen Academic Publishers 2017

Key facts

- Physical inactivity in combination with a hypercaloric diet is a root cause of the obesity epidemic.
- Excess adipose tissue invokes metabolic dysregulation, leading to 'metabolic syndrome' (MetS), type-2 diabetes, and their associated comorbidities.
- Pharmacological interventions target the clinical complications of MetS but do not provide a long term solution, as none are proven to reduce excess adipose tissue. Lifestyle interventions (diet and exercise) have been shown to be more effective in reducing MetS.
- Epidemiological evidence shows a protective effect of polyphenol intake (non-nutritive compounds found primarily in fruits and vegetables) against development of MetS, more so than pharmacological treatment. Several classes of polyphenols are being studied for their capacity as a nutraceutical for MetS treatment.
- Resveratrol (RESV), a stilbene found mostly in grapes and peanuts, shares a route of action similar to that of caloric restriction (CR). Preliminary research shows a protective effect against diet-induced obesity, where increased mitochondrial density could target excess adipose tissue, thus reversing downstream metabolic dysregulation and preventing MetS.

Summary points

- 35% of American adults are obese. As of 2011, 35% also have a form of MetS – defined by three of five possible criteria. Lifestyle modifications to target obesity have been unsuccessful because recommendations are not realistic or socially acceptable.
- Excess visceral fat leads to elevated circulating free fatty acids which in turn leads to the clinical complications of obesity and MetS: insulin resistance and increased cardiovascular health risks.
- Diet and lifestyle therapy remain the most effective measures to combat MetS, yet challenges with recidivism and compliance appear to be unsurmountable.
- Polyphenols are a diverse grouping of compounds in fruits and vegetables believed to elicit positive health benefits that include longevity and reduction of chronic disease.
- Certain polyphenols, such as flavonoids from green tea and berries, may prove a useful tool to combat obesity and MetS.
- RESV, a stilbene found in grapes and peanuts, activates the same sirtuin pathways that have been implicated in CR, which is believed to promote longevity.
- Animal studies show RESV increases lifespan in lower level organisms. Further, RESV is protective against a high-fat diet in mice: alleviating criteria of MetS and improving survival.
- Human studies of RESV and obesity are more ambiguous due to variations in study design and subject population. Despite this, the same pathways implicated in animal studies have been translated to humans.
- In both animal and human studies RESV shows the potential to alleviate the criteria used to define MetS, although human studies involving RESV administration to subjects with MetS are lacking.
- This review provides support for the study of RESV in humans with MetS with the use of a controlled feeding experiment to provide further information to this important research question.

Abbreviations

AMPK	Adenosine monophosphate-activated protein kinase
AUC	Area under the curve
CDC	Centers for Disease Control and Prevention
CR	Caloric restriction
CVD	Cardiovascular disease
EGCG	Green tea/epigallocatechin-3-gallate
HDL	High-density lipoprotein
HOMA-IR	Homeostasis model assessment of insulin resistance
LDL	Low-density lipoprotein
MetS	Metabolic syndrome
PGC-1α	Peroxisome proliferator activated receptor-gamma coactivator-1
RESV	Resveratrol
SIRT1	Sirtuin-1
TG	Triglyceride

20.1 Overview

A recent query in Pubmed that was restricted to using only the term 'resveratrol' (RESV) returned 8,776 papers. The oldest paper included in the search results was one dated 1978. Adding the term 'review' resulted in 1,204 papers. Clearly, it is beyond the scope of this review (or for that matter any review) to comprehensively survey the literature that has developed around RESV. Given this limitation, the present chapter attempts to highlight several important lines of research that chronicle the effect of RESV, a stilbenoid found in grapes, blueberries, raspberries and peanuts, and its influence on the MetS. Included in our review are several examinations of how RESV affects several components of the MetS. These include papers focused on the effects of RESV on adipose tissue, on insulin sensitivity, on glucoregulation, on dyslipidemia and on hypertension. We conclude this chapter with a call for more research on how RESV might contribute to the clinician's tools in the treatment of MetS. We propose that, under the appropriate conditions, significant improvements might be observed in insulin resistance, fasting glucose and lipids, and blood pressure with RESV administration. Further, enhanced activation of molecular targets in skeletal muscle and subsequent modulation of energy balance may be possible with RESV in obese men with MetS.

20.2 Introduction

According to a 2013 report by the CDC, heart disease, stroke, diabetes, liver disease, and kidney disease are numbers 1, 5, 7, 12, and 13, respectively (https://www.cdc.gov/nchs/fastats/deaths.htm) in their rank of diseases afflicting Americans. Poor nutrition and physical inactivity leading to excessive weight gain are the leading modifiable risk factors for these conditions (WHO/FAO,

2003). The CDC also reports that as of 2012, 69% of American adults over 20 years of age are overweight or obese (35% are obese). This staggering increase in the prevalence of overweight and obesity is a 25 year old story in the United States with no sign of improvement. However, based on current trends some experts believe the prevalence to have stabilized or that the curve has flattened out since 2011 (Ogden *et al.*, 2014).

The same behaviors leading to the current prevalence of overweight and obesity in the nation further complicate health and longevity by way of the MetS. MetS, as defined by the National Cholesterol Education Program's Adult Treatment Panel II, is a condition in which an individual/patient meets at least 3 of the 5 following criteria: waist circumference greater than 102 cm for men or 88 cm for women; serum TG above 150 mg/dl; HDL cholesterol less than 40 mg/dl for men or less than 50 mg/dl for women; blood pressure above 130/85 mm Hg or prescribed hypertension medication; and/or fasting plasma glucose above 100 mg/dl or prescribed diabetic medication (National Cholesterol Education Program Expert Panel on Detection and Treatment of High Blood Cholesterol in, 2002). Aguilar *et al.* (2015) report the overall prevalence of MetS in the United States between 2011-2012 to be 34.7% with a greater prevalence in women vs men (36% vs 30%) as well as old vs young (46.7% vs 18.3%). These statistics highlight the relationship between obesity and metabolic function: the complications of inactivity and poor diet choices. However, different criteria can satisfy the classification of MetS with ten possible different combinations of three risk factors to describe the same syndrome (see Figure 1 from Lim and Eckel (2014). Different combinations have an influence on morbidity. To illustrate this point, a person with only obesity and hyperglycemia (not technically considered MetS) has a 2 fold greater chance of becoming type 2 diabetic compared to a person who has all the criteria for MetS except hyperglycemia (Nichols and Moler, 2010). Different combinations of criteria call for individualized approaches to yield effective therapy, but a staple for the majority of cases will involve a reduction in fat mass.

Doctors and dieticians who counsel overweight/obese patients about remedial approaches typically include recommendations that their patients eat less calorically dense foods and increase their physical activity. Further, announcements to the public to adopt an active lifestyle (for example, the First Lady's 'Let's move' campaign) have gone to great lengths to bring the issue of this health crisis to the public's attention. Unfortunately, the advantages of an energy dense, nutrient poor diet – affordable, convenient, and palatable – are more captivating to the average consumer compared to the encouragement of health officials to adopt healthier eating behaviors (Drewnowski and Eichelsdoerfer, 2010). Indeed, nutritious meal plans designed to be conducive to low-income family budgets do not consider social acceptability or consumer meal patterns and are an unrealistic and ineffective solution. Nutritious meal plans, when constrained to the meal pattern of the majority of a population, more than double their cost (Maillot *et al.*, 2010). Clearly, the health community must approach this dilemma through different avenues. It is not enough to prescribe medications to treat the biochemical aberrations elicited by the obese state. Rather, researchers must discover and address the source of the dysfunctional cascade that leads to downstream comorbidities of excess weight.

Mechanistically, the complications of MetS are attributable to excess body fat which results in abnormally high concentrations of non-esterified fatty acids which then interfere with regulatory (insulin) signals in liver and skeletal muscle (Vega *et al.*, 2011). This results in a futile cycle of peripheral insulin resistance and increased hepatic release of glucose. The distribution of excess body fat determines the magnitude of this effect; excess adipose tissue surrounding the vital organs (visceral adipose tissue) is a primary insult. Excess adipose tissue also releases cytokines and plasminogen activator inhibitor-1 resulting in a chronic, low-grade inflammation and increased risk of thrombosis (Vega *et al.*, 2011). Ultimately, effective strategies designed to combat MetS (nutritional or otherwise) must address the source of the problem; an effective therapy is one that will reduce excess adipose tissue. Please refer to Figure 20.1 for a summary illustration of MetS and the interaction of factors that promote excess adiposity.

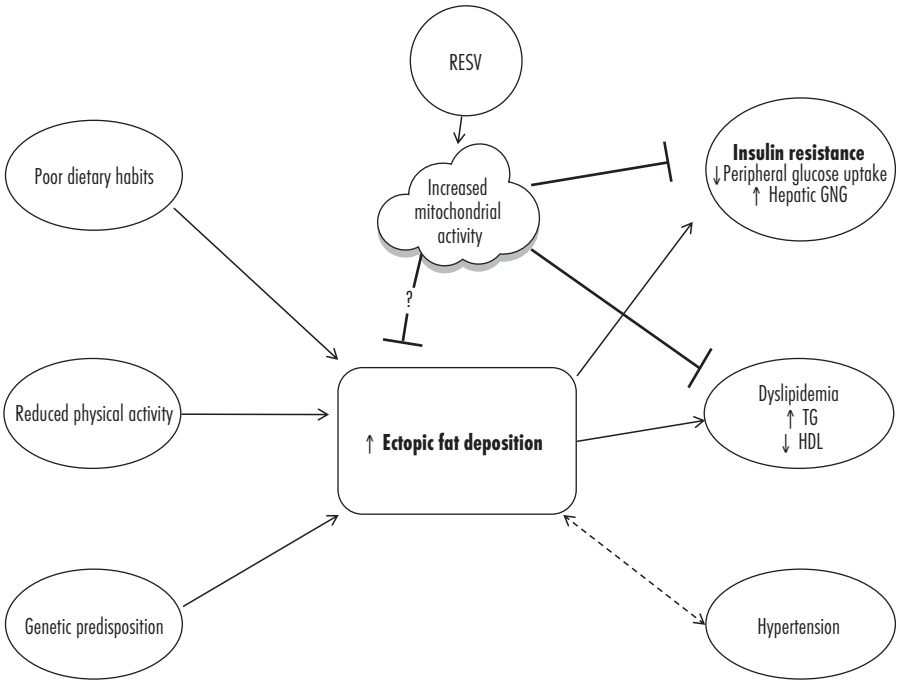

Figure 20.1. Ectopic fat interrupts normal metabolic function. Excess calories in combination with a sedentary lifestyle and a genetic predisposition (inability to store all excess calories as adipose tissue) lead to ectopic fat deposition. Fat storage in lean tissues (skeletal muscle, liver, and heart) in combination with an increase in circulating free fatty acids is believed to impede normal signaling pathways of carbohydrate and fatty acid metabolism; cornerstones of the metabolic syndrome (MetS). Resveratrol (RESV), discussed in this review, has the potential to address the complications of MetS via increased mitochondrial density and/or activity. GNG = gluconeogenesis; HDL = high-density lipoprotein; TG = triglycerides.

20.2.1 Diet therapy and metabolic syndrome

Pharmacological intervention is a standard approach to treating the clinical signs of MetS: thiazolidinedione or metformin for hyperglycemia, statins for dyslipidemia, and ACE inhibitors for high blood pressure. Anti-obesity drugs are being developed as well, but are not as commonly used because of issues with side-effects (Lim and Eckel, 2014). Thus pharmacological treatments attempt to reduce the risk of comorbidities introduced by MetS, namely, type-2 diabetes and cardiovascular disease, but safely targeting fat loss remains a challenge; lifestyle modifications (diet/exercise) remain the primary solutions. Recidivism and translation of promising lifestyle modification experiments to clinical practice are common challenges to health practitioners who are tasked with the treatment of the obese/metabolically stressed.

The potency of lifestyle over pharmacological intervention has been illustrated best in a meta-analysis by Dunkley *et al.* (2012). They identified 13 longitudinal studies that described the effects of lifestyle, drug, or combination treatments on subjects with MetS, most of whom had not developed advanced comorbidities. Lifestyle intervention resulted in a fourfold reduction in the incidence of MetS (reversal of MetS from baseline) compared to control, whereas drug therapy alone yielded a comparably modest, yet significant 60% reduction in the incidence of MetS. The results of the analysis also showed the effectiveness of anti-diabetic drugs in reversing MetS. Dunkley *et al.* noted that the heterogeneity amongst the trials prevented firm conclusions to be drawn. Although this study highlights the effectiveness of lifestyle modification, the benefit of drug therapy shouldn't be overlooked: in a sub-set of the studies with an appreciably long amount of time to follow-up, beneficial effects of metformin and statin drugs reduced incidence of diabetes and cardiovascular-related mortality at 3 to 10 year follow-up, respectively (Clearfield *et al.*, 2005; Geluk *et al.*, 2005; Ramachandran *et al.*, 2007).

Despite what may be superior effects of lifestyle treatment described above, recidivism is the greatest weakness of this intervention. For this reason, difficultly lies in translating promising experimental findings into clinical practice. Dietary approaches to promote weight loss or prevent weight gain are ubiquitous, and unfortunately there is still no consensus on what are the most effective forms of dietary treatment for this at risk population (Brown *et al.*, 2009; Meydani, 2005; Tarantino *et al.*, 2015). Conversely, diet adherence ensuring CR is more critical to success, thus comparisons regarding effectiveness of various diet compositions should be secondary and selected according to patient preference; individualized diet plans that maximize compliance will be most useful for weight loss (Dansinger and Schaefer, 2006). However, it is beneficial to determine what diet components lend to the success of certain diet patterns in managing body weight in at-risk populations, as this knowledge can then be utilized in advancing therapies that maximize the potential for weight loss or maintenance.

20.2.2 Polyphenols and metabolic syndrome

In a segment of nutrition research, scientists are exploring the capabilities of bioactive food components to quell the insult to human health caused by today's obesogenic environment.

Bioactive food components are believed to be one of the main effectors of a diet high in fruit and vegetables. It has repeatedly been found that a diet high in fruits and vegetables positively effects biomarkers of human health and reduces risk of chronic disease as outlined in epidemiologic studies (Hung *et al.*, 2004; Knekt *et al.*, 2002; Liu, 2003). The term polyphenol is used to describe a diverse spectrum of plant metabolites that can be categorized into several classes dependent on a unique chemical structure; examples include stilbenes, lignans, and flavonoids. What all of the various classes have in common is the presence of phenolic structures derived from the phenylpropanoid pathway (see Figure 1 of Hollman, 2014). The vast array of polyphenols characterized and isolated combined with the task to elucidate the effects of the high fruit and vegetable diet pattern resulted in a new facet of research for nutritionists and biochemists to investigate the effect of polyphenols on health and function. Polyphenols are being studied for their nutraceutical capabilities in the context of the metabolic dysregulation of obesity (Sae-tan *et al.*, 2011b). To demonstrate the potential for polyphenol sources to improve MetS, here we will briefly review two particularly intriguing and well-studied sources of polyphenols: catechins from tea and flavonoids from berries.

20.2.3 Green tea/epigallocatechin-3-gallate

Green tea has a rich cultural history, and has long been thought to positively contribute towards health (Wolfram *et al.*, 2006; Yang *et al.*, 2007). Polyphenols constitute approximately 40% of green tea, primarily as flavonoids, of which flavan-3-ols (sometimes referred to as catechins, though catechin is actually specific compound itself) are the main flavonoid subgroup (Cabrera *et al.*, 2003, 2006; Del Rio *et al.*, 2004; Graham, 1992). EGCG is the most abundant flavan-3-ol in green tea and has high antioxidant activity; its abilities to chelate metals, scavenge free radicals, and induce superoxide dismutase have been noted (An *et al.*, 2014; Meng *et al.*, 2008; Rice-Evans, 1999). Conversely, increased apoptosis by production of reactive oxygen species by EGCG at high doses has also been described in cancer models (Li *et al.*, 2010; Min *et al.*, 2012) as well as in adipocytes (Lin *et al.*, 2005).

Green tea or green tea polyphenol administration to animal models of obesity show a protective effect against body weight gain as well as decreases specifically to fat mass (Bose *et al.*, 2008; Choo, 2003; Fiorini *et al.*, 2005; Hasegawa *et al.*, 2003; Park *et al.*, 2011). Studies in rodent models of obesity were also able to demonstrate recapitulation of green tea's beneficial effects on fat mass after it had been decaffeinated, as confounding results attributed to caffeine were a concern (Ikeda *et al.*, 2005; Richard *et al.*, 2009). One mechanism by which green tea or EGCG are thought to reduce obesity in rodents is decreased digestibility and subsequent excretion of lipids in the feces (Bose *et al.*, 2008; Muramatsu *et al.*, 1986; Raederstorff *et al.*, 2003; Yang *et al.*, 2001), an effect that may be facilitated by EGCG's observed inhibition of pancreatic lipase *in vitro* (Grove *et al.*, 2012; Juhel *et al.*, 2000; Shishikura *et al.*, 2006; Wang *et al.*, 2014). Parallel to this intestinal effect, EGCG can increase fat oxidation and reduce activity of fatty acid synthase, of which expression of genes involved in the pathway support the findings (Friedrich *et al.*, 2012; Huang *et al.*, 2015; Ikeda *et al.*, 2005; Klaus *et al.*, 2005; Sae-Tan *et al.*, 2011a; Wolfram *et al.*, 2005).

Preliminary studies in obese or MetS human subjects have been conducted with promising findings regarding body weight and outcomes related to obesity and MetS (see Table 3 of Legeay *et al.*, 2015). For instance, a parallel-arm study of 35 obese subjects with MetS found significant reductions in body weight (-2.5 kg vs control), as well as reduced LDL and lipid oxidation after 870 mg of EGCG per day either as green tea or green tea extract for 8 weeks (Basu *et al.*, 2010). In contrast, one follow-up study, Mielgo-Ayuso *et al.* were unable to reproduce EGCG's effects in obese women fed 300 mg/d for 12 weeks, however, both the EGCG intervention group and the control group were following a diet intervention to lose weight (Mielgo-Ayuso *et al.*, 2014). In addition to the anti-obesity effects of green tea or EGCG, a plethora of work describing its ability to modulate the other criteria of the MetS exists (for useful reviews on the topic, see Legeay *et al.*, 2015; Sae-tan *et al.*, 2011b).

20.2.4 Berries/flavonoids

Dark and red pigmented berries are a good source of vitamin C, ellagic acid/ellagitannins, and flavonoids. The high density of phytochemicals found in berries results in a food source with a high antioxidant capacity. Of all of the frequently consumed beverages in the United States, blueberry and cranberry juices were shown to have the greatest antioxidant potential (Basu and Lyons, 2012; Seeram *et al.*, 2008). Because the constituents of berries are thought to elicit positive health benefits to humans beyond nutritional value i.e. by promoting normal function or preventing chronic disease, they are classified as functional foods (Brown *et al.*, 2015; Ozen *et al.*, 2012). Here we are interested in their ability to reduce adiposity or alleviate factors related to MetS.

Blueberries have high anthocyanin content; anthocyanins comprise the majority of the polyphenol profile of blueberries (Gavrilova *et al.*, 2011). Animal models of MetS that studied blueberry juice, extract, or isolated anthocyanins found varied effects on markers of MetS. In one study, anthocyanin extract didn't modulate weight gain in the presence of high-fat diet fed in mice, but still had a positive effect on insulin sensitivity (Guo *et al.*, 2012). A different study found a protective effect of blueberry juice or purified anthocyanins on fat mass, where purified anthocyanins also increased insulin sensitivity, but the juice did not (Prior *et al.*, 2010). The mechanisms by which the anthocyanins protect against a high-fat diet are not fully understood, but studies using high-fat or genetic mouse models highlight the involvement of the inhibition of sterol regulatory element binding protein 1, and down regulation of enzymes involved in fatty acid synthesis and inflammation (Tsuda *et al.*, 2003; Wei *et al.*, 2011).

In human experiments, a method to deliver a greater quantity of polyphenols from berries is to feed them as a freeze-dried powder, which concentrates the polyphenol content by approximately seven fold (Basu and Lyons, 2012). Basu and Lyons (2012) highlight several studies that have fed various freeze-dried berries, purees, cocktails, or juices to subjects with MetS (see Table 1 in Basu and Lyons, 2012). The major findings of these human trials involve decreases in blood pressure, lipid oxidation, and LDL cholesterol concentrations. Although berry consumption may not

prove applicable to reduce adipose tissue, its antioxidant and anti-atherosclerotic observations in humans prove berries a useful tool in augmenting a diet that reduces metabolic stress.

The description above of studies utilizing tea or berry sources for polyphenols to impede MetS is simply to give the reader an appreciation for how polyphenol-rich foods may improve outcome for subjects with this cluster of metabolic complications of obesity. It is not exhaustive. Other sources of polyphenols, such as apples (quercetin), coffee (chlorogenic acid), and cinnamon may prove useful in addressing the complications of MetS, although their effects on MetS specifically are not as established (Cherniack, 2011). The remainder of our discussion will focus on one candidate polyphenol, RESV, and the studies that describe its potential as a nutraceutical therapy for MetS. RESV provides a unique approach to the challenges of MetS in that it aims to alleviate the source of the dysfunction: excess adipose tissue.

20.3 Resveratrol: encouraging research points toward a nutraceutical application for the metabolic syndrome

RESV, a polyphenol abundant in red grapes, was first identified for its anti-cancer and anti-atherogenic properties *in vitro* (Jang *et al.*, 1997; Pace-Asciak *et al.*, 1995). It was not until 2003 in the seminal work by Howitz *et al.* (2003) where RESV was identified in a small molecule screening. RESV was described for its ability to activate the histone deacetylase SIRT1 in an elegant series of experiments describing its effects *in vitro* as well as *in vivo* via yeast and cell culture models (Howitz *et al.*, 2003). The implication of SIRT1 activation by RESV is above and beyond the prospect of promoting heart health and preventing cancer, as SIRT1 has been implicated in mediating calorie restriction extension of lifespan (Mercken *et al.*, 2014). Howitz *et al.* (2003) describes the ability of RESV to increase SIRT1 activity by 13 fold and increase the average lifespan of yeast by 70% compared to control conditions. The authors also demonstrate RESV's ability to increase survival of cell cultures exposed to ionizing radiation via the attenuation of the tumor suppressor gene *p53*, a downstream target of SIRT1. These findings led the authors to call for future work investigating RESV's ability to act as a CR mimetic, as CR is also known to act through similar pathways to extend lifespan in a hormetic manner (Masoro, 2000; Mattson, 2008).

CR's observed benefits on longevity date back to rat models in the 1930s, and an extensive amount of work has been completed on long term trials in non-human primates that highlight the powerful benefits of CR on extending lifespan (Colman *et al.*, 2009; Kemnitz, 2011; McCay *et al.*, 1935). Seminal work by McCay (1947) and McCay *et al.* (1935, 1939) laid the framework for the elucidation of CR without malnutrition in a series of longitudinal experiments involving rodents and dogs. Their work also included the CR effects on the intestinal microflora via parabiosis of young vs old, and fed vs calorically restricted rats reared from the same parents. The authors found the CR animals (both rats and beagles) to be smaller in size with an increase in bone fragility, but the animals lives were substantially extended; in the case of the rat, as much as a two-fold increase in lifespan was observed (McCay *et al.*, 1939). McCay, clear in his (current-day

controversial) views in how the discoveries needed to be translated to humans, was a pioneer in the topic of CR. As it stands today, the application of CR to humans is still not definitive (McCay *et al.*, 1956).

The benefits of CR are in the process of being translated to humans (Civitarese *et al.*, 2007; Heilbronn *et al.*, 2006) and long term studies investigating the translation of its benefit are underway (Stewart *et al.*, 2013). An excellent review by Mercken *et al.* (2012) addresses the challenges of connecting translational findings to humans; namely, even if a benefit of CR is identified the idea of the general public adhering to CR is optimistic at best, as the western world is steeped in its poor diet and sedentary lifestyle. Mercken *et al.* (2012) also summarizes the benefit of CR in the presence of obesity and MetS referencing studies that confirm the widely accepted belief that reduced food intake leads to a reduction in visceral adiposity and the alleviation of metabolic abnormalities which prevents or even reverses the morbidities listed above (Fontana, 2008). Although CR investigations in humans are warranted, a place still stands for the assessment of RESV to tap into these same mechanisms in humans, as a plant extract may be an easier pill to swallow than a 30% reduction in energy intake.

The CR-like effect of RESV discovered by Howitz *et al.* (2003) spurred on other researchers to confirm its effect in other short lived animal models. Several review papers have done an excellent job summarizing the connection between CR and RESV, the animal models studied, the tissue specific molecular mechanisms activated and their postulated effects on overt physiological and psychological states, as well as the physiologic conditions under which RESV is most effective (Ingram and Roth, 2015; Mercken *et al.*, 2012; Ramis *et al.*, 2015; Wright, 2014). The biggest questions one is left with after coming to appreciate the many different ways RESV elicits benefits in these translational studies are the following: Can some or any of these be translated to the human condition? Which ones? And at what dose does RESV yield such benefits and when in life should a regimen commence?

20.3.1 Life extension

Investigators have demonstrated the ability of RESV to modulate gene expression in a CR like manner: Barger *et al.* (2008) calls RESV a 'partial CR mimic' when fed low dose and started in middle age in control mice – see Figure 1 in Barger *et al.* (2008) for gene expression comparison between RESV and CR. Based on their experiment, SIRT1 protein expression is actually hindered by CR and RESV in heart and skeletal muscle tissue compared to control mice of a similar age (Figure 3 in Barger *et al.*, 2008). A study by Pearson *et al.* (2008) also showed similarities in gene expression between CR and RESV (Figures 1 and 2) but report no life extending ability in control mice (mice fed a standard diet with the addition of RESV), although several markers of health deterioration were retarded (Figure 3). Conversely, life extension was increased by 25% with the addition of RESV in the rodent groups fed a high-fat (60% fat) diet to the degree where life span was not different from the standard diet groups (Pearson *et al.*, 2008). In addition to the work by Howitz *et al.* (2003) where RESV extends lifespan in yeast, other scientists have found the same result in other animal models including worms, fruit flies, and a short-lived species of

fish (Bauer *et al.*, 2004; Valenzano *et al.*, 2006; Wood *et al.*, 2004). However, conflicting studies exist (Bass *et al.*, 2007; Pearson *et al.*, 2008). Perhaps one of the most critical reviews of RESV's life extending capabilities in animal models comes from a meta-analysis by Hector *et al.* (2012). A hazard ratio was calculated for effect size allowing the investigators to compare species with different lifespans resulting in a comparison of 6 species from 19 studies. The authors concluded that a marked increase in longevity in lower order species exists, while beneficial effects in mice and Mexican flies were less obvious; however, a prominent effect was noted in turquoise killifish (mentioned above), which the authors attribute to their exceptionally short lifespan. The authors justly concluded that RESV should not be touted as a lifespan promoter in humans. It should be noted that one failure of this meta-analysis was to not include where in the lifespan of the animals RESV treatment was initiated, as this would be a useful inclusion of the other moderators described (see Figure 2 in Hector *et al.*, 2012).

Colman *et al.* mention in the last line of their 2009 communication on the effects of CR in rhesus monkeys that its (CR's) effects in humans may never be known, but that will not dissuade their group from continuing to determine the effect in their model (they've come too far!) (Colman *et al.*, 2009). Likewise, the 'ultimate truth' of RESV's effects in these same avenues will likely never result in a definitive yes or no answer for life extension in humans, but as discussed below, other beneficial effects of RESV on human health can be gleaned along the way. As postulated by Mercken *et al.* (2012), metabolic stress may be a prerequisite condition for RESV to elicit a health benefit. A good demonstration of this is the comparison between two mice studies by Sinclair *et al.* in which one study found no age extension in adult mice on a normal diet +RESV (despite its impact on gene expression mentioned above), but the amelioration of metabolic dysregulation and increased survival were observed when adult mice were placed on a high-fat diet concurrent to RESV administration independent of weight gain (Baur *et al.*, 2006; Pearson *et al.*, 2008). A final point on longevity, CR itself has not definitively increased lifespan in all circumstances (for an excellent, exhaustive review of CR mimetics and their targets, see Ingram and Roth, 2015).

20.3.2 Metabolic dysregulation and molecular pathways

Studies mentioned above paint a murky picture on the ability of RESV to extend lifespan, especially in higher order animals as these types of studies become laborious due to the time commitment. However, mounting evidence exists for its health span promotion i.e. the alleviation of chronic morbidity. Highlighted by Ingram and Roth, broad-reaching beneficial effects of RESV include changes in stroke, heart failure, seizures, Parkinson's, and Alzheimer's disease (Ingram and Roth, 2015). As nutritionists, our interest lays in its ability to alleviate the insults of poor dietary habits, namely a high-fat, high calorie diet. Alleviation of metabolic stress as indicated by the remediation of disturbed clinical markers by RESV, independent of a poor diet, would be a powerful, simple, and inexpensive solution to an overburdened health care system in this country. If findings from animal studies can be confirmed in humans this would make RESV an attractive therapeutic tool.

A plethora of translational work exists on the mechanisms by which RESV alleviates metabolic dysfunction due to high-fat feeding (Ingram and Roth, 2015: p. 54-44; Mercken *et al.*, 2012: p. 394; Ramis *et al.*, 2015: p. 32; Wright, 2014: p. 112-113). The key findings include improvements in insulin sensitivity and blood glucose regulation. Regulatory metabolic pathways of adipose and skeletal muscle are described with a central theme on mitochondrial biogenesis. The authors highlight RESV's activation effect on the SIRT1 cascade, which includes AMPK and peroxisome proliferator activated receptor-gamma's transcription cofactor PGC-1α, although there is debate about where in this pathway RESV exerts its effect (Hoeks and Schrauwen, 2012).

These molecular pathways and their affected clinical measurements have been confirmed in mice, rats, monkeys, and humans (Baur *et al.*, 2006; Beaudoin *et al.*, 2013; Jimenez-Gomez *et al.*, 2013; Lagouge *et al.*, 2006; Timmers *et al.*, 2011). Baur *et al.* (2006) fed RESV (0.4 g/kg diet or approximately 22 mg/kg body weight/day) in combination with a high-fat (60% fat) diet to 1 year-old mice for 1 year and demonstrated a significant increase in activated (phosphorylated) AMPK protein content, activated (deacetylated) PGC1-α protein content, and mitochondria per cell in liver tissue compared to mice on the high-fat diet. The mice fed RESV also had improved insulin sensitivity and glucose tolerance. Lagouge *et al.* (2006) fed RESV (4 g/kg diet or approximately 400 mg/kg body weight/day) in combination with a high-fat (40% fat) diet to growing (4 to 8 week-old) mice for fifteen weeks. The investigators observed an increase in mitochondrial size, number, and activity (via succinate dehydrogenase staining and citrate synthase activity) in the gastrocnemius of the mice fed RESV. Mitochondria size and number were also increased in the brown adipose tissue. This study also observed a significant increase in the protein content of PGC-1α in the gastrocnemius, which confirms the molecular mechanism of the mitochondrial adaptation by RESV. To demonstrate the pivotal role of SIRT1 expression in subsequent effects on mitochondria, SIRT1 null mouse embryonic fibroblasts did not show increased mitochondrial gene expression (PGC-1α, cytochrome C, and medium-chain acetyl-CoA dehydrogenase) like that observed in positive controls when treated with RESV. Cardiac muscle PGC-1α acetylation was unchanged, likewise, a long-term feeding study of rhesus monkeys performed a comprehensive characterization of mitochondria content and activity in visceral white adipose tissue and showed no differences between treatments (a high-fat, high sugar diet with or without 480 mg RESV/day), which may indicate the tissue specific action of SIRT1 on PGC1α and subsequent effects on mitochondria. Conversely, Zucker diabetic fatty rats fed a standard diet with or without RESV (200 mg/kg body weight/day) for six weeks demonstrated increased *ex vivo* oxygen consumption and mitochondrial protein content in subcutaneous and retroperitoneal white adipose tissue with RESV treatment (Beaudoin *et al.*, 2013). This discrepancy highlights differential effects of RESV on tissue compartments across animal models which are likely also affected by study duration, different diet treatments, and RESV doses.

Timmers *et al.* (2011) translated the mechanistic effects of RESV to humans. They observed the activation of the same molecular pathways (increases in SIRT1, AMPK, and PGC1α protein content of the vastus lateralis) and subsequent effects on mitochondrial activity that were implicated in the translational studies described above. Eleven obese, but healthy male subjects were administered 150 mg of RESV or placebo every day for four weeks in a cross-over fashion with a four week

wash-out between treatments. Here, mitochondrial density was not different between RESV and placebo groups, however, *ex vivo* characterization of mitochondrial activity in permeabilized vastus lateralis muscle fibers (citrate synthase activity, oxygen flux in state 3 respiration using fatty acid substrates, and maximum capacity, uncoupled respiration) demonstrated an increase in mitochondrial efficiency with RESV treatment compared to placebo. This is the only human study to confirm the biochemical effects of RESV, thus additional studies are needed to confirm these findings. It should be noted that the review by Wright (2014) incorrectly identified the obese healthy subjects in the Timmers *et al.* (2011) study as having type-2 diabetes, and the review by Ramis *et al.* (2015) overlooked the study entirely, thus the Ramis *et al.* (2015) conclusion that there was a failure to translate RESV's effects in animals to humans is unjustified.

20.3.3 RESV's impact on specific factors of the metabolic syndrome

We have introduced RESV as a potential life/health-extending, partial CR mimetic. These exciting claims are supported by empirical evidence involving mechanisms accepted as health promoting: the activation of a sirtuin, AMPK, PGC1-α axis that is believed to increase mitochondrial density and ultimately lead to the preferential use of fatty acids as energy substrate. The exact mechanism by which RESV exerts an effect on this pathway is not fully understood, but what is clear is that somehow it has a beneficial effect on energy homeostasis (Park *et al.*, 2012; Um *et al.*, 2010). Described are studies that indicate RESV's utility in a hypercaloric environment. If this proposed pathway is activated in human subjects with MetS, this would offer the possibility of a cost-effective, simple approach that can be added to the standard of care for patients with diet-induced metabolic stress, and may lead to other research focused on expanding the knowledge of this pathway, as well as other ways in which it could be activated. Here we will review the effectiveness of RESV administration as it relates to each factor of the MetS.

20.3.4 RESV and adipose tissue

Beyond the initial discovery of sirtuin activation in yeast, another exciting development of RESV and health was the anti-obesity effects described in rodents fed a high-fat diet. In growing mice fed RESV, a dose of 4 g/kg diet (estimated as 400 mg/kg body weight/day) was protective against a high-fat diet over fifteen weeks of feeding (Lagouge *et al.*, 2006). The mice fed RESV in combination with the high-fat diet had a significant reduction in fat-pad mass as well as percentage body fat as measured by dual-energy x-ray absorptiometry. These differences were observed despite similar calorie consumption between the two treatments. The authors also reported differences in energy expenditure independent of physical activity. They were also able to demonstrate increases in mitochondrial density in skeletal, hepatic, and brown adipose tissues with commensurate increase in PGC1-α protein levels in the gastrocnemius, which was less acetylated than control high-fat fed mice, suggesting activation of the molecular axis by RESV described above (Lagouge *et al.*, 2006). The increase in energy expenditure may be explained by the increase in mitochondria in the rodents' brown adipose tissue and subsequent increase on non-shivering thermogenesis, as uncoupling protein 3 gene expression was increased (in skeletal muscle) along with core temperature. The effects on thermogenesis and molecular targets in

brown adipose tissue were confirmed in a related study, and technology is now available to assess RESV's effects on brown adipose tissue in humans *in vivo* (Andrade *et al.*, 2014; Van Marken Lichtenbelt *et al.*, 2009). A similar study using the same design as Lagouge *et al.* (2006) was able to reproduce the protective effect of RESV against weight-gain in mice fed a high-fat diet for 10 weeks (Kim *et al.*, 2011). In addition, this group demonstrated a reduction in inflammatory signaling in epididymal adipose tissue and proposed a model of RESV's actions on galanin and toll-like receptor-mediated pathways of inflammation based on changes in gene expression (see Figure 4 of Kim *et al.*, 2011).

As summarized in De Ligt *et al.* (2015), the protection by RESV to counteract increases in fat mass has not been translated to humans (see Table 1 in De Ligt *et al.*, 2015 for a summary of relevant human studies) (Bhatt *et al.*, 2012; Crandall *et al.*, 2012; De Ligt *et al.*, 2015; Movahed *et al.*, 2013; Poulsen *et al.*, 2013; Timmers *et al.*, 2011; Yoshino *et al.*, 2012). De Ligt *et al.* (2015) emphasize the high-fat feeding of the mice, dose, and duration of treatment as main differences between the mice and human studies. Age is also likely a point of disparity between animal and human studies. Indeed, the nature in which RESV has an anti-obesity effect in mice is in the context of young, growing mice fed a high-fat diet, whereas the human studies are performed with middle-aged subjects that are already obese, and none of the studies are controlling the subjects' diet. Similarly, in one longitudinal rodent study that fed RESV and a high-fat diet to middle-aged (12 months of age) mice, the investigators saw an initial difference in weight trajectory in the first few months of feeding, but differences were not observed between the two diet treatments in older mice (18-24 months of age) (Baur *et al.*, 2006). However, the survival curve of high-fat+RESV mice was normalized to the control, chow fed group (Baur *et al.*, 2006). The differences in body weight noted in other rodent studies could be transient and specific to the growing, young mouse. To recapitulate this same environment in humans is ethically impossible. Translating the appropriate dose from rodents to humans has also proven to be difficult. The metabolism of RESV is likely different between mouse and human, as Timmers *et al.* (2011) was able to achieve similar plasma concentrations of RESV using a 250-fold lower dose (150 mg/day) than the rodent studies. The dose of RESV in human studies of overweight/obese subjects ranges from 10 mg to 5 g daily (De Ligt *et al.*, 2015). Despite the disconnect from rodent to human studies regarding observable changes in fat mass, human studies have still demonstrated alleviations to the metabolic phenotype characteristic of overeating/metabolic stress (described below), as well as activation of molecular targets that indicate the potential for preferential use of fatty acids as a substrate. Well-designed studies of appropriate RESV dose and duration in the future may one day be able to address this question.

20.3.5 RESV and insulin sensitivity/glucoregulation

Impaired fasting glucose in MetS is an indicator of excess adipose tissue stores in the liver and skeletal muscle and subsequent reduction in insulin sensitivity which puts the subject at risk for developing type-2 diabetes (McGarry, 2002). Like the rationale for reduction of adiposity mentioned above, increased or normalized mitochondrial density in hepatic and skeletal muscle tissue may be one approach to alleviating metabolic stress i.e. more mitochondria available

to remove the excess fatty acids and restore normal insulin signaling. Research conducted on cardiovascular training in type-2 diabetics demonstrates an increased mitochondrial capacity parallel to increased insulin sensitivity (Meex *et al.*, 2010). Likewise, Timmers *et al.* (2011) was able to demonstrate comparable effects in healthy obese men fed 150 mg RESV for four weeks, demonstrating both reductions in fasting glucose and insulin sensitivity in addition to an increase in respiration using fatty acids in mitochondria isolated from the vastus lateralis. RESV has also been shown to preserve mitochondrial content and insulin sensitivity in a rat model of muscle atrophy (Momken *et al.*, 2011).

The rodent studies of RESV in combination with a high-fat diet found positive effects of RESV on insulin sensitivity. Lagouge *et al.* (2006) assessed insulin sensitivity with both the clamp method in C57Bl/6J mice fed respective diets for sixteen weeks as well as a meal tolerance test in KKAy mice (a rodent model of obesity and diabetes) fed respective diets for eight weeks. The C57Bl/6J mice fed high-fat + RESV had significantly lower fasting insulin concentrations and were able to tolerate a higher glucose infusion rate over the course of two hours compared to the high-fat fed mice. Similarly, the genetically obese/diabetic mice had lower fasting blood glucose as well as a trend for lower glucose AUC out to 2.5 hours from the oral glucose bolus. These changes in the genetic mouse were noted without any changes on bodyweight/adiposity which, as Lagouge *et al.* (2006) conclude, exemplifies RESV's intrinsic anti-diabetic effects. The longitudinal study by Baur *et al.* (2006) supports this claim, where their mice also responded to RESV with significant reductions in both insulin and glucose AUC after the same oral glucose bolus despite no appreciable changes in body weight after one year adherence to treatment diets.

These *in vivo* experiments of RESV and increased insulin sensitivity have also been translated to non-human primates (Jimenez-Gomez *et al.*, 2013; Marchal *et al.*, 2012). In one study, Jimenez-Gomez *et al.* (2013) fed rhesus monkeys a high-fat, high sugar diet for two years with or without RESV (80 mg/day the first year, and 480 mg/day the second) and assessed inflammation, size, and insulin sensitivity of subcutaneous and visceral adipose tissue. The authors used microarray and subsequent gene-set enrichment analysis to demonstrate a protective effect of RESV against the increased gene expression related to inflammatory pathways caused by the high-fat, high sugar feeding. Specific to the visceral white adipose tissue, they discovered a reduction in adipocyte size, and increases in SIRT1, insulin receptor substrate 1, and GLUT4 glucose transporter protein expressions. The positive effects of RESV in the visceral adipose tissue extend the beneficial effects of RESV demonstrated in diet-stressed rodents to primates, and provide further evidence of a mechanism that addresses a primary cause of the MetS. Unfortunately, this study did not perform the comparison of systemic insulin sensitivity and glucose tolerance between the two experimental animal groups after two years of feeding the respective diets. A separate study did answer this question in a longitudinal study of grey mouse lemurs, albeit the animals were not under stress of a high-fat, high sugar diet. Control animals were fed a standard diet, a second group was calorically restricted (30% less kcals than control), and the third group was fed the standard diet supplemented with 400 mg RESV/kg bodyweight/day. Compared to the control animals, both the CR and RESV groups had a lower glucose AUC after three years of treatment,

but not two. Similarly, the RESV fed animals' fasting insulin and HOMA-IR scores were not different from control lemurs after two years, but were significantly lower after three.

Human studies of insulin sensitivity and glucose tolerance in response to RESV administration are mixed; for an informative, current review on the topic refer to (De Ligt *et al.*, 2015). Three studies have performed the clamp method to assess changes in insulin sensitivity with RESV treatment with doses ranging from 75 mg to 1.5 g per day for up to twelve weeks in overweight/obese men or non-obese postmenopausal women. All three studies found no effect of RESV on insulin sensitivity or glucose infusion rates (Chachay *et al.*, 2014; Poulsen *et al.*, 2013; Yoshino *et al.*, 2012). In fact, only the Timmers *et al.* (2011) study found a significant effect of 150 mg/day RESV treatment on fasting insulin in normoglycemic, overweight subjects after 30 days. Conversely, beneficial effects of RESV are observed in subjects presenting advanced complications in glucose homeostasis: in three different clinical studies of overweight, type-2 diabetics, dose of RESV ranged from 10 mg to 1 g per day and trial duration varied from four weeks to three months (all parallel-arm) (Bhatt *et al.*, 2012; Brasnyo *et al.*, 2011; Movahed *et al.*, 2013). Significant outcomes include improvements in HOMA-IR, fasting glucose and insulin, and hemoglobin-A1c by RESV treatment. An informative meta-analysis conducted by Liu *et al.* effectively illustrates this dichotomy in glucose homeostasis response to RESV treatment between obese-healthy vs type-2 diabetics: the overall weighted mean differences of two experimental studies on subjects with type-2 diabetes demonstrates reduced fasting glucose and insulin, hemoglobin-A1c, and HOMA-IR with RESV treatment, whereas the overall weighted mean differences of five studies on overweight or obese, nondiabetic subjects does not exhibit these same improvements (Liu *et al.*, 2015).

20.3.6 RESV and dyslipidemia

Elevated TG and LDL are major modifiable risk factors in the pathogenesis of atherosclerosis and resultant cardiac and cerebrovascular diseases, the number 1 and 5 leading causes of death, respectively. Zordoky *et al.* (2015) provides an exhaustive summary of RESV's effects on the components of CVD. They provide a useful report of RESV action in a variety of rodent models of CVD (see Table 2 in Zordoky *et al.*, 2015). Studies range from two to twenty weeks in duration and RESV dose ranges from 1 to 500 mg/kg bodyweight either directly by oral gavage, IP injection, or incorporated into diet. CVD induction included high-fat/cholesterol/fructose diets and/or genetic models (apo-e deficient mice, New Zealand rabbits). Overall, the studies show a lipid and cholesterol lowering effect of RESV supporting its ability to impede 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (Cho *et al.*, 2008; Do *et al.*, 2008; Penumathsa *et al.*, 2007). Interestingly, when lipids are not affected by treatment, anti-atherosclerotic and anti-inflammatory effects are still observed which is indicative of RESV's antioxidant properties, as one study found a reduction in oxidized LDL despite no overall changes in total lipids (Rocha *et al.*, 2009). These translational findings identify at least two or three different mechanisms by which RESV could have a positive effect on atherosclerosis and that improved outcome may exist independent of effects on dyslipidemia. Further, mechanistic work describes effects of RESV on vascular smooth muscle cell migration as well as anti-platelet activity (Gocmen *et al.*, 2011; Lin *et al.*, 2014).

Effects of RESV on dyslipidemia in human clinical trials are less consistent than the aforementioned animal experiments. Like the other outcomes described in this communication, variability exists in subject population, dosing, duration, and design of the clinical trials making it difficult to confirm or refute RESV as an alleviator of metabolic dysregulation at the human level. However, improvements have been observed. In overweight or obese men with hypertriglyceridemia fed 1 to 2 g RESV for two weeks there was a significant 27% reduction in apoB-100, an indication of the reduced output of very low-density lipoprotein by the liver (Dash *et al.*, 2013). In a study by Magyar *et al.* (2012) 10 mg per day RESV for three months lowered LDL cholesterol by 14% in 20 patients with coronary artery disease. In type-2 diabetics, 1 g per day RESV for 45 days significantly increased HDL by 11% while TGs were marginally reduced (Movahed *et al.*, 2013). Alternatively, other human studies found no significant difference in circulating lipids in subjects fed RESV (Poulsen *et al.*, 2013; Yoshino *et al.*, 2012). In these studies, subjects tended to be healthier relative to the subjects in which a positive effect was noted. This is an indication of the principle described by Smoliga *et al.* (2013) where beneficial effects of RESV described in mechanistic and animal studies are most likely to be translated to a population of subjects that have the potential to benefit from the intervention; to correctly determine the action of RESV on the clinical signs of a given morbidity, the patients being studied should present said morbidity.

20.3.7 RESV and hypertension

The association between RESV and cardiovascular health was first described indirectly via the association between red wine consumption and a reduction in risk of mortality, and was thought to partially explain the 'French paradox': the consumption of a high-fat diet, but without an observable increase in mortality due to coronary complications (Kopp, 1998; Siemann and Creasy, 1992; Wang *et al.*, 2005). Subsequent research over the past 20 years from molecular pathways to clinical studies has sought to confirm and elucidate this relationship. The proposed cardio-protective mechanisms of RESV include modulation of hypertension, ischemia, angiogenesis, and atherosclerosis.

In a variety of rodent models of hypertension including surgical, chemical, and diet-induced hypertension, a range of RESV (1-800 mg/kg bodyweight) was effective in reducing systolic blood pressure when administered for two to ten weeks (see Table 1 in Zordoky *et al.*, 2015). According to Hamza *et al.,* the inter-organ mechanisms by which RESV elicits its benefit on blood pressure includes central and peripheral vascular remodeling as well as improved renal function (Figure 1 of Hamza and Dyck, 2014).

Several meta-analyses have assessed the effect of RESV on blood pressure (Hausenblas *et al.*, 2015; Liu *et al.*, 2015; Sahebkar *et al.*, 2015). Despite the same, limited collection of human clinical studies available for analysis, each author had a unique subset of studies in which to calculate an overall effect size, which is due to the main objective of each analysis: Liu *et al.* focused solely on blood pressure, Hausenblas *et al.* (2015) assessed RESV's effects specifically in type-2 diabetics, and Sahebkar *et al.* (2015) addressed inflammation and a broader examination of its cardiovascular effects. In two of the three analyses, the authors reported a weighted mean

difference that favored the effect of RESV in reducing systolic blood pressure while diastolic blood pressure appeared unaffected (Hausenblas *et al.*, 2015; Liu *et al.*, 2015). Liu *et al.* (2015) also performed sub-analyses where studies were grouped by dose, duration of treatment, and BMI of study subjects. Greater effects of RESV on systolic blood pressure were observed in studies using doses of at least 150 mg per day, of duration no greater than three months, and in overweight or obese subjects. Two of these meta-analyses (Liu *et al.*, 2015; Sahebkar *et al.*, 2015) included a study that used a modified, microencapsulated form of RESV, 'longevinex', where the authors did not report circulating levels of RESV, therefore this study may be confounding the subsequent meta-analyses as less is known about this form of RESV administration (Fujitaka *et al.*, 2011).

20.4 Conclusions

Substantial evidence exists to suggest that RESV can ameliorate several risk factors for chronic diseases facing adults, including cardiovascular disease, stroke, and diabetes. Moreover, RESV, embedded in empirical research that details a potential for correcting metabolic disturbances, could prove an effective treatment for individuals with MetS. Based on its effects of each component of the MetS detailed above, research into the efficacy of RESV on these subjects is warranted. However, there is disagreement in RESV's ability to fend off metabolic stress in the human condition. Work from Timmers *et al.* (2011) found positive effects on energy metabolism in obese men when fed 150 mg per day for four weeks. Their elegant work bridged the gap between what has been thoroughly examined in animal models and what every nutritionist who studies the polyphenol wants to know: will this compound have similar effects in humans? Poulsen *et al.* (2013) in an attempt to recapitulate the work by Timmers *et al.* (2011) found no significant effect of RESV despite a 10-fold increase in the daily dose. Discrepancies exist between the two research designs that may account for an observable effect by the Timmers group, namely the advantage of a cross-over design instead of a parallel-arm. An additional issue with both of these studies is a failure to control the subject's food intake, which may also partially account for differences in study outcomes. Nonetheless, the number of studies demonstrating positive outcomes after RESV administration warrants further research on this potentially healthful dietary component and supplement.

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Protein and energy in heart health

21. Effect of dairy products consumption on heart health and cardio-metabolic risk factors

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Abstract

Milk and dairy products have been consumed for centuries around the world and are recommended for their health benefits. However, there are studies which associated dairy products with cardio metabolic diseases because of their fat content. Previous studies showed a positive effect of dairy consumption on lipid profiles, which may be owing to its ingredients, including minerals, proteins and fats. Nevertheless, some studies found an association between high fat dairies and cardiovascular diseases. The effect of dairy products on cardiovascular risk factors including lipid profiles, body weight and blood pressure depends on the fat content and the type of product (processed or fermented). There is evidence associating the consumption of dairy products with a favorable effect on blood pressure, weight control, and lipid profiles. Although it is not apparent which component of the dairy products could contribute to the positive effects and by which mechanism this occurs, consumption of these products, specially low fat dairies has been recommended to prevent cardio metabolic risk factors.

Keywords: milk, calcium, protein, cardiovascular diseases

Key facts

- High fat dairies, are rich in saturated, mono-unsaturated and poly-unsaturated fats, medium-chain fatty acids, and conjugated linoleic acid.
- Dairy products are good sources of minerals including calcium, potassium and magnesium.
- Dairies are good sources of high quality proteins such as casein and whey that have positive effect on protein intake, satiety and energy expenditure.
- Dairy products especially high fat dairies are sources of energy which might influence the body weight.
- Other ingredients of dairy products such as probiotics may also affect heart health.

Summary points

- High fat dairies may increase serum cholesterol which is a risk factor for cardiovascular diseases.
- Low fat dairies may play a favorable role in controlling serum lipids due to their protein and minerals content.
- Some of the dairy fats such as alpha-linolenic acid and conjugated linoleic have favorable effect on blood pressure.
- Dairy calcium is associated with weight and fat loss through increasing in the excretion of fecal fat.
- Dairy minerals can affect blood pressure regulation.
- Dairy proteins have a favorable role in reducing blood pressure and controlling serum lipids.
- Dairy consumption may affect the energy intake and weight control.

Abbreviations

ALA	Alpha-linolenic acid
CLA	Conjugated linoleic acid
HDL-C	High-density lipoprotein cholesterol
LDL-C	Low-density lipoprotein cholesterol
MUFA	Monounsaturated fatty acid
PUFA	Polyunsaturated fatty acid
SFA	Saturated fatty acid
TG	Triglyceride
$1,25(OH)_2D3$	1,25-dihydroxyvitamin D3

21.1 Introduction

In recent decades due to globalization and modern lifestyle, the prevalence of chronic diseases has been increasing and has become a significant public health problem. Among dietary factors, dairy products, which have been consumed for centuries around the world, have been attracting researchers' attention. Dairy products, as an important source of nutrients, have been recommended for several health benefits. Dairy foods contain several nutrients including protein, carbohydrate, fat, riboflavin, calcium, magnesium, and selenium, all of which have important roles to provide for the body's requirements. On the other hand, the consumption of low-fat dairy products has been linked to a better cardio-metabolic situation, including increased HDL-C and decreased LDL-C (Seidel *et al.*, 2005), lower body weight (El Khoury *et al.*, 2015), lower blood pressure (Ralston *et al.*, 2012), and decreased risk of stroke (Larsson *et al.*, 2012). Furthermore, dietary guidelines advise that the consumption of low fat dairies is a good source of calcium and high quality protein to aid the prevention of cardiovascular diseases (Eckel *et al.*, 2014). These valuable effects evident from the consumption of dairy products are explained by their components, which include calcium, potassium, magnesium, and protein (McGregor and Poppitt, 2013; Xiao *et al.*, 2013). However, there are discrepancies between studies, possibly as a result of differences in study design, populations, and methodology. It is unclear which component of the dairy products, and by which mechanisms, may contribute to the protective effects. The purpose of this chapter is to review the latest studies and elucidate the association between the consumption of dairy products and cardio-metabolic risk factors, heart health, and the possible mechanisms.

21.2 Lipid profiles

Dyslipidemia has been considered as an important component of cardio-metabolic risk factors. Researchers have focused on the association of diet with lipid metabolism and, in this context, it is proposed that dairy products may play a role in lipid profile regulation (Ohlsson, 2010).

The effect of dairy products on lipid profiles depends on the fat content and the type of product (processed or fermented). Dairy products, especially high fat dairy, are rich in saturated fat and therefore, it has been speculated that consumption of dairy products is associated with unfavorable effects on serum lipoprotein profile including raised total and LDL cholesterol (Nestel *et al.*, 2005; Sun *et al.*, 2015). Thus, it has been suggested to consume low fat dairy products to prevent the cardio-metabolic risk. On the other hand, some evidence has shown that dairy products, especially low fat milk and fermented dairy products, may have cholesterol lowering effects in humans (Agerholm-Larsen *et al.*, 2000) and affect dyslipidemia (Sadeghi *et al.*, 2014). It has been suggested that dairy consumption may decrease the cholesterol concentration through increasing the excretion of bile acids (Ditscheid *et al.*, 2005). A low level of triglycerides was also reported as a result of higher dairy intake (Wang *et al.*, 2008) in addition, substituting whole milk with low fat milk can significantly decrease lipid profiles, including total and LDL cholesterol (Villalpando *et al.*, 2015). In contrast, findings from other studies could not reach the same level of significance (Abreu *et al.*, 2014; Snijder *et al.*, 2008) or even showed a positive relation between serum cholesterol and milk consumption (Biong *et al.*, 2006).

21.2.1 Dairy minerals and lipid profiles

Previous reports indicated that a higher calcium intake can reduce plasma total cholesterol and LDL-C levels (Lorenzen *et al.*, 2007; Reid *et al.*, 2002). Dietary calcium intake inhibit the absorption of fatty acids and cholesterol and increase fecal fat and consequently increase energy excretion (Jacobsen *et al.*, 2005). This increase in fat excretion might be due to the creation of insoluble soaps from calcium and dietary fatty acid or cholesterol, by the binding to bile acids, which weakens the micelles formation or by inhibition of reabsorption of bile salts (Govers *et al.*, 1994). There is also evidence regarding the inverse associations between calcium, or dairy intake, and body weight and/or body composition (Loos *et al.*, 2004; Zemel *et al.*, 2000), nevertheless, other studies failed to find this association (Lorenzen *et al.*, 2006; Rajpathak *et al.*, 2006). Besides, it has been revealed that calcium from dairy sources was more effective on body weight and/or composition than calcium supplements (Lorenzen *et al.*, 2007; Zemel *et al.*, 2000, 2004).

In spite of this, recent research showed that serum calcium might have a positive association with triglycerides and total cholesterol (Gallo *et al.*, 2016; He *et al.*, 2014). It has been hypothesized that in cases of estrogen deficiency, a calcium supplement might relate to an increase in serum cholesterol by reducing hepatic catabolism of cholesterol and stimulating lipid synthesis (Li *et al.*, 2013).

The level of serum calcium can be increased much more with calcium supplementation than dairy calcium,which is probably related to slow absorption of dairy products (Kärkkäinen *et al.*, 2001). Moreover, other ingredients of dairy products such as proteins, may also affect the association between dairy calcium and lipid profile and may occur through calcium absorption or excretion (Hunt *et al.*, 2009).

Furthermore, dairy products are a good source of other minerals, including potassium and magnesium. There is evidence that indicates low potassium intake and low serum potassium levels could affect the extracellular homeostasis and result in many metabolic disorders, such as central obesity and high level of TG, waist circumference and low level of HDL-C (Sun *et al.*, 2014). Dietary magnesium has a significant inverse association with total cholesterol, LDL-C and triglycerides but a positive association with HDL-C (Bain *et al.*, 2015; Kim and Choi, 2013; Singh *et al.*, 1991). Magnesium may increase peroxidation of lipoproteins and so, low magnesium may increase the inflammation which is related to undesirable changes in lipid profile (Bain *et al.*, 2015)

21.2.2 Dairy proteins and lipid profiles

Lipid lowering properties of dairy proteins, which consist of casein (about 80%) and whey (about 20%), have also been reported in previous studies (Graf *et al.*, 2011). These have been demonstrated that dairy proteins can reduce the postprandial increase in triglycerides (Mariotti *et al.*, 2015). Casein, due to its low solubility in acidic gastric juice, produces a separated segment with lipids that affect the digestion and absorption of dietary lipids, which finally leads to decreasing the postprandial increase in triglycerides (Mariotti *et al.*, 2015). Casein is a slowly digested protein and thus it affects the further metabolism of related nutrients.

The results of a recent meta-analysis revealed that the supplementation of whey protein can decrease the level of serum triglyceride; however it failed to show any effect on total cholesterol, LDL-C or HDL-C (Zhang *et al.*, 2016). The triglyceride lowering effect of whey might be attributed to the functions of its components. For example, beta-lactoglobulin may decrease absorption of lipid in the intestine by capturing hydrophobic molecules; moreover, other whey components, such as sphingolipids may reduce lipid absorption (Ohlsson *et al.*, 2010). Furthermore, it has been proposed that milk proteins, such as leucine, isoleucine, valine, casein and whey, downregulate the expression of cholesterol metabolism and lipogenesis genes (Chen *et al.*, 2009). Whey and isoleucine also downregulated the expression of genes which are related to cholesterol absorption and fatty acid transportation (Chen *et al.*, 2009). Dairy proteins may also decrease the postprandial lipid because of the insulinotropic effect of dairy proteins (Nilsson *et al.*, 2007). Altogether, dairy proteins play a favorable role in controlling serum lipids.

21.2.3 Dairy fat and lipid profiles

High fat milk and butter have long been associated with hypercholesterolemia because of the high content of long-chain SFA (Iggman *et al.*, 2011); however, milk fat also consists of MUFA and PUFA.

The main SFA in dairy products are palmitic acid (C16:0), myristic acid (C14:0) and lauric acid (C12:0), all associated with TG formation and increased total cholesterol (Fernandez and West, 2005; Ohlsson, 2010). Thus it is recommended to replace SFA from dairy products and other sources with PUFA to decrease total cholesterol (Mozaffarian *et al.*, 2010). On the other hand,

the presence of MUFA, such as oleic acid (C18:1), has a favorable effect on lipid profile. Dietary MUFA compared to SFA increases the size of chylomicrons and decreases cholesterol and TG levels (Perez-Martinez *et al.*, 2011). Dairy fat is also a source of trans fatty acids, however, it has been found that moderate ruminant trans fatty acids have little effect on plasma lipids and cholesterol levels (Brouwer *et al.*, 2010; Motard-Bélanger *et al.*, 2008). Medium-chain fatty acids are other forms of dairy lipids which are reported to be associated with increased lipid oxidation, thermogenesis and energy expenditure, decreased body weight, total cholesterol, LDL, and an increase of LDL particle size (Liu *et al.*, 2009). However, the effect of specific fatty acids from dairy products on lipid profile is still undetermined in regard to their concentration and the interaction with other dairy ingredients.

The results of studies on whole milk are less consistent than those on butter, suggesting additional ingredients in milk which may affect cholesterol levels. For instance, butter increases the serum cholesterol more than cheese even with similar milk fat content (Hjerpsted *et al.*, 2011). Besides milk fat globule membrane, which is a tri-layered membrane with bioactive phospholipids and proteins (Dewettinck *et al.*, 2008), has a favorable effect on lipid profiles (Spitsberg, 2005). Moreover, it has long been reported that dairy products are the major natural food sources of CLA, which is an important factor for human health and has been associated with improving dyslipidemia (Jacome-Sosa *et al.*, 2010; Reynolds and Roche, 2010). Altogether, previous studies showed that butter can increase blood cholesterol but the effects of other dairy products on lipid profiles need further studies.

21.3 Obesity

Obesity influences a number of cardiovascular risk factors, including glucose intolerance, diabetes, dyslipidaemia and hypertension (Van Gaal *et al.*, 2006). Prevalence of obesity has been increasing around the world and thus obesity has become a major health threat (organization., Updated January 2015). Therefore, identifying dietary risk factors for obesity is an urgent need. Intake of dairy has been proposed as one of the potential factors because of its dense energy and its cholesterol and SFA contents (Rolls, 2009). Moreover, dairy consumption is generally higher in most developed countries where the prevalence of obesity is higher (Mann, 2004).

Alternatively, some studies reported an inverse association between dairy consumption and obesity; however, it is suggested that this association may have a causation bias since obesity might affect dairy consumption behaviors (Dougkas *et al.*, 2011).

A meta-analysis on the effect of dairy consumption on body weight declared that dairy intake, without energy restriction, might not cause a significant reduction in body weight (Abargouei *et al.*, 2012). As a result, conventional dietary recommendations suggest low fat dairy products.

On the other hand, according to the beneficial effect of dairy product consumption on appetite and energy intake, controlling energy intake may change the results. Only a limited number

of interventional studies without energy restriction showed weight gain as a result of dairy consumption (Dougkas *et al.*, 2011). It has also been reported that the presence of dairy products in weight loss diets might cause a greater decrease in body weight, total fat mass and waist circumference and an increase in lean body mass (Abargouei *et al.*, 2012).

21.3.1 Dairy calcium and obesity

The inverse association between Ca intake and the prevalence of obesity (Bueno *et al.*, 2008; Dicker *et al.*, 2008), body weight (Davies *et al.*, 2000), fat accumulation (Zemel *et al.*, 2000) and central adiposity (Azadbakht and Esmaillzadeh, 2008) has been investigated previously. Previous studies revealed a significantly greater weight loss in participants on a milk-based diet than those on the usual hypocaloric diet with equal energy intake. It has been proposed that intracellular Ca increases energy storage, through the synthase of fatty acids and inhibition of lipolysis (Zemel, 2002). Intracellular Ca levels in adipocytes is controlled by hormones regulating Ca levels including, the parathyroid hormone and $1,25(OH)_2D3$. Inadequate intake of dietary Ca increases parathyroid and $1,25(OH)_2D3$ levels and consequently increases intracellular Ca, which increases lipogenesis and decrease lipolysis, and finally increases lipid storage (Zemel, 2003).

Additionally, in animal models, high dietary calcium increased body temperature, metabolic index and decreased the body fat storage. Conversely, low dietary calcium cause decreased thermogenesis and raised adipose tissue mass (Zemel *et al.*, 2000). Comparing calcium fortification with dairy source of calcium showed a greater weight and fat loss and increase in skeletal muscle peroxisome proliferator as a result of dairy sources of calcium (Sun and Zemel, 2004); however, the role of additional factors in dairy products was not identified.

Moreover, another anti-obesity effect of dietary calcium might be through increasing the excretion of fatty acid as fecal fat and consequently, increasing energy loss. A randomized crossover study showed a significant increase in the excretion of fecal fat, as a result of a large increases in dietary calcium (Bendsen *et al.*, 2008).

21.3.2 Dairy proteins and obesity

The important effect of a high-protein diet on body weight is through gastrointestinal hormones by increasing diet-induced thermogenesis (Halton and Hu, 2004) and satiety (Astrup, 2005) as well as decreasing hunger (Johnstone *et al.*, 2008). These effects are different in different proteins with different sources and content of amino acids (Gilbert *et al.*, 2011). Proteins have unique characteristics related to their source, the content of amino acids, and absorption kinetics. It is therefore speculated that proteins from different sources have diverse metabolic effects and some evidence exists that different protein sources vary in their satiating capacity (Acheson *et al.*, 2011).

Milk proteins, including casein and whey, are high quality proteins with all essential amino acids, however, there are differences in their digestion and absorption, as digestion and absorption of

whey are very fast but casein delays gastric emptying and amino acid absorption (Hall *et al.*, 2003). Therefore, some studies suggested that whey and casein have different effects on body weight. It has been reported that whey was more satiating in the short term but casein is more satiating in longer time (Hall *et al.*, 2003).

Furthermore, reports showed that diet-induced thermogenesis for protein is greater than for carbohydrates and fats (Halton and Hu, 2004). Diet-induced thermogenesis is the increased energy expenditure after food intake, which is the energy involved for digestion, absorption, and metabolism of nutrients (Halton and Hu, 2004). Therefore, milk, as a good source of protein, has a significant role in Diet-induced thermogenesis and subsequently weight control (Lorenzen *et al.*, 2012). In addition to the above mentioned effects of proteins, different proteins may also influence postprandial fat oxidation and dairy proteins have been reported to be more effective than other sources (Acheson *et al.*, 2011). Overall, dairy proteins have advantageous effects on energy intake and body weight.

21.3.3 Dairy fat and obesity

Dietary advice for the prevention of overweight and obesity has disapproved of high fat dairies, because of its dense energy and the high content of fat, especially SFA and cholesterol. However, dairy fat also contains other fatty acids with possible health benefits including, CLA, phytanic acid, and ALA. Most of the studies examining the relationship between dairy fat and obesity reported that consuming more dairy fat was associated with lower body weight, lower weight gain (Mozaffarian *et al.*, 2010; Te Velde *et al.*, 2011), and lower risk of central obesity (Holmberg and Thelin, 2013). However, it is difficult to separate the effect of dairy fat from other components in dairy products. On the other hand, two meta-analyses failed to show a significant effect of dairy intake on weight or body composition without energy restriction (Abargouei *et al.*, 2012; Chen *et al.*, 2012).

It has been suggested that CLA, which is produced by bacteria in the gut of ruminants and found in their milk and meat products, may reduce body fat and increase lean body mass (Whigham *et al.*, 2007). It has been hypothesized that a high CLA diet could increase thermogenesis in adipose tissues and increase energy expenditure (West *et al.*, 2000). Moreover, it has been reported that CLA causes a decrease in fat deposition through the reduced adipocyte size and decreased lipogenesis (Shen *et al.*, 2013). Although, most of the studies that showed the effect of CLA on body weight exert somewhat high doses, it is not clear that the lower amount of CLA in dairy products has a similar effect. Furthermore, medium-chain fatty acids in dairy products may have the ability to increase satiety and reduce food intake (Van Wymelbeke *et al.*, 1998). However, the effect of confounder factors is significant, for example, the possibility that overweight or obese individuals choose low fat dairy products because it is commonly perceived that they are less obesogenic (Dougkas *et al.*, 2011).

21.4 Blood pressure

Hypertension is a major risk factor for CVDs and related morbidity/mortality which is highly prevalent throughout the world (Campbell *et al.*, 2014). Lifestyle factors including diet and physical activity are effective on blood pressure levels; therefore, recognizing dietary factors that are associated with blood pressure is necessary for the prevention and control of hypertension.

It has been confirmed that some dietary patterns, such as Dietary Approaches to Stop Hypertension, which is rich in vegetables, fruit and reduced fat dairies as well as reduced total and saturated fats, could be beneficial in the prevention and control of hypertension (Sacks *et al.*, 2001). Dairy products contain high value protein and amino acids, minerals and other nutrients which may be individually, or together, involved in reducing the risk of hypertension. However, only consumption of low-fat dairy was associated with lower risk of hypertension and not consumption of whole-fat dairy (Alonso *et al.*, 2005).

21.4.1 Dairy minerals and blood pressure

Lifestyle and dietary recommendations for prevention and treatment of hypertension include enhanced physical activity, weight control, decreased sodium and sufficient calcium intake and adequate consumption of a fruits, vegetables and low fat dairies (Chobanian *et al.*, 2003). Dairy products, compared to other animal foods, have a lower amount of sodium (Demott *et al.*, 1984) and are a better choice for individuals with hypertension. There is evidence that an independent association exists between higher consumption of dairy products and lower blood pressure and it has been suggested that the association between the intake of dairy product and blood pressure may be related to their high levels of calcium (Ruidavets *et al.*, 2006). Others indicated that calcium plays a key role in blood pressure regulation and dietary calcium is inversely associated with blood pressure (Da Silva Ferreira *et al.*, 2013; Ruidavets *et al.*, 2006). It has been hypothesized that the association between dietary calcium and blood pressure is possibly through intracellular calcium levels, calcitrophic hormones and endothelial function (Van Mierlo *et al.*, 2006).

It has been revealed that increasing dietary calcium intake leads to increasing urinary sodium excretion and inhibits increase in blood pressure. On the other hand, low calcium intake increases intracellular Ca concentration and increases $1,25(OH)_2D3$, which lead to renin suppression and finally an increase in blood pressure (Li *et al.*, 2002). Moreover, low calcium intake increases parathyroid hormone levels which increase intracellular Ca and also intracellular sodium via the inhibition of sodium potassium pump activity. This subsequently increases blood pressure (Kawashima, 1990).

Additionally, dairy products are good sources of other minerals and there is a strong inverse association between blood pressure and these minerals, including potassium, phosphorous, and magnesium. It has been reported that low concentrations of potassium reduce sodium potassium pump activity, resulting in increased intracellular sodium. A potassium rich diet increases serum potassium levels and is associated with endothelium-dependent vasodilation by means

of stimulation of the sodium potassium pump and may improve hypertension (He *et al.*, 2010). The inverse association of dietary phosphorous with blood pressure has been shown previously (Elliott *et al.*, 2008). A prospective study reported an inverse association of phosphorus from dairy products, but not from other sources with baseline blood pressure and incident hypertension (Alonso *et al.*, 2010). Higher phosphorus intake might reduce $1,25(OH)_2D3$ levels which reduce blood pressure (Li *et al.*, 2002). Magnesium intake is also associated with blood pressure (Kass *et al.*, 2012). Magnesium may modify peripheral vascular resistance via regulation of responses to vasoactive agents, such as, angiotensin II and endothelin and could improve endothelial dysfunction (Kris-Etherton *et al.*, 2009). Moreover, magnesium has other properties, including antioxidants, anti-inflammatories and modulation of cell growth which could positively affect blood pressure regulation (Laurant and Touyz, 2000).

21.4.2 Dairy proteins and blood pressure

The effect of dietary protein on blood pressure is not fully understood. Some studies speculated that high protein intake, because of its unfavorable effects on renal function, could increase blood pressure (Sacks *et al.*, 1981). On the other hand, an inverse association between dietary protein consumption and blood pressure is reported by some studies (Liu *et al.*, 2002).

Previous studies indicated that dietary protein intake could increase renal absorption ability and therefore, might have a blood pressure lowering effect (Yamori *et al.*, 1979). Moreover, it was suggested that a high protein diet may increase renal plasma flow, glomerular filtration rate and sodium excretion (Cirillo *et al.*, 2015; Obarzanek *et al.*, 1996). However, the effect of proteins on blood pressure might be related to their sources and amino acids content, for example L-arginine syntheses nitric oxide, and cysteine affects metabolism of nitric oxide, which are related to blood pressure modification (Förstermann and Sessa, 2012). Another antihypertensive effect of protein and dairy peptides is through the inhibition of the angiotensin-converting enzyme (FitzGerald and Meisel, 2000) which plays a role in the renin angiotensin system and regulates blood pressure (Izzo and Weir, 2011). *in vitro* studies proposed that whey, and not casein, in dairy peptide had an anti-hypertensive property (Kawase *et al.*, 2000). However, another study revealed that either whey or casein are effective in reducing blood pressure in the long-term (Pal and Ellis, 2010), but not the short-term (Pal and Ellis, 2011). Altogether, it seems that dairy proteins are a good source of protein and may have a modulating effect on blood pressure.

21.4.3 Dairy fat and blood pressure

Although some reports showed an inverse association between low fat dairy and blood pressure (Soedamah-Muthu *et al.*, 2011), studies on the associations between consumption of dairy products and blood pressure could not find any association between the intake of high-fat dairy products and blood pressure or risk of hypertension (Wang *et al.*, 2008). Previous studies have shown that the intake of SFA is positively associated with blood pressure (Livingstone *et al.*, 2013); however, some studies did not find any association (Rasmussen *et al.*, 2006). Overall it has been concluded that blood pressure is marginally affected by SFA (Hall, 2009). On the other

hand, it has been suggested that ALA may have a blood pressure lowering effect, but, the effect has not been proven by previous studies (Wendland *et al.*, 2006).

It was hypothesized that CLA has favorable effect on blood pressure because of its effect on decreasing the adipocyte production of angiotensinogen (Zhao *et al.*, 2009), nitric oxide production and

endothelial function (DeClercq *et al.*, 2012). Although several studies reported a blood pressure lowering property of CLA supplementation (Herrera *et al.*, 2006; Zhao *et al.*, 2009), others could not find such an association (Engberink *et al.*, 2012; Sluijs *et al.*, 2010). A recent meta-analysis failed to find a significant favorable effect of CLA supplementation on blood pressure (Yang *et al.*, 2015).

21.5 Dairy products and metabolic syndrome

Metabolic syndrome consists of a cluster of cardio metabolic risk factors which is associated with increased risks of CVD, type 2 diabetes mellitus, specific cancers and all-cause mortality (Bastien *et al.*, 2014; Colangelo *et al.*, 2015; González-Santos *et al.*, 2014; Khosravi-Boroujeni *et al.*, 2015; Li *et al.*, 2014). There is a growing body of scientific evidence which linked the consumption of dairy products with reduced risk of metabolic syndrome (Babio *et al.*, 2015; Chen *et al.*, 2015; Crichton *et al.*, 2011). The mechanism underlying the association appears to be through the effects of different dairy products consumption on the components of metabolic syndrome, including hypertension, dyslipidemia and obesity (Crichton *et al.*, 2011) which has been discussed earlier. Moreover, diabetes mellitus, which is another component of metabolic syndrome, is also linked with dairy intake (Pasin and Comerford, 2015). In this association, calcium is suggested as a key element in regulating insulin mediated intra cellular processes (Gomes *et al.*, 2015; Pittas *et al.*, 2007). Insulin secretion is also dependent on calcium and changes in Ca^{2+} can unfavorably affect the beta cells secretion of insulin (Pittas *et al.*, 2007). Moreover, reduction in body weight and adiposity, as a result of consumption of dairy products may improve insulin sensitivity and influence diabetes (Eriksson-Hogling *et al.*, 2015). Therefore, dairy consumption via the influence on all of the components of metabolic syndrome, can influence metabolic syndrome.

21.6 Other ingredients

In addition to the mentioned ingredients of dairy products, the effect of other components is undeniable. Dairy products may also have vitamin D and some probiotics which affect the cardiovascular risk factors.

There is plenty of evidence related to the association between vitamin D deficiency and metabolic risk factors, including diabetes, hypertension, carotid atherosclerosis, stroke, congestive heart failure, myocardial infarction, micro albuminuria and decreased kidney function (Chiu *et al.*,

2004; Chonchol and Scragg, 2006; Cigolini *et al.*, 2006; De Boer *et al.*, 2007; Kendrick *et al.*, 2009; Krause *et al.*, 1998; Martins *et al.*, 2007; Scragg *et al.*, 2004, 2007; Targher *et al.*, 2006; Wang *et al.*, 2008). Moreover, vitamin D deficiency was associated with lipid profiles abnormality (Chaudhuri *et al.*, 2013; Ponda *et al.*, 2012), hypertension (Vacek *et al.*, 2012), increased body fat (Kremer *et al.*, 2009) and the prevalence of obesity (Mai *et al.*, 2012). Moreover, some dairy products, including yoghurt and cheese, are considered as a good source of probiotics, and their health benefits has been studied in recent years. It has been revealed that probiotics have beneficial effects on blood pressure (Khalesi *et al.*, 2014), lipid profiles (Guo *et al.*, 2011), and body weight (Omar *et al.*, 2013).

21.7 Conclusions

Most studies which examined the effect of dairy products on heart health and cardiovascular risk factors reported a beneficiary effect of these products on body weight, blood pressure and some lipoprotein levels. Although there is some discrepancy in the studies, especially regarding the fat content of dairy products, there are limited studies which reported harmful effect of dairy products on cardiovascular risk factors. Therefore, consuming low fat dairies is encouraged to prevent cardio metabolic risk factors.

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22. The French paradox revisited: cardioprotection via hormesis, red wine and resveratrol

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Abstract

Coronary heart disease (CHD), is the leading cause of death in the US and globally. With the anticipated doubling of the worldwide population of individuals age 60 and above to an estimated 2 billion by year 2040, CHD will likely witness a similarly rapid increase as the most common cause of death in Western societies and in countries currently undergoing rapid economic development. A main cause of CHD is atherosclerosis (AS), a hardening and narrowing of the arteries. It is initiated by endothelial damage which results in invasion and accumulation of macrophages capable of forming foam cells; moreover, its progression involves the interaction of the dysfunctional endothelium with cells in its immediate microenvironment and the proliferation of intimal-smooth muscle cells thus forming a plaque. Although AS is progressive; it's also preventable. Epidemiological studies suggest that the consumption of red wine (RW) is beneficial to heart health. RW can possibly reduce the frequency and/or severity of events contributing to AS which was first revealed in the early 1990s as the 'French paradox'. The cardioprotective effects of RW are frequently attributed to the grape derived polyphenol resveratrol it contains. How resveratrol mediates its cardioprotective effects remains incomplete. This chapter will provide an overview of *in vitro* and *in vivo* evidences on cardioprotection by resveratrol pertinent to the biogenesis of AS. In addition, recent advances and data pertaining to resveratrol's cardioprotective effects will be summarized, specifically: (1) control of reactive oxygen species by Keap1/Nrf2 mediated detoxification enzyme genes; (2) role of the AKT/mTOR cascade in control of atherogenesis; and (3) a hormetic response – the contribution of low/high dose resveratrol. It will also provide an overall scheme furthering the understanding of cardioprotection by RW and resveratrol via maintenance of cardiac homeostasis and control of atherogenesis.

Keywords: heart disease, atherosclerosis, phytochemical, prevention

Key facts

- 1992: the potential link between red wine (RW) and cardiovascular health publicized as the 'French paradox'.
- Resveratrol identified as likely key phytochemical providing cardioprotection.
- Development of atherosclerotic lesions via damage to blood vessel layers inhibited by resveratrol.
- Experimental evidence identifies specific molecular targets affected by resveratrol.
- Mechanistic studies determine dose level critical for cardiopreventive outcome.

Summary points

- Control of reactive oxygen species by Keap1/Nrf2 mediated detoxification enzyme genes.
- Role of the AKT/mTOR cascade in control of atherogenesis.
- Responses to low/high dose resveratrol.
- The understanding of cardioprotection by RW and resveratrol via maintenance of cardiac homeostasis and control of atherogenesis.

Abbreviations

AKT	Protein kinase
ARE	Antioxidant response element
AS	Atherosclerosis
BPAEC	Bovine pulmonary artery endothelial cells
CHD	Coronary heart disease
DRW	Dealcoholized red wine
ECs	Endothelial cells
eNOS	Endothelial nitric oxide synthase
GβL	G protein β-subunit like protein
HAEC	Human aortic endothelial cells
HPAEC	Human pulmonary aortic endothelial cells
Keap1	Kelch-like ECH-associated protein 1, a protein that complexes with Nrf2
LDL	Low-density lipoprotein
mTOR	Mechanistic target of rapamycin
NO	Nitric oxide
NQO1	NAD(P)H:quinone oxidoreductase type 1
NQO2	Quinone oxidoreductase type 2
Nrf2	A member of a small family of basic leucine zipper transcription factors, that binds to an AP1-NF-E2 tandem repeat in the DNA
PAR	Platelet aggregation rate
ROS	Reactive oxygen species
RTP	Resveratrol target protein
RW	Red wine
SMC	Smooth muscle cell
VSMC	Vascular smooth muscle cells

22.1 Introduction

Decades of epidemiological data show that the consumption of RW is beneficial to human health (Bertelli and Das, 2009; Biagi and Bertelli, 2015; Wu and Hsieh, 2011). Evidence supports that RW has the possibility of reducing clinical events in AS; the cardioprotective effects of RW having been widely attributed to its key ingredient, resveratrol (Li *et al.*, 2012; Raj *et al.*, 2015; Zordoky *et al.*, 2015). Attention in this intensely studied grape-derived phytochemical primarily stems from two key observations. In 1992, the potential link between RW and cardiovascular health was revealed by the 'French paradox' which reports that populations residing in France had a relatively low incidence of heart disease despite the preference for a saturated fat rich diet (Criqui and Ringel, 1994; Renaud and De Lorgeril, 1992). In 1997, Pezzuto and coworkers reported that resveratrol displays chemopreventive activity in breast and skin carcinomas (Jang *et al.*, 1997). Subsequently, a wealth of *in vitro* and animal model experiments, and limited results in human studies, have provided the impetus for further testing the targets and mechanisms of resveratrol

on chronic diseases (Erdogan and Vang, 2016; Hsieh and Wu, 2010; Park and Pezzuto, 2015; Shukla and Singh, 2011; Tome-Carneiro *et al.*, 2013; Vang *et al.*, 2011; Varoni *et al.*, 2016).

22.2 Atherosclerosis and coronary heart disease

AS is a major cause of CHD and clinically manifests as a fundamental pathological dysfunction affecting the circulation. The biogenesis of AS involves recurrent episodes of injury, inflammation, and repair, each contributing to the formation of cumulative lesions if left untreated. AS lesions initiate with EC injury resulting in leucocyte recruitment and lipid deposition at the site of damage. These early events are then followed by later formation of thrombi, ultimately resulting in total occlusion of the vessel.

The cellular and molecular mechanisms of AS are rooted in the conceptual framework of the response-to-injury hypothesis first introduced by Ross in a 1976 *New England Journal of Medicine* article whose seminal feature was the recognition of the highly focal nature of the distribution of vascular diseases (Aird, 2006, 2007, 2012; Ross, 1999; Ross and Glomset, 1976a,b; Ross *et al.*, 1977). The central idea advanced by Ross was that the initiation and progression of atherosclerotic lesions involve the endothelium. An intact endothelium functions not only as a physical blood barrier, but also as a dynamic biochemical platform that actively and constantly monitors for changes in physiological demands. Functional competence of the endothelium is achieved through constant surveillance, maintenance, and adaptation of hemostasis of the vasculature, and plays an integral role in AS and CHD. Thus, some inciting injurious events of the endothelium in space and time lead to alterations in endothelial permeability and expose the subintimal and medial layers to the blood elements, which culminate in interaction of EC and SMC with monocyte-macrophages, a sequence of dynamic cascading events contributing to the development of lesions.

The focus on the endothelium in AS should not only reflect its role as a simple physical barrier lining the vasculature but must also incorporate the evidence showing that EC have attributes similar to an endocrine organ, as a significant source of bioactive molecules (Cines *et al.*, 1998). Thus, EC plays a pivotal role in normal vascular activity as well as in the pathophysiology of several vascular/thrombotic disorders including AS. Functions attributed to the endothelium include: (1) provision of an antithrombotic and nonadherent surface for platelets and leukocytes; (2) secretion of platelet antagonists such as prostacyclin PGI2 and NO; (3) maintenance of vessel tone through elaboration of vasorelaxants and contractors; (4) synthesis and secretion of factors that regulate cell migration and proliferation, and angiogenesis; (5) control of the movement of nutrients and lipoprotein particles into the intima; and (6) balance between fibrin formation and breakdown.

Because of its strategic location in the vasculature, the endothelium is in constant contact with potentially damaging and/or protective biological and chemical agents and physical events, and hence is prone to injury and damages. Damage to the endothelium is considered the key CHD-

initiation event. Damages to the endothelium elicit multiple changes in the vessel architecture, blood components, and properties of hemodynamic flow, lead to increased leukocyte and platelet recruitment and attachment, promote migration of SMC to the intima, and facilitate assembly and deposition of extracellular matrix.

As an initiation event in AS, injury to the endothelium can result from repeated, sustained exposure to a multitude of CHD risk factors, e.g. cigarette smoking, hypercholesterolemia, inflammation, oxidative stress, infection, and administration of various therapeutic regimens, which, in addition to effecting disproportional synthesis and production of some or all of the bioactive molecules mentioned previously, may lead to aberrant structure and function changes in the endothelium. If such endothelial changes persist over a prolonged time period, cooperative interaction between damaged ECs and juxtaposed cell types, and the molecules they release may occur in the immediate microenvironment of the injured endothelium, resulting in atherogenesis and thrombogenesis. Over time, and with recurrent episodes of thrombus formation, AS lesions expand in physical dimension eventually leading to clinically evident obstruction of the vessel. These sequential and cascading cellular events suggest that the injured endothelium may be viewed as a microcosm of system biology marked by dynamic interaction between cells and bioactive molecules. Thus, studies of AS may offer insights and details of cell:cell interactive communication, as well as mechanisms of prevention of endothelium injury by diet-derived agents.

22.3 Red wine, resveratrol and prevention of AS and CHD

A 1979 report analyzed data from 18 developed countries and the results showed an inverse correlation of ischemic heart-disease mortality and wine consumption (St Leger *et al.*, 1979). This observation was subsequently expanded to include additional studies and the collective outcome of the analysis became known as the 'French paradox' (Renaud and De Lorgeril, 1992). This phenomenon refers to people residing in certain parts of France, where RW is customarily consumed during meals, having a low CHD mortality despite living a lifestyle considered to have comparably high CHD risks, as opposed to the outcome observed in the US and many other developed countries. Ecological studies have suggested that the effects of RW in lowering risk of mortality from heart disease may be greater than those attributed to consumption of beer or spirits due to its polyphenolic content (Chiva-Blanch *et al.*, 2013; Rimm *et al.*, 1996). It was hypothesized that regular consumption of RW, having an up to 10,000 μM total polyphenolic content (assuming an average molecular weight of 200 for polyphenols) (Brenna and Pagliarini, 2001; Pal *et al.*, 2005), was responsible for the co-existence of low incidence rate with prevalence of high risk factors for CHD in a general population (Deckert *et al.*, 2002; Falchi *et al.*, 2006). Recently the polyphenol resveratrol (3,5,4'-trihydroxy-trans-stilbene), known to be abundantly present in RW, compared to white wine or beer, has been demonstrated to elicit a broad spectrum of positive biological responses in *in vitro* and in animal studies, including effects that are compatible with the cardioprotective roles proposed for RW (Penumathsa and Maulik, 2009; Raj *et al.*, 2014; Wu

et al., 2011, 2013). Studies from our own laboratory and others, using the RW main ingredient resveratrol to elucidate the mechanism of cardioprotection attributed to RW, are described.

22.4 Previous studies on cardioprotection by resveratrol

AS is hardening and narrowing of the arteries by the formation of plaques on the inside walls. Plaques consist of LDL, platelets, macrophages, SMC and other substances. Under oxidative stress, LDL-platelet interactions induce macrophage foam cell formation which is the hallmark of early AS. Whether treatment with resveratrol could be anti-atherogenic and reduce plaque formation via inhibition of LDL oxidation and/or platelet aggregation was examined.

22.4.1 Resveratrol inhibits LDL oxidation

LDL oxidation is a key event in atherogenesis. The antioxidant activity of resveratrol prompted us to test whether it affected LDL oxidation. LDL was isolated from adult males with normolipidemic profiles. The isolated LDL was modified by oxidation using Cu^{++}, with and without addition of resveratrol. Based on monitoring of LDL oxidation, and uptake into macrophages, a high dose of resveratrol (≥50 μM) significantly inhibited LDL oxidation (Zou *et al.*, 1999b, 2000b). In addition, a recent study in which healthy subjects were fed a high fat McDonald's meal, LDL oxidation levels were found to be significantly reduced when the meal was consumed together with 250 ml of RW (Di Renzo *et al.*, 2015).

22.4.2 Resveratrol inhibits platelet aggregation

Platelets play an active role in hemostasis – a process by which injury in the endothelium can be rapidly repaired preventing compromise of the fluidity of the blood. In normal EC injury, platelets adhere to the subendothelial matrix of a damaged vessel, spread over the surface and recruit additional platelets to form a thrombus. Improper regulation or over-reactivity of this repair system can lead to pathological thrombosis. We assessed the effects of resveratrol on platelet aggregation. Platelets isolated from healthy subjects were induced to aggregate *in vitro* using collagen (5 μg/ml), thrombin (0.33 units/ml), and ADP (4 μM). Dose-dependent inhibition of platelet aggregation was observed following 10-1000 μM resveratrol treatments (Zou *et al.*, 2000b). In addition, we showed that resveratrol suppressed signaling pathways and aggregation in washed platelets, but had little effect on whole blood platelet aggregation (Kirk *et al.*, 2000). Remarkably, addition of 10 μM resveratrol was shown to decrease apoptosis in stored platelets and preserve the platelet normal hemostatic activity (assayed as aggregation and responsiveness to aggregation agonists) (Lannan *et al.*, 2016). This observation may have significant public health implications in that resveratrol may have the potential to prolong the half-life of stored platelets.

Next, using cultured EC and SMC cell models we studied the CHD-protective effects of resveratrol based on the 'response to injury' hypothesis pointing to damage in EC and proliferation of SMC as key players in the initiation and progression of AS. Whether treatment with resveratrol could

render EC less susceptible to injury, and facilitate timely and efficient repair of the damaged endothelium, was examined.

22.4.3 Modulation of EC signaling by resveratrol

The EC lining of the blood vessel could be damaged by exposure to excess ROS, which may be attenuated by production of NO. Biological functions attributed to NO include vasodilation, inhibition of platelet adhesion and aggregation, reduction of expression of adhesion molecules and chemokines, and suppression of cell proliferation and migration (Gauthier *et al.*, 1995; Radomski *et al.*, 1987; Zeiher *et al.*, 1995). eNOS is primarily responsible for the generation of NO; a functional eNOS is crucial for a healthy cardiovascular system. We tested whether resveratrol affects the level of expression of eNOS. We found that resveratrol induced eNOS expression in BPAEC as early as 6 h after exposure, and that the increase was maintained over a 4 day period (Bruder *et al.*, 2001). Thus, dietary resveratrol could induce a gradual, sustained increase in NO production, which could contribute significantly to its cardioprotective effects.

22.4.4 Modulation of EC response to shear stress by resveratrol

Shear stress is the force of the flowing blood on the endothelial surface of the blood vessel. Since arteries are largely dependent on the integrity of the EC, shear stress is a primary signal for arterial remodeling. Thus, we tested whether resveratrol modulates EC response to shear stress. Control and 100 μM resveratrol treated ECs were exposed to simulated arterial shear stress. Remaining attached cells post mechanical stress were fixed, stained with rhodamine-phalloidin and evaluated using confocal microscopy. In resveratrol treated ECs, a significantly constant number of cells remained attached following 2 and 5 min. arterial shear stress; by contrast, untreated cells became almost completely detached when shear stressed (Bruder *et al.*, 2001). These results suggest that resveratrol altered the structure/morphology of ECs in ways that render resistance to simulated arterial flow, making ECs less injury-prone to turbulent flow, additionally contributing to cardioprotection by resveratrol.

22.4.5 Resveratrol suppresses proliferation of SMC and BPAEC

Proliferation and migration of SMC into the intima of AS-susceptible blood vessels is a requisite for atherogenesis. We further investigated the effects of resveratrol on SMC proliferation and cell cycle control. Resveratrol dose-dependently reduced SMC proliferation; 50-100 μM resveratrol resulted in 70-90% reduction of the endothelin and platelet derived growth factor proliferation/mitogenic response. Antimitogenicity of resveratrol is due to G_1*S block in cell cycle traverse (Zou *et al.*, 1999a). Resveratrol also suppressed cultured BPAEC proliferation (Hsieh *et al.*, 1999). Taken together, these results suggest that resveratrol may decrease susceptibility to endothelial injury and the development of CHD (Bruder *et al.*, 2001; Hsieh *et al.*, 1999).

22.4.6 Resveratrol also exerts differential growth and cell cycle effects in HAEC and HPAEC

A remarkable feature of AS and vascular diseases is the focal nature of their distribution, suggesting that the structure and function of EC are affected by or even contingent on anatomical location, space and time (Aird, 2006, 2007, 2012). It is not surprising therefore, that a single pathological insult such as hypoxia might yield different and oftentimes opposite physiological changes depending on the vascular bed involved. For example, hypoxia induces systemic vasodilation yet imparts acute pulmonary vasoconstriction (Leach and Treacher, 1995; Lloyd, 1964). To test whether cardioprotection by resveratrol could be dependent on the anatomical origin of ECs, the responses of systemic and pulmonary artery ECs, respectively denoted HAEC and HPAEC, to resveratrol was examined with respect to suppression of proliferation, alteration in cell cycle phase distribution and induction of apoptosis. The proliferation of HAEC and HPAEC was inhibited by resveratrol in a dose-dependent manner with a more significant effect on HAEC. Analysis of the DNA content frequency histograms indicated that 10 μM resveratrol induced S phase accumulation in HAEC, doubling cell number from 10.3 to 19.6%; correspondingly, a decrease in G_1 cells from 78 to 67.8% was observed. In HPAEC, the S phase cells also accumulated in response to 10 μM resveratrol, increasing from 2% to 13% while G_2M phase cells decreased from 35.5 to 25.2%. Thus in HAEC, the effects of resveratrol largely occur at the G_1/S phase transition while in HPAEC most cells were arrested in the late portion of the S going into G_2M phase (Hsieh *et al.*, 2010).

22.4.7 Differential changes in global and specific gene expression in resveratrol-treated HAEC and HPAEC

It was of interest to determine whether treatment by resveratrol might differentially affect global and specific gene expression in the two anatomically distinct EC types. A cDNA array analysis was performed using total cellular RNA isolated from day 2 control and 100 μM resveratrol treated HAEC and HPAEC. Resveratrol up-regulated cyclin-dependent kinase inhibitor p57, Egr1 and hepatocyte nuclear factor 3 in HAEC but not HPAEC, while elevating intercellular adhesion molecule 1 expression in HPAEC cells only. Several genes, including *fibronectin* and *PIG3*, were suppressed by resveratrol in both cell types (Hsieh *et al.*, 2010).

22.5 Animal studies on cardioprotection by resveratrol from this laboratory

22.5.1 Resveratrol suppressed intimal lesions associated with endothelial denudation

We tested whether resveratrol modulates intimal hyperplasia resulting from endothelial denudation in hypercholesterolemic rabbits. In animals given resveratrol intragastrically at 4 mg/kg/day for a period of 5 weeks beginning 1 week before denudation, intimal proliferation index in the injured vascular wall (scored as the ratio of intimal to (intimal+medial) area) was reduced from 0.41±0.13 in control animals to 0.28±0.07 in resveratrol-fed animals (P<0.01). The

SMC number in the intima of resveratrol-fed animals was similarly suppressed, compared to control animals (1,100±500 vs 1,800±960, respectively, $P<0.05$) (Zou *et al.*, 2000a).

22.5.2 Studies of hypercholesterolemic rabbits fed RW, DRW, and resveratrol confirmed cardioprotection by resveratrol

Since phytochemicals and alcohol are both present in RW, we compared DRW and RW with comparable amounts of resveratrol, on formation of AS lesions and changes in plasma lipids in high cholesterol fed rabbits. Mean AS lesions in hypercholesterolemic rabbits were significantly reduced by RW, and to a similar degree, also by DRW and resveratrol. Thickness of the intima media layer in the thoracic aorta, which was markedly increased by cholesterol feeding, was reduced by RW, DRW, and resveratrol ($P<0.0001$ by ANOVA). These results suggest that RW polyphenols suffice to confer cardioprotection in an alcohol-free background (Wang *et al.*, 2005; Zou *et al.*, 2003).

22.5.6 Resveratrol inhibits platelet aggregation in hypercholesterolemic rabbits

The effects of resveratrol on platelet aggregation were also tested using hypercholesterolemic rabbits. Animals were fed a high cholesterol diet with or without concurrent gastric feeding of resveratrol (4 mg/kg/day), for 12 weeks. Platelets isolated from animals were used to determine the average PAR. PAR was significantly elevated in animals fed the high-cholesterol diet (61.0±7.0%, compared to a normal level of 39.5±5.9%, n=8, $P<0.001$). Diet-induced increase in PAR was reduced to control levels by resveratrol (35.7±6.3%, n=8, $P<0.001$). Thus, *in vivo* experiments provide evidence that resveratrol inhibits platelet aggregation (Wang *et al.*, 2002).

Collectively, *in vitro* and animal studies on cardioactive properties of resveratrol suggest that timely suppression and control of proliferation, respectively, of SMC and EC and other cells infiltrating and converging on the AS microenvironments constitute an important aspect in prevention of AS by resveratrol. In the next section, we will summarize recent advances in mechanistic studies focusing on: (1) control of ROS by Keap1/Nrf2; (2) role of the AKT/mTOR cascade in control of AS; and (3) a hormetic response of resveratrol (Figure 22.1).

22.6 NQO1 as a significant anti-ROS enzyme

Cardioprotection by resveratrol may involve suppressed production of ROS, prevention of EC injury, endothelial dysfunction and AS lesions. Recent studies have demonstrated that cardiac NQO1 protects against menadione toxicity in guinea pig atria (Floreani *et al.*, 2000) and also against oxidative and electrophile injury resulting from exposure to xanthine oxidase/xanthine, 4-hydroxy-2-nonenal or doxorubicin (Cao and Li, 2004). Immunohistochemical studies have shown that NQO1 protein is expressed in many tissues that require a high level of antioxidant protection (Siegel and Ross, 2000) including vascular endothelium.

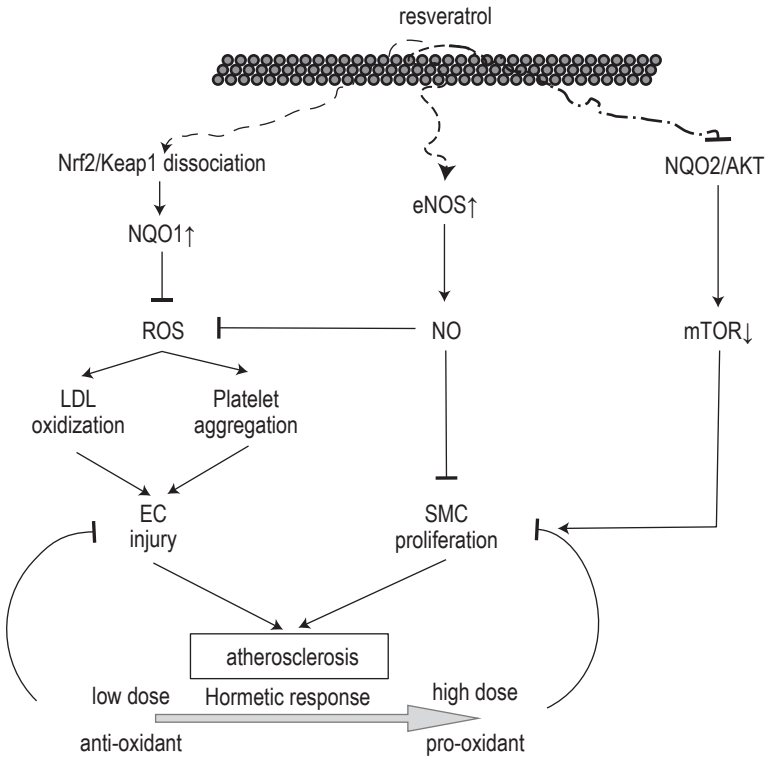

Figure 22.1. Overview of proposed cardioprotective mechanisms of action for resveratrol: (1) summarizing the progression of events resulting in atherosclerosis – endothelial cell (EC) injury, smooth muscle cell (SMC) proliferation, low-density lipoprotein (LDL) oxidation and platelet aggregation; (2) illustrating the interactions of resveratrol (arrows) resulting in control of reactive oxygen species (ROS) – Nrf2/Keap1 and stimulation of endothelial nitric oxide (eNOS); (3) quinone oxidoreductase type (2NQO2) role as interacting partner in control of the protein kinase/mammalian target of rapamycin (AKT/mTOR) cascade; and (4) moving from an anti-oxidant to pro-oxidant state as dose level increases – a hormetic response.

NQO1 is a key phase II detoxification enzyme (Prochaska and Fernandes, 1993) that protects cells against chemical carcinogens by converting NADH to NAD^+ and catalyzes the two-electron conversion of plant/vegetable byproduct quinones to hydroquinones. NQO1 thus circumvents the one-electron redox cycling of quinones, suppressing the generation of potentially harmful ROS, as well as preserving cellular glutathione concentrations. These effects of NQO1 may be viewed as secondary defenses against the damages imposed by free radicals, superoxides, and hydrogen peroxides, and as complements to the primary antioxidative defenses mentioned above. It is possible that NQO1 may actually participate in safeguarding the genetic integrity of EC and is therefore a reasonable cellular target for investigations of the mechanism of cardioprotection by resveratrol. Studies have shown that, in cultured primary human coronary arterial ECs, resveratrol dose-dependently upregulates the expression of NQO1 (Ungvari *et al.*, 2010). Induction of NQO1

by resveratrol was similarly observed in cultured human aortic SMCs (Wang *et al.*, 2006). How resveratrol induces NQO1 expression is discussed further in the following section.

22.7 Regulation of NQO1 mediated by Keap1/Nrf2

The upstream regulatory region of phase II detoxification enzyme genes including NQO1 contains the *cis*-acting ARE (Baird and Dinkova-Kostova, 2011; Hsieh *et al.*, 2006; Jaiswal, 2000). Phase II enzyme genes are induced or repressed when the transcription factor Nrf2 binds to ARE as a heterodimer with one of the small Maf proteins (Dhakshinamoorthy and Jaiswal, 2000; Hayes *et al.*, 2010; Itoh *et al.*, 1997; Katsuoka and Yamamoto, 2016; Nguyen *et al.*, 2000).

Nuclear localization of Nrf2 is an important regulatory step in the induction of phase II enzymes including NQO1. Nrf2 is normally bound to the cytoskeleton-associated Keap1 and must be translocated to the nucleus for interaction with ARE-promoter sites of antioxidant enzymes. Under basal conditions, Nrf2 in the cytoplasm is bound to Keap1. This suggests that Nrf2 is important for protecting cells and multiple tissues by coordinately up-regulating ARE-related detoxification, antioxidant genes, and molecules required for the defense system in each specific environment (Dhakshinamoorthy and Jaiswal, 2000; Hayes *et al.*, 2010; Katsuoka and Yamamoto, 2016). Thus, Nrf2:Keap1 function is to collectively and coordinately control the transcription of phase II enzymes. As mentioned, Nrf2 is normally transcriptionally inactive since it is kept in the cytosol bound to Keap1. When cells are exposed to oxidative or xenobiotic stress, Nrf2 dissociates and traverses to the nucleus. Resveratrol increases NQO1 gene transcription by affecting the expression/cellular localization/stability of the transcription factor Nrf2 (Hsieh *et al.*, 2006; Kawai *et al.*, 2011). We further postulate that resveratrol affects the cysteine-rich intervening region of Keap1, disrupting the Nrf2-Keap1 complex, and facilitating translocation of Nrf2 into the nucleus where it can heterodimerize with other transcription factors on the ARE region of phase II genes, leading to activation of transcription of NQO1 (Hsieh *et al.*, 2006).

22.8 Role of AKT/mTOR in cardiac homeostasis and control of atherogenesis

AKT is a protein kinase and exists in three forms: AKT1, AKT2, and AKT3. It acts as the major signal transducer and plays a central role in cellular processes involving inflammation, LDL cholesterol oxidation, platelet aggregation, SMC cell proliferation, and EC cell damage. AKT1 reduces stress-induced apoptosis and growth retardation, promoting physiological cardiac hypertrophy. *in vitro* studies suggest that AKT1 might play a dual role in AS: proatherogenic or antiatherogenic. AKT activation can exert pro-AS effects by enhancing macrophage survival in lesions thus promoting the formation of foam cells, enhancing expression of proinflammatory genes, particularly in an ApoE-deficient background. The persistent activation of AKT promotes cellular hypertrophy and hyperplasia, thereby promoting atherogenesis. On the other hand, AKT also exerts anti-AS activities. In mouse models using animals whose AKT1 is suppressed in VSMC in an atherogenic-stage specific manner (using the Cre/flox approach) to mimic the

initiation and progression phase of AS, results show that AKT1 knockdown in VSMC during the late stage of AS is accompanied by profound morphological changes marked by the appearance of larger AS plaques with bigger necrotic core areas and enhanced apoptosis. A reduction in fibrous cap size and collagen content were similarly observed. These results suggest that features of vulnerable plaques in humans representative of AS maturation, characterized by expanded necrotic core areas and constricted fibrous caps, may be blocked or retarded in its progression through approaches that enhance AKT1 expression specifically in VSMC (Rotllan *et al.*, 2015).

mTOR is a serine/threonine protein kinase with a central role in the regulation of cell growth and proliferation, as well as implicated in cardiovascular diseases, specifically in cardiac hypertrophy (Song *et al.*, 2010). mTOR exists in two functionally distinct multi-protein complexes called mTORC1 and mTORC2. mTORC1 is composed of mTOR, mLST8/GβL and regulatory associated protein of mTOR, which is sensitive to inhibition by rapamycin. Inhibition of mTORC1 signaling with rapamycin regresses established cardiac hypertrophy induced by pressure overload (McMullen *et al.*, 2004). mTORC1 helps maintain cellular homeostasis in EC via regulation of cell growth and proliferation by promoting anabolic processes (Laplante and Sabatini, 2009). It also limits catabolic processes from harming EC, such as autophagy (Laplante and Sabatini, 2009). mTORC2 is composed of mTOR, mLST8/GβL and rictor. Rictor prevents rapamycin from interacting with mTORC2 or inhibiting mTORC2 activity. mTORC2 regulates cell survival, metabolism, proliferation, and cytoskeletal organization by phosphorylating AKT at serine 473, thereby activating AKT. Thus, both complexes of mTOR help AKT promote cell survival through regulating transcription and translation. The role of resveratrol in cardioprotection via control of AKT-mediated phosphorylation and mTOR is discussed next.

22.9 AKT/mTOR and cardioprotection – effects of resveratrol

Study has shown resveratrol at low doses (0.1 and 1 μM) to protect cardiac myoblast cells from damage caused by oxidative stress via induction of autophagy, evidenced by enhanced formation of autophagosomes and its component LC3-II and by the suppression of autophagy using the autophagic inhibitors, wortmannin and 3-methyladenine. Importantly, induction of autophagy occurs secondary to the induced expression of rictor, a component of mTORC2, the sequential phosphorylation of serine 473 and the activation of its downstream survival kinase AKT (Gurusamy *et al.*, 2009). Similar results were also found using Sprague-Dawley rats; induction of autophagy was observed with low dose resveratrol alone (2.5 mg/kg) or combined with γ-tocotrienols (Lekli *et al.*, 2010). In addition to AKT and mTOR, multiple other resveratrol targets have been identified. In the following paragraph we focus on one, a RTP identified in our laboratory which may have impact on AKT/mTOR mediated cardioprotection.

22.10 AKT control by NQO2 – effects of resveratrol

Because resveratrol shows broad cell type dependent dose efficacy and has low bioavailability, we hypothesized that it acts by binding to specific cellular targets denoted RTPs; thus, qualitative and quantitative differences in RTPs could explain the diverse dose-, organ- and cell type-specific effects it displays (Buryanovskyy *et al.*, 2004). By using resveratrol-affinity chromatography, cloning, and X-ray crystallography we discovered NQO2 as a high affinity ($K_D \leq 50$ nM) binding protein (Buryanovskyy *et al.*, 2004). Our most recent studies provide evidence that resveratrol and NQO2 both participate in the control of AKT (Hsieh *et al.*, 2014). Binding to resveratrol induced conformational changes in AKT favoring a forced Pleckstrin homology domain-in or PH-out that may switch off/on conformation to inactivate or activate AKT, favors the thesis that resveratrol may enhance either pro- or anti-atherogenic activity via AKT by a dose-dependent mechanism.

22.11 Role of AKT/mTOR in cardiac homeostasis and control of atherogenesis – working hypothesis

Resveratrol interaction with AKT/PKB impinges on the mTOR kinase and its downstream signaling events in regulating key EC, SMC, LDL-oxidation and platelet aggregation, thus affecting functions that include cell proliferation, migration, survival, and other functions of the vasculature. In addition, the RTP NQO2 plays a significant role in cardioprotection as an interacting partner in the control of the AKT-mTOR cascade. In conclusion, our current working hypothesis is that resveratrol, by interacting at the plasma membrane, modulates membrane fluidity changes, in turn integrating NQO2/AKT control and thus be linked to the NQO2/AKT-mTOR cascade.

22.12 A Hormetic response – the contribution of low/high dose of resveratrol

A biphasic dose-response result showing positive biological effects at low to moderate dose and toxic or even lethal effects at high dose is known as hormesis. More than six centuries ago, more broadly, Paracelsus stated 'the right dose differentiates a poison from a remedy'. Plants produce a variety of bioactive phytochemicals, e.g. resveratrol, in response to environmental stresses which in turn have the ability to provide stress resistance to the animals consuming them. This is described as xenohormesis, a form of mutualism where the active compounds, although detrimental to plant pests, at the subtoxic dose levels consumed by humans, beneficial adaptive stress responses are activated leading to increased expression of genes mostly encoding cytoprotective proteins including antioxidant enzymes, phase-II detoxifying enzymes, protein chaperones, growth factors and mitochondrial proteins (Surh, 2011).

Well studied examples of hormesis include low to moderate consumption of alcohol and reduction in development of cardiovascular diseases, as opposed to the toxic and lethal effects

associated with excess consumption. This phenomenon, known as the 'French paradox', is based on the premise that in spite of a diet high in saturated fats, moderate RW consumption at meals appears to provide cardioprotection. The *in vitro* and animal studies from our laboratory, as discussed earlier, have found low levels of resveratrol to be most efficacious in affecting the targets while high dose affect the survival pathways. Overall, resveratrol mediated cardioprotection is determined via its hormetic response. Other research supporting the hormetic response of resveratrol is listed below.

In a study with Sprague-Dawley rats fed pure resveratrol for up to 30 days at doses of 2.5, 25 and up to 100 mg/kg, a cardioprotective effect was reported at lower doses as evidenced by improved aortic flow, left ventricular developed pressure and maximum first derivative of developed pressure, whereas a detrimental effect with deteriorated ventricular function was noted at >25 mg/kg (Juhasz *et al.*, 2010).

A recent study showed oxidative stress induced by resveratrol when acting as a pro-oxidant, thus demonstrating a hormetic shifting of cellular defense towards a more reductive state to improve resilience to oxidative stress in a manner that can be exactly defined by the redox environment of the cell. In this study increased cellular fitness was observed up to about 50 μM resveratrol in treated keratinocytes, whereas higher concentrations tended to produce toxic effects, leading to a typical bi-phasic, hormetic dose-viability curve. Similar bi-phasic dose-viability curves were observed in fibroblasts and liver but with varying susceptibility to oxidative products derived from resveratrol treatment. Increased expression of molecular markers for oxidative stress response, such as catalase, could be observed up to 100 μM resveratrol with a maximum at 50 μM resveratrol. However, too high concentrations of resveratrol (>100 μM) resulted in toxic effects. At the molecular level, the hormetic effects of oxidative products derived from resveratrol were shown to be driven by the activation of Nrf2 (Plauth *et al.*, 2016). In summary, such data are in line with a large body of reported hormetic cellular effects of resveratrol (Calabrese, 2004; Plauth *et al.*, 2016).

22.13 Conclusions

The rapidity of the population's aging has made it more urgent for the adoption and implementation of countermeasures to prevent atherogenesis and cardiac aging. As a multi-tasking molecule, resveratrol exhibits a variety of effects on different cell types known to play important roles in atherogenesis. The use of resveratrol could be beneficial to retard cardiac degeneration, and may have the potential to be developed as an adjunctive therapeutic tool to enhance the maintenance of cardiac homeostasis by fine-tuning its target proteins. Given the variety of functions and biological mechanisms regulated by resveratrol and considering the rapid progress in our knowledge of resveratrol, it is likely that this grape-derived polyphenol will affect cardiac and other aspects of aging broadly. It is therefore highly desirable to develop novel strategies for time-phase and cell-type specific delivery of resveratrol mimics, in efforts to resist/inhibit age-dependent decline in cardiac function, and with suppression of atherogenesis to broaden the

scope of physiological homeostasis. Additionally, recognition of the hormetic dose response has the potential to open up new opportunities for understanding basic biological processes to be applied in the development of new therapeutics for the improved treatment of patients.

Acknowledgments

SI and LP were participants in STAR (Summer Trainees in Academic Research), a summer program at New York Medical College aimed at enhancing the research experience of high school students.

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Microbes in heart health

23. The gut microbiota in heart health – do probiotics and prebiotics have a role?

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Abstract

In 2016, the WHO reported that cardiovascular disease (CVD) is the number one cause of death globally – both in developed and developing countries. This non-communicable disease accounts for 31% of deaths worldwide, and contributes significantly to the burden of healthcare costs. Given the social and economic consequences of CVD, researchers are actively searching for new therapies and approaches to manage key risk factors of the disease prior to and during the onset of CVD. One new and emerging area that shows promise is positively shifting the gut microbial balance to benefit key aspects of heart health, e.g. cholesterol metabolism, blood pressure, and inflammatory processes. Indeed, intestinal bacteria currently hold center stage for their role in maintaining digestive health. Now, emerging data suggest associations between the gut microbiota and several facets of CVD, including atherosclerotic plaque formation, myocardial infarction, heart failure, obesity, type 1 and type 2 diabetes, and non-alcoholic fatty liver disease. The use of sophisticated molecular techniques including high-throughput sequencing and metabolomics is providing deeper mechanistic insights to understand the relationship between intestinal microbial dysbiosis and CVD risk factors. As a result, these combined clinical and mechanistic studies are yielding new approaches to the management of CVD risk through the use of probiotics and synbiotics. This non-pharmacological therapeutic intervention holds important promise in light of emerging clinical evidence, lack of side-effects and adverse events as well as its cost-effectiveness.

Keywords: microflora, cardiovascular, synbiotics, cholesterol, inflammation

Key facts

- According to the Human Microbiome Project the number of bacteria in the human body is over 100 trillion representing at least 5,000 different species.
- The lower gut harbors the largest number of bacteria in the human body wherein it performs a multitude of functions (protective, structural, metabolic) beneficial to the host.
- Cardiovascular disease (CVD) is the number one cause of death globally and more people die annually from CVD than from any other cause.
- The metabolic crosstalk between the gut microbiota and host extends 'geographically' far beyond the gut and can contribute to heart health.
- Clinical studies are focusing on the use of probiotics alone or in combination with prebiotics as dietary interventions to manage key risk factors associated with CVD.

Summary points

- A number of non-modifiable (age, genetics) and modifiable (early life, diet, lifestyle, health status, medication, supplementation with pre/probiotics) factors influence gut microflora composition therefore contributing to overall health.
- Changes in gut microflora affect metabolic and immunologic processes and can predispose humans to diseases such as intestinal infections, inflammatory diseases, metabolic diseases, cancer and CVD.
- Emerging data suggests associations between the gut microbiota and several facets of CVD, including atherosclerosis, diabetes and obesity.
- Mechanistically, alterations in the gut microbiota may compromise gut barrier function, intestinal and systemic inflammation and thereby contribute to CVD.
- Research continues to explore the mechanisms by which the microbiota influences heart health, e.g. the role of short-chain fatty acids in mediating satiety and inflammatory processes.
- Clinical studies are focusing on the use of probiotics alone or in combination with prebiotics as dietary interventions to manage CVD risks.
- While certain specific probiotic strains and prebiotics have been associated with significant lowering of cholesterol, more studies are needed to support clinical recommendations.
- Manipulation of the gut microflora to minimize risk factors associated with CVD represents a promising therapeutic approach to support heart health and minimize its social and economic costs.

Abbreviations

BA	Bile acids
CVD	Cardiovascular disease
GLP	Glucagon-like peptide
HDL-C	High-density lipoprotein cholesterol
LDL-C	Low-density lipoprotein cholesterol
SCFA	Short-chain fatty acid
TAG	Triacylglycerol
TC	Total cholesterol

23.1 Introduction – why is the gut microbiome relevant to health?

The gut, skin, vagina, urinary tract and oral cavity are among several colonization sites in which microbial communities exist in a specific equilibrium to support proper biological function and health. The Human Microbiome Project has estimated the number of microbes in the human body at over 100 trillion representing at least 5,000 different species, and this number will continue to evolve as we learn more about this vast and multifaceted system (Gevers *et al.*, 2012; Turnbaugh *et al.*, 2007). Shaped by millennia of co-evolution, some host-bacterial associations have developed into beneficial relationships creating an environment for mutualism.

A key example of such an environment is the lower gastrointestinal tract which harbors the largest number of bacteria in the human body. These bacteria are involved in a multitude of functions encompassing the transformation of BA and the breakdown of insoluble fibers, as well as the production of specific vitamins and cofactors (Ettinger *et al.*, 2014; Tremaroli and Backhed, 2012) (Table 23.1).

Despite the huge inter-individual variability in gut microbial compositions, a core group of more than fifty taxa has been reported in nearly half of the human population. Furthermore, microbiota of most individuals can be clustered into three predominant 'enterotypes' characterized by three different genera: *Bacteroides*, *Prevotella*, *Ruminococcus*, independently of age, sex, nationality and body mass index (Power *et al.*, 2014). The main commensal organisms that populate the human gut are dominated by 4 main phyla: *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*. *Firmicutes* is the most populous bacterial phylum, capable of producing several SCFA and includes 250 genera, such as *Lactobacillus*, *Streptococcus*, *Mycoplasma*, and *Clostridium*. *Bacteroidetes* includes 20 genera, the most abundant of which is *Bacteroides* (Ettinger *et al.*, 2014; Singh *et al.*, 2016).

A number of factors influence the composition of the gut microflora including genetics, early events in life, anatomical structure and physiology of the digestive tract, age, diet, lifestyle (e.g. stress, exercise, smoking), health / disease status, use of medications, supplementation with pre- and probiotics, etc. (Graf *et al.*, 2015; Power *et al.*, 2014; Zhang *et al.*, 2015) (Figure 23.1).

Table 23.1. Functions of the gut microflora (Conlon *et al.*, 2015; O'Hara *et al.*, 2006).[1]

Protective functions	Structural functions	Metabolic functions
• pathogen displacement • nutrient competition • production of anti-bacterial factors, e.g. bacteriocins, lactic acid • induction of immunoglobulin A	• gut barrier fortification • apical strengthening of tight junctions • immune system development	• ferment non-digestible dietary residue and endogenous epithelial-derived mucus • production of SCFA (SCFA reach the circulation and impact immune function and inflammation the body) • production of vitamins (e.g. K, B12, biotin, folate, thiamine) • ion absorption (e.g. magnesium, calcium, iron) through action of bacterial phytases • salvage of energy • BA detoxification

[1] BA = bile acids; SCFA = short-chain fatty acids.

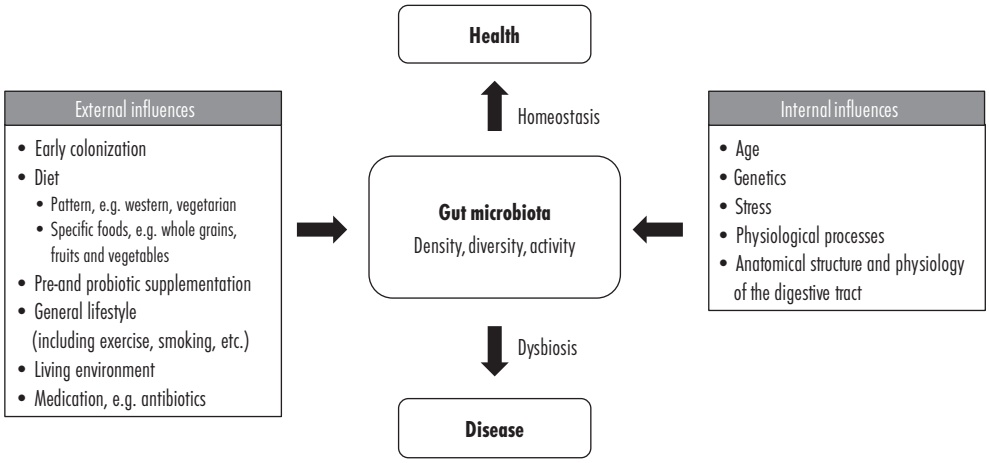

Figure 23.1. Factors influencing the composition of the human gut microflora (GI = gastrointestinal).

23.1.1 Age

Microbes colonize the human gut immediately after birth and proliferate to high numbers in the trillions, hence vastly outnumbering host cells. The gut microbiota starts to shape its future composition from birth to 2-3 years of age. Thereafter it's relatively stable until ~65 years of age. Some changes still occur in this period, for example *Proteobacteria* predominate in the gut of neonates, but are then substantially reduced from childhood (approx.16% in neonates) to adulthood (approx. 4.6%). Finally, the diversity and composition of the gut microbiota declines in the elderly (Aron-Wisnewski and Clement, 2016; Singh *et al.*, 2016).

23.1.2 Diet

Variation in diet composition is a major contributing factor in determining the relative abundance of different gut microorganism and their metabolic output. Diet-derived carbohydrates that are not fully digested in the upper gut (i.e. non-digestible carbohydrates), provide a major source of energy for gut bacteria in the human large intestine. Dietary intake of non-digestible carbohydrates influences microbial fermentation and total bacterial numbers in the colon. Recent evidence from molecular ecology has also shown that the amount and type of non-digestible carbohydrates (e.g. resistant starch, non-starch polysaccharides, and prebiotics) influences the species composition of the intestinal microbiota both in short-term dietary interventions and in response to habitual long-term dietary intake (Conlon and Bird, 2015; Graf *et al.*, 2015). Preliminary evidence suggests that dietary patterns are associated with distinct enterotypes (Conlon and Bird, 2015; Graf *et al.*, 2015). For example Western diets result in significantly different microbiota compositions than traditional diets. This was shown in studies comparing the diversity and phylogenetic composition of gut microbiota from individuals consuming a western-type diet (rich in fat and animal protein) and indigenous people from Africa and South America (living on a diet dominated by plant-based polysaccharides) which have demonstrated that the former is associated with an increase in the abundance of *Bacteroides*, whereas the latter is associated with increased *Prevotella spp.* and overall diversity (Hansen *et al.*, 2015).

While there is a strong body of knowledge on the effects of various dietary carbohydrates, the impacts of dietary fats and protein on the gut microbiota are just starting to be understood. It is important to underline that both short- and long-term dietary changes can influence the microbial profiles. Of particular importance is infant nutrition since it may have life-long consequences through microbial modulation of the immune system (Conlon and Bird, 2015; Graf *et al.*, 2015).

23.1.3 Medications

Numerous drugs have been reported to impact the gut microbiota. For example antibiotics reduce microbiota diversity and specifically impact selected genera. Depending on the antibiotic type and duration of treatment, the complete recovery of gut microbiota can take several weeks and up to several months (Aron-Wisnewski and Clement, 2016; Hansen *et al.*, 2015). In addition drugs used in type 2 diabetes mellitus (e.g. metformin), or proton pump inhibitors, also alter gut

microflora composition (Aron-Wisnewski and Clement, 2016). Finally, prebiotics and probiotics interventions may modulate microbial composition along with their potential to maintain a beneficial microbial balance to promote health as discussed later in this chapter.

The metabolic interaction between the gut microbiota and host extends far beyond the gut, since the gut microbiota perform various metabolic activities lacking in the host that influence host physiology, including stimulating the release of gut hormones (e.g. incretin and peptide YY) (Horwitt and Garrett, 2012). Some authors have described the gut microbiota as a 'multicellular organ within the organ' (O'Hara and Shanahan, 2006). Furthermore, the combined genomes of the gut microbiota (the so called microbiome) contain >100-fold more genes than are encoded in the human genome. These microbial genes play a role human physiology and metabolism (Karlsson *et al.*, 2013).

Due to all the above-mentioned properties of the gut microbiota, it makes sense for the host to control the own microbiota. In fact, it has been demonstrated that an imbalance of the intestinal microbiota can lead to local and/or systemic disease state. Changes in the composition and abundance of commensal bacteria affect the entire immune system and can predispose humans to a variety of diseases, including intestinal infections, inflammatory diseases (e.g. inflammatory bowel syndrome), metabolic diseases (e.g. obesity and diabetes), cancer, neurological diseases and finally CVD (Engen *et al.*, 2015; Round and Mazmanian, 2009). Alternatively, manipulation of the gut microflora to enhance its beneficial components represents an interesting therapeutic approach (O'Hara and Shanahan, 2006).

One emerging field of research is around the role of gut microbiota in heart health. Compelling evidence linked to the interactions between microbiota, obesity, and the metabolic syndrome axis indicate that the gut microflora plays a role on the initiation and progression of CVD and its risk factors (Singh *et al.*, 2016). The next sections further highlight the physiological relevance of the gut microflora specifically in the context of heart health and addresses the following key topics: (1) widespread prevalence of CVD, a disease well-established to be an inflammatory condition; (2) alterations in the gut microflora associated with key risk factors of CVD; (3) mechanisms by which the microflora influences these risk factors; and (4) the use of probiotics and synbiotics as non-pharmaceutical approaches to positively shift the microflora patterns in the gut and thereby reduce the risk to CVD.

23.2 The burden of cardiovascular disease

The nutrition-transition toward higher fat and refined carbohydrate diets occurring worldwide combined with increasingly sedentary lifestyles plays a central role in the current global epidemic of obesity and the associated non-communicable conditions such as diabetes type 2, hypertension, CVDs, stroke, some types of cancer, and osteoarthritis. Chronic diseases are costly to individuals, families, and public budgets, but many of them are preventable or can be postponed (WHO, 2003).

CVD is a leading cause of death worldwide and is rapidly increasing in both low- and middle-income countries. CVD is a set of disorders of the heart and blood vessels supplying the heart and includes coronary heart disease and cerebrovascular disease (WHO, 2016). In general, CVD encompasses certain conditions that result from the accumulation of atherosclerotic plaques in the coronary arteries, which restricts blood flow to the heart and brain. This restriction can lead to heart failure, angina pectoris, acute myocardial infarction, other ischemic heart diseases, and sudden death. Those individuals who have suffered a CVD event and survive face a life of ischemic heart failure, increased risk of stroke, and overall lower quality of life (WHO, 2015).

Recently updated WHO (2016) data indicate that CVDs are the number one cause of death globally. It has been reported that more people die annually from CVDs than from any other cause. An estimated 17.5 million people died from CVDs in 2012, representing 31% of all global deaths. Of these deaths, an estimated 7.4 million were due to coronary heart disease and 6.7 million were due to stroke. Over three quarters of CVD deaths take place in low- and middle-income countries. Out of the 16 million deaths under the age of 70 due to non-communicable diseases, 82% are in low and middle income countries and 37% are caused by CVDs.

Nearly 787,000 people in the USA died from heart disease, stroke and other CVDs in 2011, corresponding to one of every three deaths in America. About 2,150 Americans die each day from these diseases (one every 40 seconds). CVDs cause more deaths than all forms of cancer combined. About 85.6 million Americans have some form of CVD or the after-effects of stroke. Direct and indirect costs of CVDs and stroke total more than $320.1 billion and are projected to triple by 2030. That includes health expenditures and lost productivity (Mozaffarian *et al.*, 2015).

In Europe, according to hospital utilization statistics provided by the WHO (2015), over 38.0 million CVD-attributed hospital events occurred from 2011 to 2015 in the EU among adults aged 55 and older and it is expected that 24% of the population of 55 years and older will experience a CVD-attributed hospital event in the next 5 years. The total cost of addressing CVDs in the EU will be € 1,328 billion over the next 5 years, or € 34,637 per event over the same period (Frost and Sullivan, 2016).

People with CVD or who are at high cardiovascular risk (due to the presence of one or more risk factors such as hypertension, diabetes, hyperlipidemia or already established disease) need early detection and management using counselling and medicines, as appropriate (WHO, 2016).

CVD is mediated by both non-modifiable and modifiable risk factors and most CVDs can be prevented by addressing behavioral risk factors such as tobacco use, unhealthy diet and obesity, physical inactivity and harmful use of alcohol using population-wide strategies. Adopting healthy and therapeutic lifestyle habits is an important part of managing CVD risk and reducing costs associated with the disease (WHO, 2016).

23.3 Cardiovascular disease – an inflammatory disease

Inflammation, the immune response of body tissues to injury or infection, is an important component of innate immunity. The inflammatory process involves a complex biological cascade of molecular and cellular signals that alter physiological responses, ultimately resulting in the familiar clinical symptoms of pain, swelling, heat, and redness (Calder, 2006; Libby, 2007). At the site of the injury, cells release molecular signals that cause a number of changes in the affected area: vasodilation, increased blood flow, increased vascular permeability, secretion of fluids containing antibodies, and attack by several different types of leukocytes, including granulocytes, monocytes, and lymphocytes (Scott *et al.*, 2004).

Acute inflammation is a normal process that protects and heals the body following physical injury or infection. Once the infection or the causing factor are eliminated, or at least controlled, mechanisms come into play to terminate the inflammation process to dampen additional damage to the host and to start tissue repair. This active process is termed resolution of inflammation. Failure to 'resolve' inflammation may permit the normally acute inflammatory processes to become chronic. Chronic inflammation can result from a viral or microbial infection, environmental antigen (e.g. pollen), autoimmune reaction, or persistent activation of inflammatory molecules (Calder *et al.*, 2013; Scott *et al.*, 2004). Chronic inflammation is abnormal and is detrimental to the body; in fact, chronic inflammation has been reported to be involved in a number of disease states (Calder *et al.*, 2013). Chronic inflammation can also be of a 'low grade' with overt clinical manifestations being minimal or absent. Low-grade asymptomatic inflammation can occur in adipose tissue as a feature of obesity (Calder *et al.*, 2013) (Table 23.2).

Several human diseases are inflammatory in nature, including asthma, Crohn's disease, rheumatoid arthritis, polymyalgia rheumatica, tendonitis, bursitis, laryngitis, gingivitis, gastritis, otitis, celiac disease, diverticulitis, and inflammatory bowel disease. More recently, it has become clear that diseases such as atherosclerosis, type 2 diabetes and obesity also have an inflammatory component, albeit low-grade (Calder *et al.*, 2013).

When it comes to CVD, inflammation plays a role in the development and propagation being involved in processes encompassing arterial plaque formation, plaque rupture and thrombosis, reduced blood flow distal to atherothrombotic blockage, all of which can lead to myocardial infarction (Lowe, 2005; Roifman *et al.*, 2011). Specifically, the trigger is usually an inflammatory change involving cytokines and T-cells and through these inflammatory processes the initial lesion of atherosclerosis, the fatty streak, is formed. Furthermore, inflammation is central to the progression from fatty streak to complex plaque. As the plaque evolves, T cells activate macrophages to secrete an array of molecules, including cytokines and matrix metalloproteinases that make up the collagen that forms the fibrous cap, which ordinarily protects the plaque. As a result, the fibrous cap becomes thin and friable and can rupture, thus creating a thrombus that can lead e.g. to a myocardial infarction or other complications (Libby, 2006; Lowe, 2005; Roifman *et al.*, 2011).

Table 23.2. Characteristics of main inflammation types (Calder *et al.*, 2013).

	Acute inflammation	Chronic inflammation	Low-grade chronic inflammation
Cause	viruses, bacteria, allergens, injuries	failure to resolve acute inflammation, autoimmune reactions	metabolic disturbances, chronic infections
Involved immune cells	neutrophils and other granulocytes, monocytes, macrophages; T cells later	monocytes, macrophages, T cells, B cells, neutrophils, fibroblasts	monocytes, macrophages, T cells, B cells, neutrophils, adipocytes
Main mediators	eicosanoids, cytokines, chemokines	cytokines, chemokines, eicosanoids, growth factors, reactive oxygen species, hydrolytic enzymes	cytokines, chemokines, adipokines, eicosanoids, reactive oxygen species, hydrolytic enzymes
Timing	a few days	long-term	long-term
Outcomes	resolution, abscess formation, chronic inflammation if unresolved	tissue destruction, fibrosis, necrosis	no overt pathology, tissue (vascular) damage, increased insulin resistance, intracellular lipid accumulation

Inflammatory biomarkers have been consistently associated with the presence of CVD in multiple studies from different populations (Roifman *et al.*, 2011). Epidemiological and clinical studies have shown strong and consistent relationships between markers of inflammation and risk of future cardiovascular events. These markers include cell adhesion molecules, cytokines, pro-atherogenic enzymes and C-reactive protein (Blake and Ridker, 2002; Calder *et al.*, 2013; Pearson *et al.*, 2003).

Taken together these data indicate that inflammation plays a critical role in CVD, and the inflammatory cascade is particularly important in the atherosclerotic process. In this context it's important to mention that patients with significant systemic autoimmune diseases have a higher risk and prevalence of CVD. Observational research has linked CVD with autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, ankylosing spondylitis, psoriatic arthritis, polymyositis/dermatomyositis. Both CVD and autoimmune diseases involve immune system activation and endothelial dysfunction (Calder *et al.*, 2013; Roifman *et al.*, 2011).

Finally, recent studies have identified intestinal microbiota imbalance as a new factor that may contribute to both inflammation and CVD. Indeed, the microbial inhabitants of the gut may affect the body's metabolic processes and should be considered an environmental factor that contributes to obesity and its comorbidities such as insulin resistance, diabetes and CVD (Mafra

et al., 2014; Musso *et al.*, 2011; Tremaroli and Backhed, 2012). The role of intestinal microbial dysbiosis in modulating cardiovascular risk factors is discussed in the next sections.

23.4 Gastrointestinal microbial modulation of traditional cardiovascular risk factors

As discussed previously, CVD maybe mediated by different risk factors, including age, sex and genetics. These represent non-modifiable risk factors which directly influences an individual's likelihood of developing CVD. There are also modifiable risk factors that shape the likelihood of developing CVD. Many of these risk factors are environmental in nature and frequently linked to diet and lifestyle, e.g. smoking, chronic low-grade systemic inflammation (sometimes called metabolic endotoxemia), dyslipidemia, high blood pressure, diabetes and insulin resistance, metabolic syndrome, overweight/obesity. Recent studies in animal models and humans have identified the gut microbiota as another contributor to CVD risk. The gut bacteria both in terms of diversity at the genus and species level as well as their respective amounts have received and continue to receive significant attention given their role in maintaining digestive health and immune health. There is now preliminary evidence suggesting associations between the gut microbiota and several facets of CVD, including atherosclerotic plaque formation, myocardial infarction, heart failure, obesity, type 1 and type 2 diabetes, and non-alcoholic fatty liver disease (Aron-Wisnewsky and Clement, 2016; Rak and Rader, 2011; Tuohy *et al.*, 2014). The role of the gut microflora on modulating key risk factors, namely atherosclerosis, obesity and diabetes mellitus, are further discussed below and summarized in Figure 23.2.

23.4.1 Atherosclerosis

Distinct alterations in microbial patterns have been observed in individuals with atherosclerosis. For example, the bacterial species from the genera *Chryseomonas*, *Veillonella*, and *Streptococcus* have been identified in the gut, oral cavity as well as in atherosclerotic plaques. Moreover, in

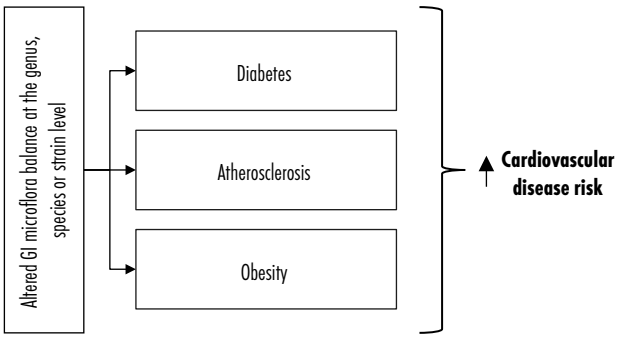

Figure 23.2. Characterization of key microflora changes, at the genus and species levels, in association with key risk factors of cardiovascular disease.

comparison to healthy control subjects, higher levels of *Collinsella* and lower levels of *Eubacterium* and *Roseburia* were measured in individuals who had experienced an atherosclerotic event. In addition to changes in the gut microbiota patterns, these subjects also exhibited an increase in proinflammatory peptidoglycan genes and a decrease in genes involved in the synthesis of anti-inflammatory molecules, e.g. butyrate. These alterations in the functional metagenome may be linked with the changes in the gut microbial pattern and could together influence the progression of atherosclerosis (Aron-Wisnewsky and Clement, 2016; Karlsson *et al.*, 2012; Tuohy *et al.*, 2014). The microbiome-metagenome relationship is currently being further explored to identify mechanisms of heart disease as well as implications for dietary and pharmacological interventions.

23.4.2 Obesity

As alluded to earlier, overweight or an obese phenotype represents another risk factor of CVD while weight-loss is proposed to reduce risk. Similar to individuals with atherosclerosis, alterations in the composition of the composition of the gut microbiota have also been observed in overweight and obese populations. As per data from human and mice studies, excess body weight is associated with changes in the composition of the gut microbiota, for example, an enrichment in *Firmicutes* and a corresponding decrease in *Bacteroidetes* levels. Interestingly, the *Bacteroidetes*-to-*Firmicutes* ratio shifted to that observed in observed in lean individuals after weight loss. An increased ratio of *Firmicutes* to *Bacteroidetes* has also been observed in mice genetically predisposed to obesity (ob/ob). This observation however has not been a consistent finding across studies (Aron-Wisnewsky and Clement, 2016; Karlsson *et al.*, 2012; Tuohy *et al.*, 2014).

In addition to possible alteration in the *Bacteriodetes*-to-*Firmicutes* ratio, Karlsson *et al.* (2012) reported that *Christensenellaceae* and associated bacteria are less abundant in individuals with an increased body mass index. Other studies have been focusing on the beneficial gut microbe, *Akkermansia muciniphila*. Herein, it was observed that individuals with high levels of *A. muciniphila* experienced a more favorable metabolic profile characterized by lower lipid levels, higher insulin levels and overall improved metabolic outcomes upon weight loss intervention compared to those with lower levels of *A. muciniphila* (Karlsson *et al.*, 2012). The mechanism by which *A. muciniphila* favorably influence metabolic outcomes including body weight may be related to its production of propionate, which affects intestinal L-cells and GLP-1 production via receptors expressed in the gut (Aron-Wisnewsky and Clement, 2016).

Prospective studies are obviously warranted in order to fully address whether changes in the gut microbiota contribute to obesity or whether obesity changes the gut microbiota. For example, a prospective Finnish study of 49 infants sampled at 6 and 12 months of age showed that children who were overweight at 7 years of age had higher levels of *Staphylococcus aureus* and lower levels of *Bifidobacteria* during infancy. Additionally, standardized study protocols should also be implemented to confirm cross-comparisons between studies as well as to support taxonomically

detailed descriptions of changes at the species and strain levels versus phylum level changes (Karlsson *et al.*, 2012).

23.4.3 Diabetes

Diabetes is another well-established risk factor for CVD and there is now accumulating evidence to support a link between altered gut microbiota and insulin resistance in humans. This is best demonstrated by a study which showed that subjects with the metabolic syndrome experienced higher insulin sensitivity and higher levels of butyrate-producing bacteria after undergoing transplantation with intestinal microbiota from lean healthy donors (Karlsson *et al.*, 2012; Olmstead, 2015).

At the species levels, the following bacteria have been associated with the incidence of diabetes: *Faecalibacterium prausnitzii*, *Roseburia* spp. and *Faecalibacterium* spp. It has been reported that individuals with metabolic syndrome or overt type 2 diabetes, had lower levels of *F. prausnitzii* compared to levels in healthy controls. Interestingly, *F. prausnitzii* levels are reduced in obese patients with type 2 diabetes, but increased after bariatric surgery – a procedure that promotes weight-loss and improves glycaemia and inflammation (Aron-Wisnewsky and Clement, 2016). Using molecular-based approaches, shotgun sequencing of the gut metagenome indicated that butyrate-producing bacteria, known to be anti-inflammatory (e.g. *Roseburia* spp. and *Faecalibacterium* spp.), are less abundant in type 2 diabetics versus healthy controls.

The microbiota from patients with type 2 diabetes is typically characterized by an increased level of bacteria involved in sulphate reduction, resistance against oxidative stress, and a decrease in butyrate-producing bacteria compared to those of normoglycemic individuals. These data are compiled from a study involving 345 individuals with normoglycemia or type 2 diabetes (Aron-Wisnewsky and Clement, 2016).

Through sophisticated molecular technology and software, a comparison of metagenomic data from Chinese and Swedish subjects, showed that type 2 diabetes associated metagenomes encode similar functions, but the species involved are markedly different. Interestingly, children who progress to develop type 1 diabetes have a markedly altered serum metabolome that could already be detected in the cord blood, and many of these metabolites are microbially regulated. Taking these data one step further, it is also plausible that exposure of the fetus to the mother's microbiome in-utero, could likely influence metabolic programing of the baby as it relates to insulin sensitivity and glucose metabolism. A study of four children with newly developed type 1 diabetes and four matched control children found differences in the composition of the gut metagenome between the groups and reduced diversity in type 1 diabetes-associated metagenomes. Studies in non-obese diabetic mice have shown that germ-free mice or those housed in specific-pathogen free conditions are more likely to develop diabetes, suggesting that the gut microbiota are involved in the development of autoimmune diabetes (Karlsson *et al.*, 2012).

Given the strength of the data demonstrating an association between intestinal dysbiosis in association with key risk factors of heart disease, there is interest in exploring the mechanisms by which the microflora mediates these risk factors. This is discussed in the next section.

23.5 Novel microbiota mechanisms of action for cardiovascular disease

In light of the accumulating evidence that the gut microbiota could contribute to CVD development, research is focusing on sophisticated molecular techniques and metabolomics analysis to support a deeper mechanistic understanding into the relationship between the gut microbiota and CVD.

Microorganisms within the human intestine ferment carbohydrate sources into the SCFA acetate, propionate and butyrate, and which finally make their way to the colon. These SCFA have been shown to influence satiety and food intake through the secretion of shown incretin or gut hormone production, e.g. GLP-2. GLP-2 may also be involved in maintaining gut barrier function, a defense mechanism, which can limit the absorption of inflammatory compounds such as lipopolysaccharide and which can trigger the low-grade chronic inflammation and subsequent insulin resistance associated with obesity and CVD. SCFA have been shown to modulate adipocyte hormone production, the obesity hormone, and to regulate inflammatory processes in adipose tissue, which oftentimes is at the 'heart' of CVD risk. Thus, SCFA produced by our gut bacteria may influence the way energy is stored or burnt through the processes of adiposity and thermogenesis (Singh *et al.*, 2016; Tuohy *et al.*, 2014).

However, less food intake has been observed in colonized mice vs their germ-free counterparts, so increased food intake cannot be blamed for the obese phenotype of colonized mice. In the same vein, it is plausible that individuals with a tendency for obesity may be more efficient in extracting energy from carbohydrate digestion and absorption. Indeed, an analysis of the metagenome of twins concordant for obesity showed that obese individuals harbor more genes for phosphotransferase systems involved in carbohydrate processing, suggesting an increased capacity to degrade polysaccharide-rich diets (Karlsson *et al.*, 2012).

Another potential mechanism as to how altered gut microbiota can promote obesity is through the AMP-activated protein kinase and expression of angiopoietin-like protein 4 (also known as fasting-induced adipose factor). Both these proteins are associated with reduced energy expenditure and can be suppressed by the gut microbiota. (Karlsson *et al.*, 2012).

With respect to mechanisms regulating cholesterol levels and atherosclerotic plaque formation, gut microbiota are involved in microbial deconjugation of BA and the enterohepatic circulation of BA. This is considered to a primary mechanism as to how gut microbiota regulate cholesterol levels. Usually, conjugated BA are secreted into the small intestine to aid micelle formation and fat absorption but ~5% of BA may pass to the distal ileum and colon. Herein, the gut microbiota

deconjugates them to reduce their absorbility and increases their excretion in the feces so that less BA are available for cholesterol synthesis (Singh *et al.*, 2016; Tuohy *et al.*, 2014).

Certain gut bacteria, most notably the *Bifidobacteria*, have been shown to produce folate, a key metabolite in carbon one metabolism which lowers circulating levels of homocysteine, an independent risk factor of CVD. Animal studies have shown that feeding folate-producing *Bifidobacteria* can increase plasma folate concentrations, and that simultaneous administration of the *Bifidobacterium* strain with a the prebiotic inulin, can further increase plasma folate concentrations (Singh *et al.*, 2016; Tuohy *et al.*, 2014).

In addition to these ongoing mechanistic studies, there is interest in understanding the role of probiotics alone, or in combination with prebiotics, to support heart health. The next section summarizes the clinical data in this regard.

23.6 The role of probiotics and synbiotics in modulating cardiovascular risk factors

Probiotics and fermented foods have been a part of the traditional human diet for multiple hundreds of years. Maintenance of cholesterol levels among African tribes despite their high-fat dairy intake sparked the theory that live bacteria present in the fermented foods may benefit the gut microflora and subsequently reduced CVD risk. Studies conducted in the 1990s did indeed provide clinical evidence for this theory and demonstrated the ability of different lactic acid bacteria species and strains for a cholesterol-lowering benefit (Di Rienzo 2013; Tuohy *et al.*, 2014).

Although a variety of lactic acid bacteria have been clinically tested for their cholesterol-lowering benefit, results appear to be more promising for *Lactobacilli reuteri* NCIMB 30242, *Eubacterium faecium*, and the combination of *Lactobacillus acidophilus* LA5 and *Bifidobacterium lactis* BB12, compared to placebo. Data to support the efficacy of each of these strains is briefly summarized below and tabulated in Tables 23.3 and 23.4. It is apparent that the majority of the clinical studies have focused on the cholesterol-lowering benefit of probiotics and little work has been conducted in evaluating their benefits on other risk factors, e.g. insulin sensitivity and body fat accumulation.

23.6.1 *Lactobacilli reuteri* NCIMB 30242

Two randomized, placebo-controlled, double-blind, parallel-arm, multicenter studies provide support for the ability of *L. reuteri NCIMB 30242* in both yogurt and capsules to significantly lower LDL-C and TC compared with placebo. The yogurt study involved 114 hypercholesterolemic men and women who consumed 250 ml of yogurt containing this probiotic (2.8×10^9 cfu/day, microencapsulated) for 6 weeks. Significant reductions in LDL-C and TC of 4.8 were measured (Jones *et al.*, 2012a). The study using capsules included 127 hypercholesterolemic men and women who consumed two capsules (200 mg; 4×10^9 cfu/day) for 9 weeks. Participants receiving the

Table 23.3. Summary of clinical studies supporting a lowering of low-density lipoprotein cholesterol with select probiotic strains.

Reference	Study design	Probiotic intervention
Jones *et al.* (2012a)	randomized, double-blind, placebo-controlled, parallel, multi-center; hypercholesterolemic men and women (n=114)	*Lactobacilli reuteri* NCMB 30242 @ 2.8×10^9 cfu/day in 250 g yogurt – 6 weeks
Jones *et al.* (2012b)	randomized, double-blind, placebo-controlled, parallel, multi-center; hypercholesterolemic men and women (n=127)	*L. reuteri* NCMB 30242 @ 4×10^9 cfu/day in capsules – 9 weeks
Bertolami *et al.* (1999)	randomized, double-blind, cross-over; hypercholesterolemic (n=32)	*Eubacterium faecium* @ 10^{5-9} cfu/ml in 200 g yogurt/d – 8 weeks
Agerbaek *et al.* (1995)	randomized, double-blind, parallel; hypercholesterolemic (n=57)	*E. faecium* @ 2×10^8 cfu/ml in 200 g yogurt/d– 6 weeks
Hlviak *et al.* (2005)	randomized, double-blind, placebo-controlled, parallel; hypercholesterolemic (n=43)	*E. faecium* @ 2×10^9 cfu/d in capsules – 60 weeks
Ejtahed *et al.* (2011)	randomized, double-blind, placebo-controlled, parallel; type 2 diabetes (n=60)	*Lactobacillus acidophilus* LA5 and *Bifidobacterium lactis* BB12 @ 4×10^6 cfu/d in 300 g yogurt – 6 weeks

Table 23.4. Summary of clinical studies supporting a lowering of low-density lipoprotein cholesterol with a combination of probiotics and prebiotics (synbiotics).

Reference	Study design	Synbiotic intervention
Ooi *et al.* (2010)	randomized, double-blind, parallel study; hypercholesterolemic (n=32)	*Lactobacillus acidophilus* CHO 220 @ 1×10^9 cfu plus 0.2 g inulin daily in capsules – 12 weeks
Schaafsma *et al.* (1998)	randomized, double-blind, placebo-controlled cross-over; hypercholesterolemic (n=30)	*L. acidophilus* (2 strains not specified) @ 107-8^9 cfu plus 2.5% fructooligosaccharides in fermented milk (125 ml) g inulin daily in capsules – 12 weeks

probiotic supplements achieved significant reductions in LDL-C and TC as well as in apoB-100. Additionally, the ratios of LDL-C/HDL-C and apoB-100/apoA-1 were reduced relative to placebo (Jones *et al.*, 2012b). Concentrations of serum TAG and HDL-cholesterol were unchanged in both studies.

23.6.2 *Eubacterium faecium*

A meta-analysis of five randomized, controlled studies with *E. faecium* in milk products involving about 400 male and female subjects and different initial LDL-C levels found a significant decrease in LDL-C and TC versus placebo (Agerholm-Larsen *et al.*, 2000). Seperate from this meta-analysis, outcomes from individual randomized, placebo controlled, double-blind trials were mixed, with studies showing decreased LDL-C (Agerbaek *et al.*, 1995) or no effect (Richelsen *et al.*, 1996; Sessions *et al.*, 1998). In a study using capsules, 43 hypercholesterolemic men and women consumed *E. faecium* M-74 (2×10^9 cfu/day) for 60 weeks. Changes from baseline in LDL-C and TC, but not HDL-C or TAG, were reported (Hlivak *et al.*, 2005).

23.6.3 *Lactobacillus acidophilus* LA5 and *Bifidobacterium lactis* BB12

Two randomized, placebo-controlled, double blind, parallel-arm studies showed a considerable reduction in LDL-C levels for this probiotic combination. One study involving 60 people with type 2 diabetes, who consumed 300 g of yogurt per day (4×10^6 cfu), for 6 weeks reported a significant reduction in LDL-C and TC (Etjahed *et al.*, 2011). A similarly designed trial involving 59 normocholesterolemic women who consumed 300 g of yogurt per day (3.9×10^7 cfu) for 6 weeks reported no changes in LDL-C and TC (Sadrzadeh-Yeganeh *et al.*, 2010). Thus, a mixture of LA5 and BB12 may be a potential therapeutic dietary option to help people with type 2 diabetes manage their LDL-C and TC levels.

23.6.4 Synbiotic studies

Commonly used prebiotics such as the fructans, inulin, oligofructose, and galatooligosaccharides have been evaluated and shown to increase the relative abundance of *Bifidobacteria* within the human gut microbiota and provide beneficial effects, e.g. production of SCFA and increased barrier function (Tuohy *et al.*, 2014). Given the bifidogenic effect of prebiotics, researchers wanted to understand if combining prebiotics with probiotics (synbiotics) could serve as another therapeutic option to manage CVD risk, particularly in the case of cholesterol management.

Two randomized, placebo-controlled double-blind synbiotic studies were shown to decrease LDL-C. The first study was a parallel-armed study including 32 hypercholesterolemic men and women and examined the combination of *L. acidophilus* CHO-220 plus inulin. Subjects consumed four capsules per day containing 1×10^9 cfu of *L. acidophilus* CHO-220 and 0.2 g of inulin for 12 weeks and achieved reductions in LDL-C and TC over placebo (Ooi *et al.*, 2010). The combination of *L. acidophilus* (strains undefined) plus fructo-oligosaccharides was examined in 30 normocholesterolemic men in a crossover study. Subjects consumed 125 mL of a fermented

milk product containing 10^7-10^8 cfu/g of *L. acidophilus* and a 2.5% fructo-oligosaccharide mixture daily for 3 weeks at breakfast, lunch, and dinner. Significant reductions in LDL-C, TC, and LDL-C:HDL-C were achieved, compared with placebo (Schaafsma *et al.*, 1998). No differences in HDL-C or TAG were found in either study.

Not all probiotic and or synbiotic studies showed consistent cholesterol-lowering benefits. For example the studies by Greany *et al.* (2008) and Kiessling *et al.* (2002), were unable to show a cholesterol-lowering benefit with synbiotic supplementation. These inconsistent findings may be attributed to factors such as different types and doses of probiotics and prebiotics, clinical characteristics of participants, length of the study time as well intervention, sample size and study design. In general, there have been more probiotic studies conducted vs prebiotic vs symbiotic studies. Despite this, large cohort studies involving early interventions with probiotics have seldomly been conducted likely because of the costs related to fecal microbiological testing. As a result, probiotic and similarly, symbiotic studies, have not always been able to achieve statistical power necessary to adequately test the cholesterol-lowering potential of even the most promising strains. Moreover, it has become apparent that probiotics effects are strain specific with particular probiotic health effects, e.g. immune modulation, production of antimicrobial compounds or the ability to lower cholesterol being present in one strain and absent in another strain belonging even to the same species (Ejtahed *et al.*, 2010; Ooi and Liong, 2010). Not surprisingly, additional human studies need to be conducted to confirm and fully understand the benefit of probiotics and synbiotics in positively modulating the key risk factors of CVD.

23.7 Conclusions

The wide-spread prevalence of CVD is reaching pandemic proportions resulting in significant social and economic burden. Not surprising, there is significant interest in identifying treatment and preventive options to manage risk. One such approach is the gut microflora. Data accumulated over the past decade has consistently shown an association of distinct changes in gut microbiota composition and function with key risk factors of CVD, namely obesity, type 2 diabetes and atherosclerosis. While the exact mechanisms have yet to be elucidated, emerging cell-culture and animal studies point to the beneficial role of the microbiota in metabolic, structural, and immunological processes. Researchers have also been searching for ways to reverse or minimize this intestinal dysbiosis and, consequently lower the risk to this non-communicable disease.

Probiotics and fermented foods have played an important role in human diets for thousands of years, wherein certain African populations had reduced blood cholesterol levels despite a high-fat dairy intake. As a result, several clinical studies have conducted to ascertain the benefit of probiotics alone or in combination with prebiotics (i.e. synbiotics) on CVD risk. Results of meta-analysis and systematic review studies reveal significant lowering of cholesterol, specifically LDL-C in association with four probiotic strains compared to placebo: *L. reuteri* NCIMB 30242, *E. faecium*, and the combination of *L. acidophilus* LA5 and *B. lactis* BB12. With respect to symbiotic studies,

L. acidophilus plus inulin or fructo-oligosaccharides appear to have a cholesterol-lowering effect but studies need to be conducted in this regard to fully understand the benefit of combining both.

In closing, the use of probiotics presents an attractive and promising non-pharmacological approach to reduce the risk of CVD, in light of its promising clinical data, lack of side-effects and adverse events as well as its cost-effectiveness. These points certainly warrant further investigation into this therapeutic opportunity for CVD.

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24. Heart health and microorganisms: the unexpected beat

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Abstract

The human body is colonized by an extremely complex ecosystem composed of many commensal organisms such as viruses, bacteria and fungi, which together are called microbiota. The microbiota, in particular the gut microbiota, has important metabolic functions which influence the integrity of mucosal barrier and homeostasis of the immune system. Microbial imbalance or dysbiosis has been identified as a potential risk factor for susceptibility to several chronic metabolic diseases, including diabetes mellitus, obesity and cardiovascular disease (CVD). Diets based on high intake of whole-plant foods, e.g. the Mediterranean diet, and the current prevailing Western-style diet have different effects in the gut microbiota composition influencing the abundance of different bacterial communities in the gut. The advances in science are showing a growing importance of the gut in the regulation of the immune system and its effects are related to development and also to protection against several diseases. In CVD, as in other disorders, the immune system plays at least a partial role in its pathology. Recently gut microbiota alterations, mainly during obesity induced by high fat diet have been linked to metabolic syndrome due systemic low grade inflammation and consequently, might play a role in CVD development. In this chapter, we discuss the role of nutrition influencing gut microbiota and the consequences on the immune system for cardiovascular health.

Keywords: nutrition, gut microbiota, immune system, cardiovascular disease

Key facts

- The microbiota corresponds to commensal organisms including viruses, bacteria and fungi that colonize the skin and mucosal surfaces of the body.
- Cardiovascular diseases (CVD) are a group of diseases related to heart and blood vessel injuries.
- Nutrition is the science that investigates the relationship between the nutrients obtained by food with growth, health and disease of an organism.
- Diet is what an organism eats which is determinate by availability and palability of foods.
- The Human Microbiota Project launched in 2008, was created with the mission of generating research resources enabling characterization of the human microbiota and analysis of their role in human health and disease.

Summary points

- Host microbiota influences health by exerting important metabolic functions and effects on mucosal barriers to maintain homeostasis of the immune system.
- Diet is an important factor that can alter the microbial composition, in which certain nutrients favor the development of determined microorganisms.
- The microbiota can be viewed as a metabolically active, complex organ, producing many metabolites that can directly influence host phenotype.
- Microbiota is important in the development and regulation of the immune system, thus influencing the course of heart disease
- Bacterial products can induce systemic low-grade inflammation which is associated with metabolic syndrome.
- Atherosclerosis is considered to be a chronic inflammatory disease that can be triggered by consumption of high saturated-fat diet, smoking, hypertension, hyperglycemia and obesity.
- The immune system is a double edged sword, a certain degree of inflammation is beneficial to the reparative process of an injured heart, while excessive inflammation can be damaging.
- Understanding how microbial diversity affects clinical phenotypes and risk of CVD will be beneficial to develop personalized approaches to nutrition and medical therapy.

Abbreviations

Ang	Angiotensin
AS	Atherosclerosis
CCR2	C-C chemokine receptor 2
CRP	C-reactive protein
CVD	Cardiovascular disease
EAE	Experimental autoimmune encephalomyelitis
GIT	Gastrointestinal tract
HDL	High-density lipoprotein
HSP	Heat shock proteins
IBD	Inflammatory bowel disease
IFN-γ	Interferon gamma
IL-10	Interleukin-10
IL-17	Interleukin-17
IL-6	Interleukin-6
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein-1
MI	Myocardial infarction
NF-κB	Nuclear transcription factor kappa B
NLR	NOD-like receptor
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
PVD	Peripheral vascular disease
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
SCFA	Short-chain fatty acid
SFB	Segmented filamentous bacteria
TAG	Triacylglycerol
TLR	Toll like receptor
TMAO	Trimethylamine N-oxide
TNFR	Tumor necrosis factor receptor
Tregs	Regulatory T cells
VCAM-1	Vascular cell adhesion molecule-1

24.1 Microorganisms and the host

The human body is colonized by an extremely complex ecosystem composed of many commensal organisms such as viruses, bacteria and fungi, which together are called microbiota. If only the bacterial fraction is analyzed, we will still be examining trillions of bacteria, scattered throughout our skin and mucosa (Hooper *et al.*, 2012). Within the body, the colon contains the most diverse

and numerous microbial populations, there are 10^{10}-10^{12} organisms per gram of luminal content (Zoetendal *et al.*, 2008). Most of the bacteria found in the colon belong to five major phyla: *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Verrucomicrobia* (Van Baarlen *et al.*, 2013), however, the proportions of these phyla can vary greatly between individuals and even in the individual himself over the time (Rajilic-Stojanovic *et al.*, 2007).

In the past the relationship between microbiota and the host was largely unknown; recently, it is becoming clear that the maintenance of a healthy state is influenced by the host microbiota, which has important metabolic functions and effects on mucosal barriers that balance the homeostasis of the immune system.

The human body starts being colonized by microorganisms present during passage through the birth canal and with the breastfeed milk while feeding (Sekirov *et al.*, 2010; Yatsunenko *et al.*, 2012). The microbial signature begins to emerge between 1-2 years of age, when the microbiota begins to differ from the mother's microbiota under the influence of genetic and environmental factors (Yatsunenko *et al.*, 2012), such as, solid food feeding, daycare, school, among others. It is already very well described and it has been shown by several groups that the microbiota contributes to the physiological processes of the host while the host provides the necessary nutritional environment for its survival (Hooper and Macpherson, 2010).

The metabolic capacity of the intestinal microbiota is comparable to the liver metabolism and it may therefore to be regarded as an additional organ. An imbalance in microbial composition also termed as dysbiosis can cause diseases, and in turn several disorders have been associated with dysbiosis (Gerritsen *et al.*, 2011).

In a well-balanced system, microorganisms perform functions that are essential for the maintenance of homeostasis of the immune system and do not represent a risk, however, when the intestinal barrier is impaired, they can lead to several chronic metabolic diseases, including diabetes mellitus (Larsen *et al.*, 2010), obesity (Turnbaugh *et al.*, 2008) and CVD (Karlsson *et al.*, 2012). A recent line of investigation views the microbiota as a metabolically active, complex organ, producing many metabolites that can directly influence host phenotype (Borthakur *et al.*, 2012; Wang *et al.*, 2016).

When we better understand how the interaction between commensal microorganisms and the host modulates the body´s vital systems, such as the immune and metabolic systems, we will be able to explain the origin and mechanism of various diseases. In this chapter, we will provide an overview of the current understanding of the role of nutrition on intestinal microbiota, immune system and its impact on heart health.

24.2 Diet and microbiota: the perfect relationship

Decades of research have already been spent on the effort to understand the relationship between nutrition and CVD, an overwhelming amount of observational/epidemiological studies and hundreds of thousands of participants have been instrumental in linking diet to health (Ferguson *et al.*, 2016; Knowler *et al.*, 2002; Sacks *et al.*, 1995). Nowadays, it is known that both nutrients and genes play a role in determining health, yet the complex interactions among genes, diet and downstream networks are still not well understood.

In recent years, the interactions between diet and genome have been demonstrated through 'omics' technologies which measure perturbations by RNA expression (transcriptome), epigenetic modifications (epigenome), metabolites (metabolome), lipids (lipidome), proteins (proteome), and resident microbial communities (microbiome) (Ferguson *et al.*, 2016). It is already well accepted that diet is an important factor that can alter the microbial composition, in which certain nutrients favor the development of some microorganisms (Dominguez-Bello *et al.*, 2010).

The contribution of gut microbiota to host nutrition and energy metabolism can be characterized by production of SCFA, amino acids and vitamins (Morowitz *et al.*, 2011). The microbial-derived metabolites can be distributed well beyond the GIT and influence the physiology of the host, enabling this complex network of microorganisms to act as a virtual endocrine organ and playing an important role in host health and disease status (Fujimura *et al.*, 2010; Sekirov *et al.*, 2010). This close interaction can probably occur because the human genome has evolved closely with its microbial counterpart over the course of evolution, resulting in many shared or co-metabolic pathways.

Concerning the critical influence of diet to both the relative abundance of different gut microorganisms and their metabolic output, we have to take into account the differences in dietary habits in the various regions of world to better understand how diet-microbiota interaction can impact on chronic disease incidence. In fact, diets based on high intake of whole-plant foods, e.g. the Mediterranean diet, is radically different from the current prevailing Western-style diet characterized by foods rich in saturated fat, animal protein, flavoring agents, sugars, salt and monosodium glutamate, and low in fiber, plant phytochemicals, beneficial fats, minerals and vitamins (Tuohy *et al.*, 2014). Animal and human studies have shown that diets rich in fat or animal protein can radically remodel the gut microbiota, reducing the relative abundance of *Bifidobacteria* and butyrate-producing bacteria, considered beneficial to health, and increasing the concentration of harmful microbe-derived metabolites (Fava *et al.*, 2013; Wang *et al.*, 2011). In addition, there is growing recognition of the importance of the gut in the regulation of the immune system and subsequent metabolic effects (Burrows *et al.*, 2015; Shen *et al.*, 2013). Thus, it is no wonder that the chronic diet-associated diseases, both metabolic and autoimmune in nature, are reaching epidemic proportions in the populations following Western life style in diverse regions of the world.

Reports have highlighted interactions between the microbiota and metabolism of dietary components such as phosphatidylcholine and carnitine on modulating CVD risk through TMAO (Tang *et al.*, 2013; Wang *et al.*, 2011). TMAO is a naturally occurring small organic dietary compound that is abundant in fish, or can be generated from other nutrients including choline (abundant in eggs) and carnitine (abundant in beef). It is proposed that gut bacteria generate trimethylamine (trimethylamine; volatile, fish-odor compound) (Barrett and Kwan, 1985) which can be subsequently enzymatically converted to TMAO metabolite in a reversible reaction catalyzed in the liver (Cho *et al.*, 2017).

Recent studies have shown that elevated TMAO levels strongly predict Coronary Artery Disease, at least partly reflecting the ability of increased levels to enhance atherogenesis (Tang *et al.*, 2013; Troseid *et al.*, 2015). In this regard, some researchers have advocated for the restriction of animal source foods that raise circulating TMAO concentrations (Tang *et al.*, 2013). However, some animal foods sources are enriched in nutrients are beneficial for health, such as fatty fish, that contain omega-3 and vitamin D, well-known for its cardio and immune protective attributes in humans (Baeke *et al.*, 2010; Galli and Rise, 2009). Thus, caution is warranted when proposing dietary recommendations that restrict the intakes of animal source foods because of their TMAO-raising characteristics.

It is important to note that alteration in the gut microbiota arising from dietary habits are observed even during childhood, which enables us to speculate that the risk factor for developing chronic disease can be detected early. In a study using 16S rRNA metagenomics to characterize the composition and metabolic output of the fecal microbiota from children growing up in urban Florence, Italy, and rural Burkina Faso in Africa, it was found that the bacterial profile differed greatly between healthy, age-matched children. In the African children whose diet contained abundant whole-plant foods, cereals and fermented fruits, supplemented occasionally with bush meat and insect protein, the gut microbiota was dominated by *Bacteroidetes* phylum, especially the *Prevotella* group, indicative of high capacity for carbohydrate fermentation (De Filippo *et al.*, 2010). In addition, the African children appeared to have enhanced production of SCFA, with higher concentration of acetate, butyrate and propionate in their feces compared to Italian children, and recent studies have shown the potential of SCFA to modulate disease mechanisms linked to CVD (Schwiertz *et al.*, 2010; Teixeira *et al.*, 2013).

Microorganisms within the human intestine ferment carbohydrate sources and reach the colon into the SCFA acetate, propionate and butyrate (Conterno *et al.*, 2011). Prebiotics are selectively fermented ingredients, good source of SCFA, and result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefits to host health (Gibson and Roberfroid, 1995; Petschow *et al.*, 2013). A study using an animal model showed that propionate was an efficient inhibitor of cholesterol biosynthesis in rat hepatocytes when acetate was the main substrate available (Demigne *et al.*, 1995). Common prebiotics include mainly the fructans, inulin, oligofructose, and β-glucan and all of which have been shown to increase the relative abundance of *Bifidobacteria* genera within the human gut microbiota (Wang *et al.*, 2016). Dyslipidaemia is characterized by elevated TAG levels together with elevated total cholesterol

(high LDL-cholesterol and low HDL-cholesterol), which represent an important modifiable risk factor for CVD. Animal studies have consistently shown that dietary intervention with prebiotics, especially the fructans inulin and oligofructose can reduce serum TAG (Delzenne *et al.*, 2002) and it is another example of the potential role of diet-microbiota axis to host health.

Whole-grain oats comprise a number of different classes of biologically active molecules capable of modulating cholesterol metabolism in mammals, including mono- and di-unsaturated fatty acids, fibers such as β-glucan (Borneo and Leon, 2012; Ryan *et al.*, 2007). β-glucan is the major soluble fiber in oat and barley and was shown to have physiological benefits including lowering of plasma cholesterol (AbuMweis *et al.*, 2010; Whitehead *et al.*, 2014), reduction of postprandial glycemic responses (Braaten *et al.*, 1991), and weight control by increasing satiety (Maki *et al.*, 2010). Since humans lack enzymes for the digestion of β-glucan, like other dietary fibers, these compounds are fermented in the lower GIT via carbohydrate active enzymes harbors by gut microbiota, which also result in compositional and functional shifts in the microbiota (Cantarel *et al.*, 2012).

Gut health in part is associated with diversity and stability of gut microbiota. It is believed that individuals with low richness of microbiota composition are more vulnerable to obesity, insulin resistance and dyslipidemia compared to those with high richness of microbiota (Le Chatelier *et al.*, 2013). Thus, understanding how microbial diversity and specific microbial species affect clinical phenotypes and risk of CVD will be beneficial to focus on personalized approaches to nutrition and medicine.

24.3 Gut microbiota in health and disease

A few years ago, in 2008, the Human Microbiota Project, was created with the mission of generating research resources enabling characterization of the human microbiota and analysis of their role in human health and disease (Turnbaugh *et al.*, 2007). In this project, researchers from all over the world are connected to study how gut microbiota can influence the human body development and functions. Since then, there is mounting evidence that microorganisms have different effects in the GIT of the host.

Under normal conditions, the immune system is instructed by commensal microbiota to not respond to luminal antigens. Moreover, metabolites secreted by commensal microbiota are responsible for processing nutrients, to prevent infection by pathogenic bacteria, providing signals for the development of the immune system and stimulating the innate and adaptive immune responses to maintain homeostasis. Furthermore, in certain circumstances, the microbiota can also cause disease in genetically susceptible and/or immunodeficient individuals (Honda and Littman, 2012).

The commensal bacteria maintain the integrity of epithelial cells, stimulate the secretion of mucus and antimicrobial peptides, and thus contribute to the maintenance of a basal level of the host

defenses in a regular state. However, when there is dysbiosis, non-invasive bacteria are transported to key immune sites such as the mesenteric lymph nodes (Abt and Artis, 2013) triggering immune responses against these micro-organisms that would normally not be considered dangerous. This shows that certain components of the microflora can trigger inflammatory responses, whereas others lead to anti-inflammatory mechanisms according to the site where they are found (Hooper *et al.*, 2012).

Much of the impact of the gut microbiota in the host is mediated by pattern recognition receptors of the innate immune system, particularly toll-like (TLRs) and nod-like (NLRs) receptors. Both TLRs and NLRs recognize a wide variety of conserved microbial components and allow the innate immune system to recognize a wide range of bacteria, viruses, fungi and parasites (Hooper *et al.*, 2012). Several studies have shown the role of TLR in the establishment of the microbiota and host interaction. TLR2 in CD4+ T cells are important for the colonization of *Bacteroides fragilis* and the maintenance of gut homeostasis. The commensal bacteria somehow exploit TLRs pathway to suppress immunity (Round *et al.*, 2011). Further, TLRs polymorphisms are correlated with various intestinal diseases (Torok *et al.*, 2004). Moreover, TLR5 plays an important role in metabolic homeostasis keeping a healthy gut microbiota. It was shown by Vijay-Kumar *et al.* (2010) that in mice lacking TLR5 increased glucose intolerance and insulin resistance was correlated with gut microbiota alterations in absence of TLR5.

In addition, NLR receptor family is also important in maintaining the antimicrobial responses in the intestine, while the recognition of the intestinal microbiota by NOD2 is important to keep homeostasis of intra epithelial lymphocytes (Jiang *et al.*, 2013). For instance, experiments have shown that the absence of NOD2 correlates to the development of intestinal diseases (Comalada and Peppelenbosch, 2006). Furthermore, deficiency of NLRP6, another member of NLR family, in mouse colonic epithelial cells resulted in altered fecal microbiota characterized by expanded representation of the bacterial phyla *Bacteroidetes* (*Prevotellaceae*) and TM7 (Elinav *et al.*, 2011).

Another important role of the microbiota in the development of the immune system is the induction of IgA and maintenance of homeostasis of different populations of T-lymphocytes, including Tregs, Th2 and Th1 (Gaboriau-Routhiau *et al.*, 2009). Some populations of commensal bacteria preferentially lead to the development of Tregs such as *Bifidobacterium breve*, *Bacteroides fragili*s, *Clostridium*, and *Lactobacillus* species of the phylum *Firmicutes* (Atarashi *et al.*, 2011; Jeon *et al.*, 2012; Mazmanian *et al.*, 2008). The idea that microbiota is able to induce the expression and expansion of Tregs in the gut derived from studies in which conventional mice were shown to have an expression of IL-10 and Foxp3 ten times higher than germ-free mice (Gaboriau-Routhiau *et al.*, 2009; Ishikawa *et al.*, 2008). In addition, colonization of germ-free mice with *Bacteroides fragilis* induced Tregs (Zhang *et al.*, 2010) while germ-free mice were shown to be more susceptible to infection by pathogenic bacteria (Sommer and Backhed, 2013).

Moreover, a limited number of SFB such as *Candidatus arthromitus*, *Firmicutes* and *Clostridium*, and also *Candida albicans*, *Staphylococcus aureus Lactobacillus acidophilus* and *Bacteroides distasonis* appear to induce Th17 cells, which play a key role in host defense as well as in the

development of autoimmune diseases (Geuking *et al.*, 2011; Korn *et al.*, 2009). Th17 cells are capable of producing IFN-γ and IL-25, however, the exact mechanism by which SFB are able to induce the differentiation of Th17 in the intestine is not well known; however, flagellins present in the bacteria could be a possible mechanism for Th17-induction (Prakash *et al.*, 2011; Zielinski *et al.*, 2012).

While the role of the microbiota in intestinal inflammation has been appreciated, more recent studies point out that bacterial products can also induce low-grade systemic inflammation which is associated with metabolic syndrome. The association between microbiota and metabolic syndrome was first suggested by Cani and colleagues, who demonstrated that obesity can result in loss of function of the epithelial barrier, leading to activation of TLR4 by LPS, induction of inflammation and subsequent insulin resistance development (Cani *et al.*, 2007).

The composition of intestinal microbiota has also been associated with the development of other diseases, such as cancer. For example, several species have been linked to colon cancer, namely; *Streptococcus bovis*, *Streptococcus* spp., *Escherichia coli* and *Fusobacterium nucleatum* (Kostic *et al.*, 2012). A hypothesis to explain this association is that inflammation caused by some strain of commensal microbiota could contribute to the development of colon cancer in mice.

In addition to cancer, allergic diseases are also correlated with changes in intestinal microbiota. Lately, the incidence of allergic diseases has increased in industrialized countries, which suggests that changes in the environment could be an important factor in the development of this malady (Beasley *et al.*, 2000). The 'hygiene hypothesis' suggests that better sanitary and medical conditions could be associated with the increased prevalence of allergic and autoimmune diseases in developed countries due to decreased exposure to pathogens that can shift immune responses towards a Th2 profile or immunoregulation (Okada *et al.*, 2010). The hygiene hypothesis allied with use of antibiotics and diet could also lead to alterations in intestinal microbiota thus aggravating immunological imbalance (Noverr and Huffnagle, 2005). Pre-clinical studies in which mice were administered *Lactobacillus* and *Bifidobacterium* have demonstrated modulation of allergic responses in the respiratory tract by induction Tregs, suggesting that the microbiota plays an important role in development of these diseases and the combination of probiotics may be an effective treatment (Feleszko *et al.*, 2007; Karimi *et al.*, 2009).

Along with insulin resistance, obesity, cancer and allergies, microbiota has been associated with the development of autoimmune diseases, including IBD, RA, EAE and lupus (Cerf-Bensussan and Gaboriau-Routhiau, 2010; Toivanen, 2003). However, the mechanisms by which commensal bacteria could trigger autoimmunity are still poorly understood. In the case of IBD, colitis models have shown that the use of antibiotics ameliorates symptoms and this also translates to patients, suggesting that bacteria play an important role on the pathogenesis of IBD (Packey and Sartor, 2008; Wu *et al.*, 2009). In addition, recent studies have pointed that bacteria are also involved in the development of EAE (Lee *et al.*, 2011). It was demonstrated that germ-free mice develop less severe EAE compared to conventional mice, lower stringency was associated with reduced IL-17 and IFN-γ production, and high levels of Tregs. Also, colonization of germ-free mice with SFB

restored susceptibility of these mice to EAE, which strongly suggests that microbiota is important for disease severity (Lee *et al.*, 2011). Moreover, germ-free mice also have attenuated development of RA, presenting decreased autoantibodies and IL-17 levels in serum. Interestingly, colonization of these mice with SFB induced rapid development of RA, which correlated with IL-17 expression in lamina propria (Wu *et al.*, 2010). IBD patients often have increased IL-17 levels and specific inhibition of IL-17 decreases disease severity (Weaver *et al.*, 2013). The production of IL-17 seems to have a dual role in IBD, while this cytokine is important to boost immune responses that clear pathogens, overproduction could also lead to damaging inflammation (Fujino *et al.*, 2003; O'Connor *et al.*, 2009).

The role of gut microbiota regulating CVD has also gained interest of the scientific community. Changes in gut microbiota and inflammation in the colon can increase intestinal permeability, allowing microbial compounds, such as LPS, to enter the systemic circulation, in turn, this systemic endotoxemia induces expression of pro-inflammatory cytokines that contribute to endothelial damage and foam cell formation (Howell *et al.*, 2011; Rogler and Rosano, 2014) (Figure 24.1). These factors are strongly correlated to atherosclerosis (AS), since LPS activates macrophages and also stimulates LDL oxidation, which is toxic to human endothelial cells, both processes could thereby accelerate AS (Maziere *et al.*, 1999; Wiesner *et al.*, 2010).

As observed in obesity-induced insulin resistance, TLR2 and TLR4 may also mediate the microbial effect on atherogenesis, since these receptors are responsible for recognizing bacterial products and are increased on circulating monocytes during acute MI compared to healthy controls (Ashida *et al.*, 2005; Satoh *et al.*, 2006). More recently, there is an increasing evidence that microbiota plays an important role during coronary artery disease, since a recent study in rats showed that partial depletion of gut microbiota using antibiotics, led to smaller myocardial infarcts and improved recovery of postischemic mechanical function compared to untreated controls (McCafferty *et al.*, 2012).

However, periodontal microbiota is also associated with CVD. Genomic DNA of numerous oral microorganisms have been detected in atherosclerotic plaques, suggesting a strong correlation between AS and oral microbiota dysbiosis (Lockhart *et al.*, 2012).

Since nutrition is directly correlated to gastrointestinal tract, gut microbiota and to CVDs, there is a particular interest to understand how this correlation works.

24.4 Inflammation and heart disease

The immune system is a highly complex defense system whose mission is to protect us against pathogens, recognizing and responding to diverse environmental and endogenous stimuli. AS and its complications arise from the combination of environmental, genetic and immune dysregulation, thus do not the result from simple arithmetic of imbalanced dietary lipid consumption. In CVD, like in every other disorder, the immune system plays at least a partial

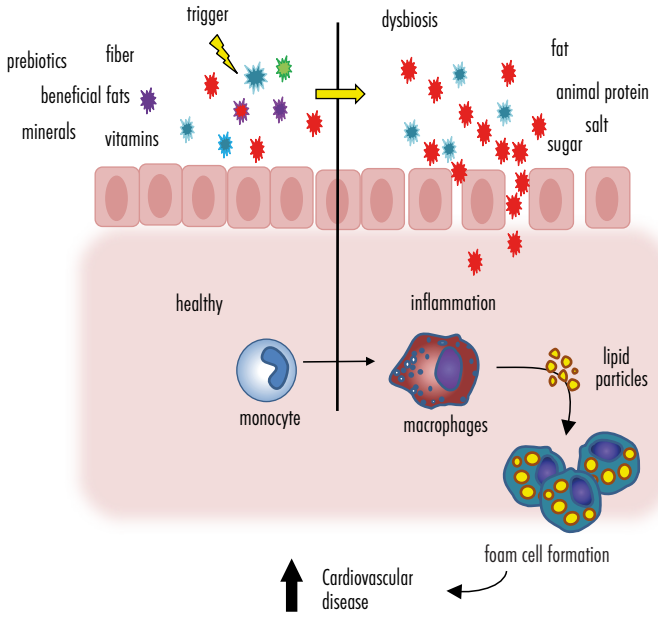

Figure 24.1. Environmental triggers or unhealthy diets may induce dysbiosis which can increase intestinal permeability, allowing microbial compounds, such as lipopolysaccharide, to enter the systemic circulation, in turn, this systemic endotoxemia can stimulate differentiation of monocytes into macrophages, which engulf lipid particles in the vessel wall and become lipid-laden foam cells that drive the inflammatory milieu contributing to cardiovascular disease (CVD).

role in its pathology. AS is a multifactorial process that can lead to life-threatening complications such as, stroke, MI and heart failure. It is considered to be a chronic inflammatory disease that can be triggered by consumption of high saturated-fat diet, smoking, hypertension, hyperglycemia and obesity (Swirski and Nahrendorf, 2013).

For the past thirty decades, arteries were viewed as inanimate conduits in which lipid deposits were formed and build up until eventually blood supply to tissues became obstructed, resulting in MI or stroke (Libby, 2006). However, earlier observations that pro-inflammatory markers were elevated in patients with myocardial ischemia and correlated with severity of cardiomyopathies prompted studies on the interaction between the immune and cardiovascular system (Briasoulis *et al.*, 2016; Entman *et al.*, 1991).

The immune system is a double edged sword in disease, while some leukocytes are viewed as atheroprotective and a certain degree of inflammation is necessary to ensure the beneficial reparative process of an injured heart, excessive inflammation can lead to further heart damage (Frangogiannis, 2012). Immune responses involve a vast repertoire of leukocytes and the various classes of myeloid and lymphoid cells participate in CVD.

Both the humoral and cellular arms of the innate immune system are main players in the initiation and perpetuation of inflammatory responses following injuries to the cardiac muscle. Damaged cardiac myocytes as well as the extracellular matrix deliver 'danger' signals that activate and recruit immune cells. HSPs, chromatin-binding protein high-mobility group box-1, adenosine triphosphate and reactive oxygen species have been described as initiator signals (Briasoulis *et al.*, 2016; Frangogiannis, 2012). NO which is released from vascular endothelium has anti-inflammatory and anti-apoptotic properties, however in an environment of high oxidative stress present in ischaemic heart disease and cardiomyopathies, NO can interact with superoxide ion to form reactive oxygen species which promotes cell damage and perpetuates inflammation and apoptotic pathways (Cotton *et al.*, 2002; Ishida *et al.*, 1996).

Furthermore, anti-microbial responses involving TLRs in particular TLR2 and TLR4 as well as the complement system have been implicated in the signaling cascade of NF-κB. In turn, the NF-κB signaling pathway generally leads to transcription of cytokines, chemokines and cell adhesion molecules (Tak and Firestein, 2001). Many infectious diseases have been associated with AS, viruses, bacteria and even parasites haven been implicated in CVD. Amongst these associations, infections with *Chlamydia pneumoniae* have been most commonly reported to MI, CVD and PVD (Shoenfeld *et al.*, 2001; Watson and Alp, 2008). Other AS-associated pathogens are *Helicobacter pylori*, Epstein-Barr virus and cytomegalovirus. Chronic infections could result in accelerated AS formation either by nonspecific mechanisms such as hypercoagulability, and increased adhesion molecule and elevated CRP levels or by induction of HSP-60 expression and pathogenic anti-HSP-60 antibody production (Shoenfeld *et al.*, 2001). High serum CRP levels correlate with an increased incidence of MI, Cerebrovascular Accident and PVD, it might play a pathogenic role in atherogenesis through the induction of expression of adhesion molecules and chemokines, such as MCP-1 (Shoenfeld *et al.*, 2001; Yousuf *et al.*, 2013).

Several inflammatory cytokines have been implicated in CVD, tumor necrosis factor alpha has a dual role: binding to TNFR1, promotes inflammatory and pro-apoptotic effects while it has the opposite effect via the TNFR2 (Hamid *et al.*, 2009). In addition, increased levels of IL-6 are found in AS patients, while in murine models, inhibition of IL-6 signalling in has led to better functional status of ventricular function in the acutely ischaemic myocardium (Kobara *et al.*, 2010). Furthermore, activation of the inflammasome in inflamed myocardium promotes generation of IL-1β, leading to leukocyte recruitment, cell death, adverse cardiac remodeling, and cardiac dysfunction (Van Tassell *et al.*, 2015).

The cardiac renin-angiotensin system has several components which exert pleiotropic effects and is traditionally known to regulate blood pressure and water-electrolyte homeostasis (Pfeffer *et al.*, 1985). Ang II can induce hypertension via oxidative stress and endothelin, inhibition of Angiotensin-converting Enzyme, which converts Ang I into Ang II lowers mean arterial pressure and improves survival in MI and HF (Bolterman *et al.*, 2005; Briasoulis *et al.*, 2016).

Under normal healthy conditions the inner surface of the artery wall are not prone to adhesion by leukocytes (Libby, 2006). Therefore, the active role of arteries in the process of acute and chronic

inflammatory responses is highly dependent on the expression of chemokines and adhesion molecules by endothelial cells. Consumption of a high-saturated-fat diet, smoking, hypertension, hyperglycemia, obesity, or insulin resistance, can trigger the expression of adhesion molecules such as, VCAM-1 and P-selectin that mediate the attachment of circulating monocytes and other leukocytes to the arterial wall (Libby, 2006; Packard *et al.*, 2009). Expression of VCAM-1 can be induced by oxidized lipoprotein and is linked to the induction of the atherogenic process, also VCAM-1 is expressed in human coronary atherosclerotic plaques and in animal models of hypercholesterolemia (Gimbrone and Garcia-Cardena, 2016). Although, various types of immune cells participate in AS and its complications, monocytes and macrophages are the most prominent cellular type in AS, particularly, pro-inflammatory $CCR2^{+}Ly6C^{high}Gr\text{-}1^{+}$ monocyte subset is present in all stages of disease (Tacke *et al.*, 2007). CCR2 is the receptor for MCP-1 and it has been shown that in murine models the genetic absence of MCP-1 or CCR2 inhibits monocyte recruitment and dramatically decreases atherosclerotic disease (Boring *et al.*, 1998; Gu *et al.*, 1998). Although challenged, a model has been proposed in which monocytes transform into macrophages, which engulf lipid particles in the vessel wall and become lipid-laden foam cells that drive the inflammatory milieu in the atherogenic vessel (Wolf *et al.*, 2015).

Adaptive immunity also regulates the magnitude of the atherogenic pro-inflammatory response, T cells will join macrophages during lesion evolution and secrete cytokines and growth factors that can promote the migration and proliferation of smooth muscle cells. Although the frequency of T cells is relatively rare in atherosclerotic lesions, there is a body of evidence that T cells participate in AS and studies suggest that Th1 and Th17 cells are atherogenic by producing pro-inflammatory cytokines that propagates inflammation (Wolf *et al.*, 2015). T cells also secrete cytokines that inhibit collagen production by smooth muscle cells while it stimulates macrophages to produce collagen-degrading enzymes that weakens the fibrous cap, which protects against thrombogenic lipid core of the plaque, thus increasing the chances of plaque rupture and formation of a life-threatening thrombus (Andersson *et al.*, 2010; Libby, 2006). There is even a school of thought supporting the view that AS could be an autoimmune disease and some evidence points towards this direction; T cells that infiltrate the aorta have a restricted repertoire of T cell receptor, activation of T cells in the plaque requires presentation of specific antigens by plaque resident antigen-presenting cells, and the immunization against some known antigens can modulate the outcome of murine AS (Kimura *et al.*, 2015). B cells, on the other hand, particularly the B2 subtype contributes to disease, presumably by interacting with other leukocytes and/or secreting inflammatory cytokines (Briasoulis *et al.*, 2016; Kyaw *et al.*, 2011).

Importantly, the role of immune cells in CVD is not restricted to mediating disease but also regulating it. For instance, the M2 macrophage subtype could be important for the regression of the atherosclerotic plaque by; secreting anti-inflammatory cytokines such as IL-10 and reducing the production of damaging reactive nitrogen species, promoting tissue remodeling and repair by promoting collagen formation and the clearance of dying cells and debris and increasing efferocytosis of dying macrophages (Moore *et al.*, 2013). Tregs could also dampen inflammation by secretion of IL-10 and transforming growth factor-β. Finally, the innate-like B1 B cells is linked

to protection, possibly by production of natural IgM antibodies that mark lipids for Fc receptor-mediated removal (Kyaw *et al.*, 2011, 2012; Swirski and Nahrendorf, 2013).

Recent research has disputed the traditional view of the 'cardiovascular continuum' postulated by Dzau and Braunwald which focused only on arteries and hearts (Libby *et al.*, 2016). New insight on the participation of diverse components of the immune system in both sustaining and controlling inflammation in CVD has expanded the realms beyond myocardium and vessels. Better understanding of cellular and molecular arms of immunity involved in AS and its complications can lead to the development of more accurate strategies to prevent and treat CVD.

24.5 Concluding remarks

In this chapter we have viewed how our diet can influence the composition of microbiota and in turn how the microbiota is important in the maintenance of health by affecting immunological equilibrium. Heart and vessels are no longer seen as inert receptacles that fill and become obstructed by lipid deposits, we now know that myocardial and endothelial cells directly interact with the immune system. Thus, understanding mechanisms by which commensal microorganisms changes the host, we can access the importance of microbiota in the modulation of various systems, such as the cardiovascular system. Although many of the microorganisms present in our body perform essential functions for the maintenance of homeostasis, perturbations in the composition of our microbiota can lead to many complications. Further investigation is needed to improve our knowledge on how nutrition and dietary metabolites affects microbiota and consequently the immune system homeostasis and how this modulates the course of CVDs and the health state of the heart.

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25. Health perspectives of medicinal macrofungi of southwestern India

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Abstract

Several macrofungi are traditionally known for health benefits or health-promoting potential in Southwestern India. This study compares bioactive components and antioxidant potential of methanol and aqueous extracts of four macrofungi (*Amauroderma conjunctum*; *Daldinia concentrica*; *Ophiocordyceps nutans*; *Pycnoporus cinnabarina*). Total phenolics (TP) and flavonoids content were significantly higher in *P. cinnabarina* than the rest of macrofungi ($P<0.05$), while vitamin C content was almost uniform. Methanol extract showed significantly higher total antioxidant activity (TAA), ferrous-ion chelating capacity (FCC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity and reducing power (RP) than aqueous extract in all macrofungi. The TAA and FCC of methanol extract were significantly higher in *P. cinnabarina* followed by *A. conjunctum*, while the DPPH radical-scavenging activity was significantly higher in *A. conjunctum* followed by *P. cinnabarina*. The RP (at 1 mg concentration) of methanol extract was the highest in *D. concentrica* followed by *A. conjunctum*, *P. cinnabarina* and *O. nutans*. TP, flavonoids and vitamin C of these macrofungi were associated with at least two antioxidant properties denotes their ability in combating the cardiovascular diseases. Species-dependent (*A. conjunctum*, *D. concentrica*, *O. nutans* and *P. cinnabarina*) and extract-dependent (methanol and aqueous) antioxidant potential of these wild macrofungi facilitates to utilize selectively in favor of combating cardiac diseases.

Keywords: antioxidant activities, cardiovascular diseases, disease prevention, health-promotion, wild mushrooms

Ronald Ross Watson and Sherma Zibadi (eds.) Handbook of nutrition and heart health
Human Health Handbooks no. 14 - DOI 10.3920/978-90-8686-853-7_25, © Wageningen Academic Publishers 2017

Key facts

- Nowadays attention has been focused on derivation of natural bioactive compounds to remedy several life threatening diseases.
- In addition to conventional sources of bioactive compounds (plant-, animal- and microbe-derived), several wild macrofungi possess promising health-promoting potential.
- Macrofungi constitute one of the major non-conventional sources to derive health benefits.
- Bioactive components and antioxidant potential of four macrofungi of Southwest India have been addressed to understand their therapeutic significance.

Summary points

- Four wild macrofungi (*Amauroderma conjunctum*; *Daldinia concentrica*; *Ophiocordyceps nutans*; *Pycnoporus cinnabarina*) were evaluated for bioactive components and antioxidant potential.
- The antioxidant potential of macrofungi has been compared between methanol and aqueous extracts.
- Total phenolics and flavonoids were significantly higher in *P. cinnabarina* than the rest of macrofungi, while vitamin C content was almost uniform.
- Compared to aqueous extract, methanol extract showed significantly higher total antioxidant activity, ferrous-ion chelating capacity, 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity and reducing power.
- Species-dependent and extract-dependent antioxidant potential of these macrofungi facilitates their utilization in combating cardiac-related ailments.

Abbreviations

DPPH	2,2-diphenyl-1-picrylhydrazyl
FCC	Ferrous ion-chelating capacity
FL	Flavonoids
PCA	Principal component analysis
RP	Reducing power
TAA	Total antioxidant activity
TCA	Trichloroacetic acid
TP	Total phenolics
VC	Vitamin C

25.1 Introduction

Exploration and utilization of natural products derived from biological sources is more advantageous than the synthetic drugs in combating several human diseases. Macrofungi constitute the largest volume of non-timber forest products and serve as important segments of biodiversity and ecosystem functions especially in wood degradation (Ehlers *et al.*, 2003). Macrofungi being biologically, nutritionally and medicinally versatile, have historical intimacy with human beings since Neolithic and Paleolithic eras (Samorini, 2001). In China, macrofungi were traditionally in use for human health promotion since 100 AD, however, their importance was realized from 1960 onwards (Gunde-Cimmerman, 1999). Many macrofungi have been designated as 'medicinal mushrooms' and considered generally regarded as safe for nutritional (food or food supplements) or medicinal (cure diseases or health-promotion) or nutraceutical (nutritional and pharmaceutical) applications (De Silva *et al.*, 2013). There seem to be differences in bioactive potential between wild and cultivated macrofungi especially in their flavor, pigments, vitamins, amino acids, fatty acids and antioxidant properties. Thus, necessity arises to relay on wild macrofungi to derive desired benefits.

A conservative estimate by FAO (2004) indicates that 1,069 mushroom species are being used as a food source worldwide. Approximately 283 species of wild mushrooms are used by the ethnic groups in India (Purkayastha and Chandra, 1985). Diversity, nutritional qualities and medicinal potential of Indian mushrooms has been reviewed recently by Thatoi and Singdevsachan (2014). A large segment of tribals in the Himalayas and Western Ghats are dependent on wild macrofungi for their livelihoods like nutrition, health and trade (e.g. Christensen *et al.*, 2008; Karun and Sridhar, 2013; Pahlevanlo and Janardhana, 2012; Pavithra *et al.*, 2015; Rai *et al.*, 2007; Sharma *et al.*, 2009). Compared to Northern and Central India, studies on mushrooms in Southwest India are scanty. In addition, ethno-pharmacological knowledge on macrofungi is fragmentary and confined to the ethnic tribes distributed in different parts of India similar to the aboriginals of Africa, Brazil and Australia. As the Western Ghats and west coast of India are known for a variety of macrofungi (Farook *et al.*, 2013; Karun and Sridhar, 2016; Mohanan, 2011; Pavithra *et al.*,

2016), the present study focuses on four medicinal macrofungi to link their bioactive potential in combating human ailments especially cardiac diseases.

25.2 Macrofungi

Four macrofungi were collected during monsoon and post-monsoon seasons from the west coast and Western Ghats (July-August, 2013). *Amauroderma conjunctum* (Lloyd) Torrend (Basidiomycotina) (Figure 25.1A-B) and *Daldinia concentrica* (Bolton) Ces. & De Not. (Ascomycotina) (Figure 25.1C) were sampled from Mangalore University Campus, Mangalore of the west coast region (12°48'N, 74°55'E; 112.4 m asl). *Ophiocordyceps nutans* (Pat.) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora (Ascomycotina) (Figure 25.1D-E) and *Pycnoporus cinnabarina* (Jacq.) P. Karst. (Basidiomycotina) (Figure 25.1F) were collected from Kadnur coffee agroforest near Virajpet of the Western Ghats (12°13'N, 75°46'E; 891 m asl) (Figure 25.1D-F). Among them, *Daldinia* and *Pycnoporous* were common, *Amauroderma* was frequent and *Ophiocordyceps* was rare. The former three species grow on decomposing wood logs, while the latter grow on pentatomid bugs (*Halyomorpha halys* Stål).

Figure 25.1. Wild macrofungi assessed for bioactive components and antioxidant potential: (A) young and (B) mature *Amauroderma conjunctum*; (C) *Daldinia concentrica*; (D and E) *Ophiocordyceps nutans*; and (F) *Pycnoporus cinnabarina*.

After sampling, *Amauroderma*, *Daldinia* and *Pycnoporus* were transferred to the laboratory, their whole fruit bodies were separated and cleaned to remove debris. After two rinses in distilled water, they were blotted and chopped into pieces, spread on aluminium foil to dry at 55-60 °C in a hot-air oven. Whole fruit bodies of *Ophiocordyceps* grown on dead pentatomid bugs were separated from insect remains, cleaned and processed like other macrofungi. Dried fungi were milled (Wiley Mill, mesh # 30) and refrigerated (4 °C) in airtight containers for assessment of bioactive compounds and antioxidant potential. Mushroom powder extract was prepared in methanol (1 mg/ml; w/v) as well as water (1 mg/ml; w/v) to assess antioxidant potential.

25.3 Assay of bioactive components

Bioactive components like TP, FL and VC of mushroom powder were assessed using standard methods.

25.3.1 Total phenolics

TP was assessed based on the method by Rosset *et al.* (1982). Fifty mg samples of mushroom powder were extracted in methanol (50%, 5 ml) on a water bath (95±1 °C; 10 min) followed by centrifugation (1,500 rpm) to collect the supernatant. Extraction was repeated on dissolving the pellet and pooled supernatant was made up to 10 ml. Aliquot of extract (0.5 ml) was mixed with distilled water (0.5 ml) and treated with sodium carbonate (sodium hydroxide, 0.1 N; 5 ml) followed by incubation (10 min) and addition of Folin-Ciocalteu's reagent (diluted, 1:2, v/v; 0.5 ml). The absorbance was read (725 nm; ultraviolet-visible spectrophotometer-118; Systronics, Gujarat, India). The tannic acid was used as standard and the quantity was expressed as milligram tannic acid equivalents per gram mushroom powder (mg TAEs/g).

25.3.2 Flavonoids

To assess FL content of mushroom powder, method outlined by Chang *et al.* (2002) was followed. The mushroom powder was extracted in methanol (1 mg/ml), aliquots of extract (0.5 ml) was blend with methanol (1.5 ml), aluminium chloride (10%; 0.1 ml), potassium acetate (1 M; 0.1 ml) and distilled water (2.8 ml). The mixture was incubated at laboratory temperature (30 min) and absorbance was read (415 nm). Quercetin served as standard and FL content was expressed in milligram of quercetin equivalents per gram mushroom powder (mg QEs/g).

25.3.3 Vitamin C

The VC content of mushroom powder was determined based on method proposed by Roe (1954) with a slight modification. Mushroom powder (1 g) was extracted with TCA (5%, 10 ml). Aliquots (0.2 ml each) were diluted up to 1 ml using (5%) followed by addition of 2,4-dinitrophenylhydrazine (1 ml). The mixture was boiled (10 min), cooled, sulfuric acid was added (65%, 4 ml) and incubated (30 min) at laboratory temperature followed by measurement of

absorbance (540 nm). To prepare the standard curve, ascorbic acid (Sisco Research Laboratories, Mumbai, India; purity, 99.8%) was used and VC content was expressed as ascorbic acid equivalents in milligram per gram mushroom powder (mg AAEs/g).

25.4 Antioxidant assays

Antioxidant potential of macrofungi were evaluated by standard methods: TAA by reduction of Mo(VI) to Mo(V) by antioxidant compounds (Prieto *et al.*, 1999); FCC was detected by ferrous ion-ferrozine complex formation (Hsu *et al.*, 2003); DPPH radical-scavenging activity on exposure to radical scavengers (Singh *et al.*, 2002); RP was evaluated by conversion of Fe^{3+}/ferricyanide complex to the ferrous form (Oyaizu, 1986).

25.4.1 Total antioxidant activity

To determine the TAA, the mushroom extract (0.1 ml) was mixed with reagent mixture (sulfuric acid, 0.6 M + sodium phosphate, 28 mM + ammonium molybdate, 4 mM, 1 ml) followed by incubation (95 °C, 90 min) and cooled to laboratory temperature to measure absorbance of phosphomolybdenum complex (695 nm) with methanol as blank. The TAA was expressed as micromole AAE per gram of mushroom powder (μM AAEs/g).

25.4.2 Ferrous ion-chelating capacity

To determine the FCC, mushroom extract (1 ml), ferric chloride (2 mM, 0.1 ml) and ferrozine (5 mM, 0.2 ml) were mixed and made up the volume to 5 ml using methanol. On incubation of mixture (10 min) at laboratory temperature, the absorbance of Fe^{2+}-ferrozine complex was measured (562 nm). Control consists of sample without extract to calculate ferrous ion chelating capacity:

$$\text{Ferrous-ion chelating capacity (\%)} = [1 - (A_{s562}/A_{c562})] \times 100 \quad (1)$$

Where, absorbance of the control is A_c and absorbance of sample is A_s.

25.4.3 DPPH radical-scavenging activity

To determine the DPPH free radical-scavenging activity, DPPH (0.01 mM, 4 ml) was added to mushroom extract (1 mg in 1 ml methanol) to react at laboratory temperature (20 min). Reagents in absence of mushroom extract served as control to measure absorbance (517 nm) to calculate the DPPH radical-scavenging activity:

$$\text{DPPH radical-scavenging activity (\%)} = [1 - (A_{s517} - A_{c517})]/(A_{s517}) \times 100 \quad (2)$$

Where, absorbance of the control is A_c and absorbance of sample is A_s.

25.4.4 Reducing power

To determine the RP, increasing concentrations (0.21 mg in 0.2-1 ml) of mushroom powder were transferred to the phosphate buffer (0.2 M, pH 6.6, 2.5 ml) followed by potassium ferricyanide (1%, 2.5 ml). After mixing, incubated (50 °C, 20 min), added TCA (10%, 2.5 ml) and centrifuged (3,000 rpm, 10 min). For supernatant (2.5 ml) distilled water (2.5 ml) was added, mixed and on addition of ferric chloride (0.1%, 0.5 ml) the absorbance (700 nm) was measured. Increased absorbance with increased concentration reveals increase in RP.

25.5 Data analysis

The differences in bioactive components between macrofungi and antioxidant activities between methanol and aqueous extract were assessed by *t*-test (StatSoft Inc., 2008). The PCA was employed to find out the relationship between bioactive components (TP, FL and VC) against antioxidant potential (TAA, ferrous-ion chelating capacity, DPPH radical-scavenging activity and RP) of methanol and aqueous extracts (SPSS 16.0: www.spss.com). The score of PCA plots for methanol and aqueous samples were separately grouped for bioactive components against antioxidant activities.

25.6 Observations and discussion

25.6.1 Bioactive components

Among the four macrofungi studied, the TP was significantly higher in *Pycnoporus* (55.1 mg/g) than the rest (22.8-26.1 mg/g; $P<0.001$; Figure 25.2A). The TP is higher than several wild edible mushrooms (Keleş *et al.*, 2011), while comparable with uncooked, cooked and fermented (*Rhizopus oligosporus*) seeds of *Canavalia* spp. (Niveditha and Sridhar, 2014). The quantity of FL was significantly higher in *Pycnoporus* (128 mg/g), followed by *Amauroderma* (31.4 mg/g; $P<0.001$) and present in low quantities in others (2.2-4.3 mg/g; $P>0.05$; Figure 25.2B). The TP content of all macrofungi and FL content of *Amauroderma* and *Pycnoporus* were higher than many edible mushrooms (Hussein *et al.*, 2015; Karun *et al.*, 2016). Among FL, flavones intake is known for risk management of cardiovascular disease (McCullough *et al.*, 2012). However, besides flavones in FL family, anthocyanidins, catechins, flavanones, flavonols, isoflavones and proanthocyanidins are also known to be beneficial in prevention of cardiovascular diseases (Gross, 2004). The VC content of macrofungi was ranged between 0.46 mg/g (*Daldinia*) and 0.41 mg/g (*Ophiocordyceps*) without significant difference ($P>0.05$; Figure 25.2C). Its content in macrofungi studied is lower than edible mushrooms (e.g. *Auricularia* and *Termitomyces*) (Karun *et al.*, 2016), while higher than seeds of *Canavalia* (Niveditha and Sridhar, 2014).

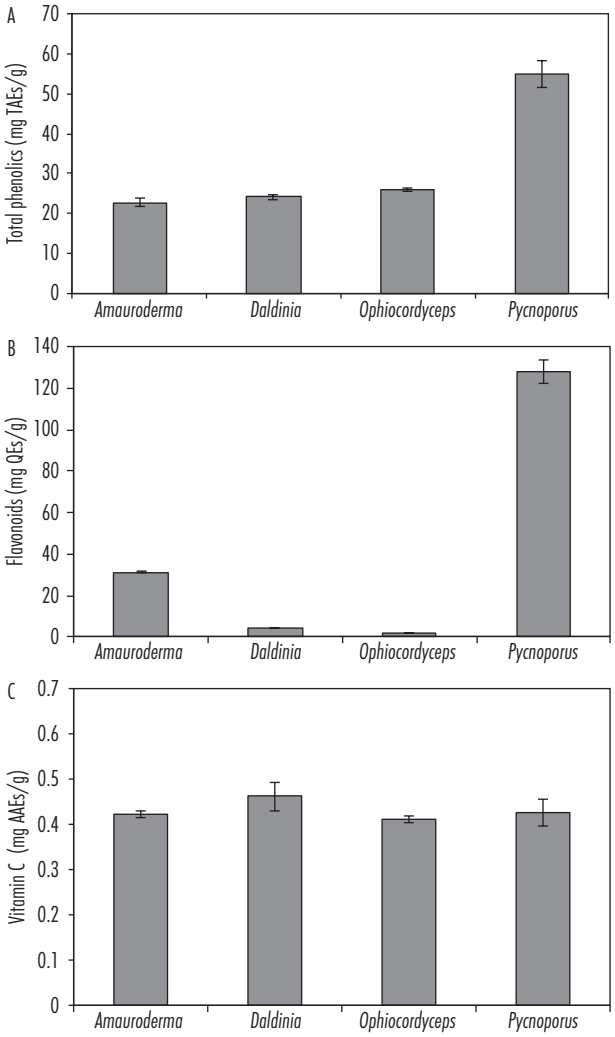

Figure 25.2. Bioactive principles of four wild macrofungi: (A) total phenolics (TAE = tannic acid equivalents); (B) flavonoids (QE = quercetin equivalents); and (C) vitamin C (AAE = ascorbic acid equivalents).

25.6.2 Antioxidant potential

All the four antioxidant potential of methanol extract were significantly higher in all macrofungi than in aqueous extract ($P<0.01$; Figure 25.3). The TAA of methanol extract was significantly higher in *Pycnoporus* (506.3 μM/g) compared to *Amauroderma* (389.5 μM/g), *Ophiocordyceps* (203.4 μM/g) and *Daldinia* (192.4 μM/g; $P<0.001$; Figure 25.3A). The TAA in aqueous extract was significantly higher in *Ophiocordyceps* than the rest (128.7 vs 58.7-79.5 μM/g; $P<0.05$). The

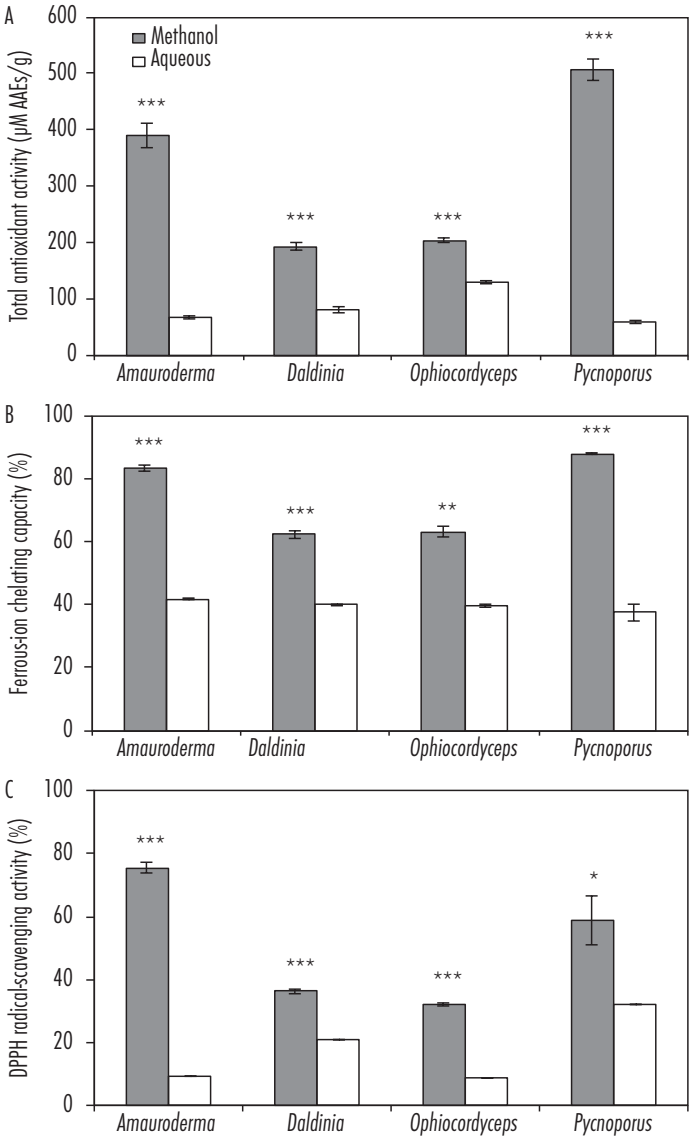

Figure 25.3. Antioxidant activities of methanol and aqueous extracts of four wild macrofungi: (A) total antioxidant activity (AAE = ascorbic acid equivalents); (B) ferrous ion-chelating capacity; and (C) DPPH radical-scavenging activity (**P*<0.05; ***P*<0.01; ****P*<0.001).

TAA of macrofungi studied is comparable to edible mushrooms (*Auricularia* and *Termitomyces*) (Karun *et al.*, 2016) and extracts (bark and leaf) of many medicinal plant species (Iqbal *et al.*, 2015). Similar to TAA, the FCC was highest in *Pycnoporus* (87.8%) followed by *Amauroderma* (83.2%), *Ophiocordyceps* (63%; *P*<0.05) and *Daldinia* (62.2%; *P*>0.05; Figure 25.3B). The FCC

in aqueous extract ranged between 37.6-41.7% without significant difference ($P>0.05$) and comparable to edible mushrooms (*Auricularia* and *Termitomyces*) (Karun *et al.*, 2016). The DPPH radical-scavenging activity was significantly higher in *Amauroderma* (75.5%) followed by *Pycnoporus* (58.8%; $P<0.001$), *Daldinia* (36.4%) and *Ophiocordyceps* (32.1%; $P>0.05$; Figure 25.3C). In aqueous extract, *Pycnoporus* and *Daldinia* showed significantly higher DPPH radical-scavenging activities than *Amauroderma* and *Ophiocordyceps* ($P<0.001$). The DPPH radical-scavenging activity of macrofungi studied is higher than wild edible mushrooms (Karun *et al.*, 2016), while comparable with other edible wild mushrooms (Hussein *et al.*, 2015; Keleş *et al.*, 2011).

The RP (at 1 mg concentration in methanol) was highest in *Daldinia* followed by *Amauroderma*, *Pycnoporus* and *Ophiocordyceps* (Figure 25.4A). It was significantly higher in methanol than aqueous extract of *Daldinia* ($P<0.001$) followed by *Pycnoporus* ($P<0.01$) and *Ophiocordyceps* ($P<0.01$; Figure 25.4B). The TAA, FCC, DPPH radical-scavenging activity and RP of macrofungi are comparable or higher than uncooked, cooked and fermented (*Rhizopus oligosporus*) seeds of *Canavalia* spp. (Niveditha and Sridhar, 2014).

25.6.3 Principal component analysis

The PCA of bioactive principles of four macrofungi against antioxidant potential of methanol extract resulted in two components with 100% variance (Eigen value <1; PC1: 63.79%; PC2: 36.21%). The first cluster composed of bioactive components TP (DTP and PTP), FL (DFL) and VC (OVC) with TAA (ATA and DTA), FCC (AFC, DFC, OFC and PFC) and RP (PRP) (Figure 25.5A). The second cluster composed of bioactive components TA (OTP), FL (PFL) and VC (AVC and DVC) with DPP (ADP and DDP) and RP (ARP and DRP).

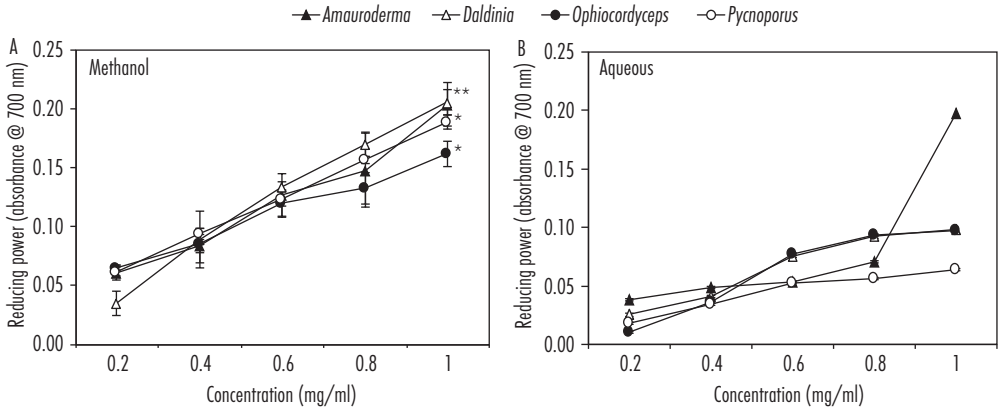

Figure 25.4. Reducing power of methanol (A) and aqueous (B) extracts of four wild macrofungi (*$P<0.01$; **$P<0.001$).

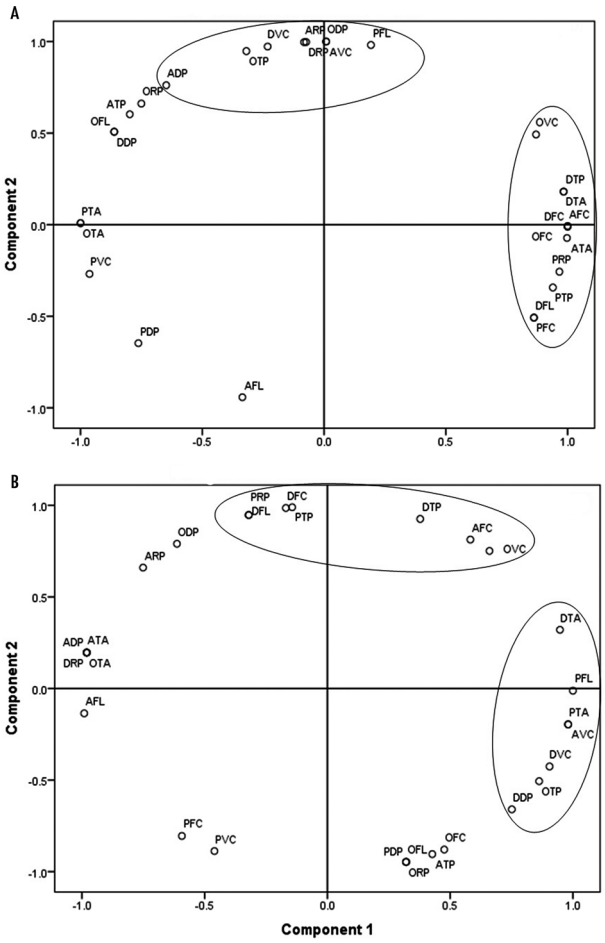

Figure 25.5. Principal component analysis of four wild macrofungi given with first letter (A = *Amauroderma conjunctum*; D = *Daldinia concentrica*; O = *Ophiocordyceps nutans*; P = *Pycnoporus cinnabarina*) followed by bioactive components with two letters (TP = total phenolics; FL = flavonoids; VC = vitamin C) and antioxidant potential with two letters (TA = total antioxidant activity; FC = ferrous-ion chelating capacity; DP = DPPH radical-scavenging activity; RP = reducing power) of methanol (top panel, A) and aqueous (lower panel, B) extracts.

The PCA of bioactive principles of four macrofungi against antioxidant potential of aqueous extract resulted in two components with 100% variance (Eigen value <1; PC1: 62.35%; PC2: 37.65%). The first cluster composed of bioactive components TP (OTP), FL (PFL) and VC (AVC and DVC) with antioxidant activities TAA (DTA and PTA) and DPP (DDP) (Figure 25.5B). The second cluster composed of bioactive components TP (DTP and PTP), FL (DFL) and VC (OVC) with FCC (AFC and DFC) and RP (PRP).

All the bioactive principles evaluated (TP, FL and VC) in macrofungi irrespective of methanol and aqueous extracts were clustered with at least two antioxidant properties strengthens the assumption that these macrofungi have potential role in combating the cardiovascular diseases.

25.6.4 Therapeutic potential

Ismail *et al.* (2014) demonstrated that aqueous and methanol extract of *Amauroderma* sp. has a high antioxidant potential and is devoid of cytotoxic, antivirus and antibacterial activities and therefore suitable for therapeutic purposes. Based on antioxidant potential and anti-inflammatory properties of *Amauroderma rugosum*, Chan *et al.* (2013) concluded that the mycelial extract possesses ethyl linoleate and ergosterol, which contribute in mitigating inflammatory disorders leading to epilepsy. Unsaturated fatty acids in *Amauroderma subresinosum* amount to 80% of total fatty acids is an added advantage for its therapeutic value (Quang *et al.*, 2011). The medicinal mushroom *Amauroderma rude* showed powerful anti-cancer activities (Jiao *et al.*, 2013).

D. concentrica is used in treatment of stomach ulcer, skin disease, whooping cough and prevention of excessive growth of fetus to ease the delivery in Nigeria (Oyetayo, 2011). *Daldinia* sp. has the potential to inhibit *Salmonella* spp. (Ribeiro *et al.*, 2012), while methanol and aqueous extracts of *D. concentrica* was inhibitory against several pathogenic bacteria (e.g. *Salmonella typhi*) and fungi (e.g. *Aspergillus flavus*) (Kavitha *et al.*, 2011). The metabolite concentricolide derived from *D. concentrica* is known to inhibit HIV-1 (Qin *et al.*, 2006).

The caterpillar fungi *Cordyceps* and *Ophiocordyceps* are known for excellent medicinal value in China (Lo *et al.*, 2013; Paterson, 2008). The *Ophiocordyceps sinensis* besides traditionally used in medicine in Himalayas (Shrestha *et al.*, 2012), it has also potential to reduce acute and chronic rejection associated with cardiac transplantation (Jordan *et al.*, 2008) and its metabolite butylated hydroxytoluene has a major role in prevention of atherosclerosis (Jilal and Devraj, 1996; Yu *et al.*, 2012).

P. cinnabarina is well known for several value-added products like flavor, pigment and β-glucan (e.g. Boonyanuphap and Hansawasdi, 2010; Zheng *et al.*, 2007). High quantity of β-glucan in *P. cinnabarina* was linked to the specific habitat characteristic features (e.g. high altitude, crown cover and rainfall) helps in management of forest ecosystem in favor of medicinally important component in a given forest ecosystem (Boonyanuphap and Hansawasdi, 2010). It has the capacity to convert p-coumaric acid into caffeic acid as a strong natural antioxidant, which involve in strengthening resistance of human low-density lipoprotein to oxidative modifications aids in remediating atherosclerosis (Alvarado *et al.*, 2003). The *P. cinnabarina* is also a strong producer of lignin degrading laccases (Camarero *et al.*, 2012; Eggert *et al.*, 1997).

25.7 Conclusions

In the recent past, it is known that cardiovascular diseases and diabetes are the major causes for human mortality in the western and Asia-Pacific countries (Celermajer *et al.*, 2012). Edible and medicinal mushrooms owing to their high fiber and low fat contents serve in prevention of cardiovascular diseases including atherosclerosis (De Silva *et al.*, 2013). In addition, TP content has a major role in limiting the risks of cardiovascular and related diseases (Pandey and Rizvi, 2009). All the macrofungi in our study possess a higher quantity of TP than leafy vegetables and other edible mushrooms which serve as an added advantage. Similarly, high quantities of FL especially in *A. conjunctum* and *P. cinnabarina* needs further insight to quantify flavone and flavonol which are most strongly associated with lowering the risk of coronary heart diseases (Peterson *et al.*, 2012). The present study provides preliminary account on antioxidant potential of four wild macrofungi in turn helpful in combating cardiovascular diseases. The bioactive potential of a macrofungus may also be dependent on the habitat (e.g. old growth forest, sacred grove, forest reserve and monoculture forest) as well as the host on which it establishes (e.g. wood, insect, caterpillar and dung) which needs further investigation. As the reproductive structures of macrofungi have been evaluated in our study, it is essential to follow whether their vegetative stages also possess similar bioactive potential. Owing to their long history of utilization in Chinese medicine, further precise studies are warranted to follow their potential in alleviating reactive-oxygen-scavenging, cholesterol lowering, membrane-stabilizing and glycoprotein-modulating capabilities in prevention of cardiovascular diseases.

Acknowledgments

Authors are grateful to Mangalore University for permission to carry out this research in the Department of Biosciences. CNA, GAA and SDG acknowledge the INSPIRE Fellowship award by the Department of Science and Technology, New Delhi, India. KRS is grateful to the University Grants Commission, New Delhi, India for the award of UGC-BSR Faculty Fellowship.

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About the editors

Ronald R. Watson, PhD, earned his PhD in biochemistry from Michigan State University. His postdoctoral schooling in nutrition and microbiology was completed at the Harvard School of Public Health. Dr Watson was assistant professor of microbiology and immunology at the Indiana University Medical School and associate professor of nutrition at Purdue University. Dr Watson joined the faculty at the University of Arizona Health Sciences Center in the Department of Family and Community Medicine of the School of Medicine. He is a member of the Sarver Heart Center. His primary appointment now is professor of health promotion sciences in the Mel and Enid Zuckerman Arizona College of Public Health. He has 14 patents on dietary supplement and health promotion. He continues to do research in animals and in clinical trials on dietary supplements and health. Dr Watson has published over 450 articles in peer-reviewed journals and has an h-index of 25. He and Dr Zibadi have collaborated extensively on cardiac and immunology research.

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