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# **BASIC AND CLINICAL ENDOCRINOLOGY UP-TO-DATE**

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Edited by **Fulya Akin**

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## **Basic and Clinical Endocrinology Up-to-Date**

Edited by Fulya Akin

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## Preface

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This book provides the most up-to-date information on the basic and clinical aspects of endocrinology. It offers both researchers and clinicians experts, gold-standard analysis of endocrine research and translation into the treatment of diseases such as insulinoma, endocrine disease in pregnancy and steroid induced osteoporosis.

Investigates both the endocrine functions of the kidneys and how the kidney acts as a target for hormones from other organ systems.

Presents a uniquely comprehensive look at all aspects of endocrine changes in pregnancy and cardiovascular effects of androgens. Clear translational presentations by the top endocrinologists in each specific hormone or functional/systems field.

This book contains many important innovations.

I would like to thank our authors who have helped in the preparation of this book. We hope it would be useful as an up-to-date resource for endocrinology.

We are particularly grateful for support of our publishers. Their efforts have been essential in ensuring the successful publication of this high quality book.

As always, we would like to acknowledge the debt we owe to our families for their support during the inevitable periods when 'book work' intruded on family life.

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# **Part 1**

## **Thyroid Disease**



# Thyroid Disruptors: How They Act and How We React

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Raquel Bueno Barbieri, Marjory Alana Marcello, Lucas Leite Cunha,  
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## 1. Introduction

### 1.1 Actual thyroid disease demographics

Thyroid diseases increase in a ubiquitous global phenomenon suspected to further rise in the upcoming decades (Dua et al. 2008). Thyroid cancer is the most common endocrine malignancy, representing 2% of all malignancies. A rapid global rise in its incidence has been seen in recent decades, especially concerning the papillary type, which has been increasing in several countries (Liu et al. 2001; Reynolds et al. 2005; Davies and Welch 2006; Enewold et al. 2009; Kilfoy, Devesa et al. 2009; Kilfoy, Zheng et al. 2009). An estimated 44,670 new cases of thyroid cancer are expected to be diagnosed in the United States of America in 2010, with three out of four cases occurring in women. The incidence rate of thyroid cancer has been increasing sharply since the mid-1990s, and it is the fastest growing cancer in both men and women, as well as the ninth most common human malignancy in the USA (American Cancer Society: Cancer Facts and Figures 2010).

The reasons for the increase in occurrences of this type of cancer are still unclear and controversial. Earlier studies suspected that the upward trend was caused by the widespread use of radiation therapy for benign conditions of the head and neck among children and adolescents from the early 1920s to the late 1950s (Weiss 1979; Pottern et al. 1980). Other studies suggested that this trend could be associated with atmospheric nuclear fallouts (Catelinois et al. 2004; Gilbert et al. 1998; Kazakov, Demidchik, and Astakhova 1992; Kerber et al. 1993; Takahashi et al. 2003) or constant diagnostic X-ray exposures (Prokop 2001; Ron et al. 1995) suffered especially by children (Brenner et al. 2001; Golding and Shrimpton 2002; Maitino et al. 2003). More recent studies suggested that the prevalence of thyroid nodules depends on the screening method and population evaluated according to geographic regions and racial groups, indicating that the widespread use of ultrasonography and socioeconomic indicators of health care access could be the major players in this new demography of the thyroid cancer (Morris and Myssiorek 2010; Sprague, Warren Andersen, and Trentham-Dietz 2008).

The steep increase in thyroid cancer is certainly related to better cancer detection by ultrasonography and other imaging techniques in a population growing older, as well as a larger and easier access to health care and robust laboratory diagnostic tools such as TSH and thyroid antibody dosages (Davies and Welch 2006; Ward and Graf 2008). However,

there are strong indicators that other causes may be involved. Better techniques and larger access to health care cannot be held responsible for the increasing incidence rates of larger tumors, which have been similar to papillary microcarcinoma (Hughes et al. 2011).

In addition, an epidemiological study of thyroid cancer cases in a highly radiated territory in Poland – i.e. in the province of Opole after 1986 – indicate a significant increase in thyroid cancer incidence in males and females during the period 1995-2002, when comparing with the years 1987-1994. The data comprises all the thyroid cancer cases registered in Opole province in the years 1987-2002, originated from the Provincial Cancer Registry in Opole (Tukiendorf, Miszczyk, and McEwan 2010). Finally, the proportion of local staged thyroid cancer increased by 24% in the Black group, 14.4% in the Hispanic white group, 14.3% in the non-Hispanic white group, and only 4.0% in the Asian group between the periods 1992-1996 and 2000-2004. Five-year survival rates of patients with papillary tumor were approximately 95%; however, that of anaplastic tumor ranged from 5.6% to 11.4% among REGs (Race/Ethnicity Groups) (Yu et al. 2010).

Autoimmune thyroid disorders, comprising Graves' disease and Hashimoto's thyroiditis, are considered polygenic, multifactorial diseases characterized by an abnormal activation of the immune system as a result of interactions between genetic predisposition and environmental factors, the former accountant for approximately 70 – 80% of liability to develop autoimmune thyroid disorders (Wiersinga 1999). Accumulating data confirm that the burden of autoimmune diseases is rising as well, affecting 5 to 10% of the world population. They are a significant cause of morbidity and mortality accounting for soaring health care costs, comparable to those of cancer and heart disease (Cooper, Bynum, and Somers 2009; Eaton et al. 2007; Shoenfeld et al. 2008).

Hashimoto's disease and Graves' disease are the two most common forms of autoimmune thyroiditis, the archetypal organ-specific autoimmune disease in humans. Both are characterized by lymphocytic infiltrate and autoreactivity against thyroid autoantigens (Michels and Eisenbarth 2010; Rapoport and McLachlan 2001; Zenewicz et al. 2010). There are strong evidences for a major role of heredity in Graves' disease, as demonstrated with a population-based study of two Danish twin cohorts (Brix et al. 2001). This study indicated that 21% of the causes involved in Graves' disease development can be attributed to environmental factors (Brix et al. 2001; Prummel, Strieder, and Wiersinga 2004). However, recent analysis of thyroid disease demographics indicate that environmental factors probably have been playing an important role in the observed rise in thyroid disease incidence all over the world (Morris and Myssiorek 2010). Among these environmental causes, viral infections have been repeatedly demonstrated related to type 1 diabetes mellitus and multiple sclerosis (Cabrera-Rode et al. 2003; Dahlquist et al. 1995; Hiltunen et al. 1997; Hyoty and Taylor 2002; Lonnröt, Korpela et al. 2000; Lonnröt, Salminen et al. 2000). In addition, other factors including ionizing radiation, dietary iodine intake, reproductive factors like estrogens, and cigarette smoking might be considered as thyroid disruptors, working as triggers for thyroid disease.

## **2. Main disruptors**

### **2.1 Ionizing radiation**

Ionizing radiation has been being described as the known major risk factor for thyroid cancer, especially papillary carcinoma, the most common thyroid cancer type (Nikiforov 2010). Convincing evidence exists of an association between exposure to external radiation

and an increased risk of thyroid cancer (Mushkacheva et al. 2006; Pottern et al. 1990; Ron et al. 1995; Ron et al. 1989; Schneider et al. 1993; Shore et al. 1993; Shore et al. 2003; Yoshimoto et al. 1995); however, risks from internally deposited radioactive iodine were not well studied until recently (Zablotska et al. 2011; Kazakov, Demidchik, and Astakhova 1992; Shibata et al. 2001; Takahashi et al. 2003; Dobyns et al. 1974).

Well-documented examples include medical therapeutic external beam radiation and accidental exposure to c-radiation and radioiodine as a result of nuclear weapon explosions or nuclear reactor accidents (Ron et al. 1995).

Both radiations (Ostroumova et al. 2009) and thyroid benign nodules (Lyon et al. 2006; Brent 2010) are related to autoimmune processes.

## **2.2 Radiation and thyroid nodules**

The growing incidence of thyroid cancer over the last decades is entirely due to papillary carcinomas, whereas the incidence of other thyroid cancer types, including the follicular type, remains unchanged or is decreasing (Nikiforov 2010). A better understanding of the biology and etiologic factors responsible for the development of papillary carcinomas is particularly important for unraveling the reasons behind the general increase in thyroid cancer incidence.

Mahoney et al (Mahoney et al. 2004) have described that marked increases in the incidence of thyroid cancer after the Chernobyl incident have occurred in all areas of the Republic of Belarus and among all age groups. However, the greatest increases have occurred in children, suggesting that a high prevalence of pre-existing iodine deficiency in combination with unique susceptibility among younger people might have contributed to potential carcinogenic exposures to the thyroid.

Ron et al. (Ron et al. 1995) presented a pooled analysis of five cohort and two case-control studies concerning external radiation and thyroid cancer. The study reported a statistically significant excess relative risk per Gy for individuals exposed before 15 years of age. There was no evidence of increased thyroid cancer risk for individuals exposed at older ages. More recent analyses of atomic bomb survivors confirmed a strong inverse association between ERR/Gy and age at exposure, but suggested that adult exposure is associated with a small increase in thyroid cancer risk (Preston et al. 2007). In 2011, Schonfeld et al. (Schonfeld, Lee, and Berrington de Gonzalez 2011) presented an overview of the use of radiation for medical purposes and its significance for thyroid cancer, especially among children, who seem to be more susceptible to the effects of ionizing radiation. In their study, Schonfeld et al analyzed data concerning x-ray use for dental purposes and did not find significant differences among patients that were more exposed to that type of radiation. In what concerns nuclear medicine, there are no data relating that segment to thyroid cancer, and the authors link that to the fact that kids are hardly ever exposed to those types of exams. To analyze data on diagnostic computed tomography, Schonfeld et al used Analytica software for the calculations to estimate means and 95% uncertainty limits. Using this methodology, they calculated that about 1000 future thyroid cancers could be related to computed tomography scans conducted in the United States of America during the year 2007.

There are also some reports on the effects of radiotherapy for cancers and development of thyroid cancer. Thyroid cancer is one of the most common second cancers after radiotherapy during childhood for Hodgkin lymphoma, and significant increased risks of thyroid cancer have been observed from 5 to more than 40 years after childhood radiotherapy (Ng et al. 2010). In addition to cancer treatment, radiotherapy for the treatment of benign conditions,

including (but not restricted to) *tinea capitis*, hemangioma, and enlarged thymus, has been clearly associated with increased thyroid cancer risk (Ron 2003).

Hayashi et al (Hayashi et al. 2010) found a statistically significant radiation dose-response for the prevalence of these tumors, with an excess odds ratio (OR) per gray of radiation (Gy) of 0.57. The excess risk was limited to women, and among them, the excess OR was 2.4 Gy. These data raise the possibility that the incidence of thyroid microcarcinomas may in fact be related to radiation exposure and point to another interesting issue on the fact that this effect is more important in females.

In fact, many studies have been associating thyroid cancer with exposure to  $^{131}\text{I}$ , which can cause several molecular alterations in genes such as *RET*, *NTRK1*, *RAS*, and *BRAF* (Nikiforov et al. 1997). Remarkably, the mechanisms of activation of these genes are quite different in tumors associated with radiation exposure when compared with those from the general population. The dominant mutation mechanism in radiation-induced tumors consists of chromosomal rearrangements, such as those leading to the formation of oncogenic *RET/PTC*, *TRK*, and *BRAF/AKAP9* fusions. A very different mutation mechanism, i.e. a point mutation, is the most prevalent in sporadic tumors, which typically carry point mutations of *BRAF* and *RAS* genes (Nikiforov 2010). Correlation between radiation exposure and high frequency of *RET/PTC* in papillary carcinomas has been seen after accidental radiation exposure from Chernobyl, in patients treated by external beam radiation and, more recently, in atomic bomb survivors in Japan (Hamatani et al. 2008). In the study reported by Hamatani et al, the presence of *RET/PTC* rearrangement had direct correlation with increasing radiation dose, whereas *BRAF* point mutation had strong inverse correlation with the dose.

### 2.3 Radiation and thyroid autoimmune diseases

The most common thyroid manifestation of radiation is hypofunction, in addition to thyroid nodules and thyroid cancer. Autoimmune thyroid disease has been linked to therapeutic medical radiation, as well as environmental radiation exposure (Brent 2010; Sklar et al. 2000; Dunkelmann et al. 2004; Simon et al. 2009). Both the atomic bomb detonations in Japan and nuclear contamination from the Chernobyl nuclear power plant accident were associated with an increased risk of autoimmune thyroid disease (Hamatani et al. 2008; Sklar et al. 2000; Brent 2010). This association, however, has not been a consistent finding, with several studies showing no effect (Imaizumi et al. 2006; Agate et al. 2008). Radiation effects are generally associated with greater radiation exposure and can be tracked with position of the exposed individual at time of the accident or, in the case of the Chernobyl exposure, the pattern of the wind currents dispersing radiation. The initial study of atomic bomb survivors in Japan showed an increase in the incidence of thyroid autoimmunity (Nagataki et al. 1994), but another study, with longer follow-up, did not confirm initial data (Sklar et al. 2000). The effect of radiation may, in fact, be related to the iodine intake by exposed individuals. The risk of both thyroid cancer and autoimmunity seems to be enhanced if diets deficient in stable iodine result in chronic thyroid stimulation. Exposure to  $^{131}\text{I}$  alone appears to be associated with a lower risk when compared with acute exposures to the atomic bomb or medical radiotherapy, perhaps due to the associated low dose rate and nonuniform distribution of dose in the gland. A study of children exposed to radiation from Chernobyl showed an increase in thyroid size, higher serum TSH levels, and a greater incidence of thyroid autoantibody positivity in areas where the iodine intake was low (Vermiglio et al. 1999). A more recent study of those exposed to radiation from the Chernobyl accident showed an increase in thyroid autoantibodies, but



not in the incidence of hypothyroidism (Agate et al. 2008). These discrepant data may also be related to the study population. A study regarding exposures to radiation of Hiroshima and Chernobyl (Boice 2006) found disagreeing data on the effects of those exposures and their correlation with thyroid diseases and also suggested that the radiosensitivity of the young thyroid gland is high and most likely relates to subsequent proliferative activity of the gland during puberty and growth, but the reasons for the absence of risk following adult exposures are not entirely clear (Lyon et al. 2006).

A recent publication also confirmed antithyroid antibody production and autoimmune thyroid disease in patients submitted to external radiation for Hodgkin's disease (Brent 2010). In addition, patients receiving  $^{131}\text{I}$  for toxic goiter, later develop Graves' disease and, in some cases, Graves' ophthalmopathy (Dunkelmann et al. 2004). In patients treated with radioiodine for thyroid autonomy, a sensitive TSH receptor antibody measurement showed that low level thyroid autoantibody positivity was associated with the development of Graves' disease after using radioiodine (Dunkelmann et al. 2004).

Although there are several studies reporting the relationship between radiation and thyroid disorders, the mechanisms and the pathways involved in that relationship remain not well understood and should be investigated (Ostroumova et al. 2009) The unfortunate recent events in Japan, i.e. the successive earthquake, tsunami, nuclear plants problems, as well as its weather conditions, led a large population to be exposed to radiation effects, demonstrating the urgent need to adopt international contingency measures related to radiation accidental exposure.

## 2.4 Iodide

The following text is a report of travelers who visited Brazil between the years 1873 and 1875 (Wells 1886). They traveled throughout different regions in the country and were able to report some interesting aspects of life of Brazilians in the Nineteenth century. Their observations demonstrate the ravaging effect of iodide deficiency, especially on certain segments of the population.

*"Goiter is very prevalent amongst the very poor peasants, but is rarely seen in the more well-to-do farmers. The sex seems to make no difference, neither does the colour, for the lightcoloured, brown, and black appear to be equally disfigured. In some instances the excrescence attains an enormous size, like a huge feather pillow hung round the front of the neck and extending to the shoulders on each side, hanging over the chest and forcing the head backwards; they apply various herbs and burnt sponge, but admit that it is almost useless to treat it; a change of locality will often stop its continual development, and very rarely cause it to become reabsorbed. The presence of lime in the waters of the streams and a humid atmosphere are considered the primary causes of the evil; but a poor diet, indolent habits, and an absence of all hygiene and cleanliness, either in person or houses, are undoubtedly great promoting causes." In.: "Exploring and travelling three thousand miles through Brazil from Rio de Janeiro to Maranhão".*

It was already clear in these early years that the mineral composition of water, diet profile, and habits were related to the presence of goiter. In addition, the travelers reported that these risk factors, but not gender and color, seemed to be associated with goiter. However, more than one 100 years after this publication, we still do not fully understand the mechanisms involved in goitrogenesis or thyroid disorders overall. Epidemiological evidences suggest that iodide play a key role not only as a major determinant of thyroid disorders but also influencing other factors affecting the thyroid, as previously explained concerning the effect of ionizing radiation.

## 2.5 Thyroid physiology is closely related to iodide homeostasis

Thyroid hormones are the only iodine-containing compounds with established physiologic significance in vertebrates. The thyroid gland produces two hormones, L-thyroxine (tetraiodothyronine, T<sub>4</sub>) and L-triiodothyronine (T<sub>3</sub>). Iodine is an indispensable component of these hormones. Ingested iodine is absorbed through the small intestine and transported in the plasma to the thyroid, where it is concentrated. Thyroid hormone synthesis requires the uptake of iodide by active transport, thyroglobulin (Tg) biosynthesis, oxidation and binding of iodide to thyroglobulin, and within this protein matrix, oxidative coupling of two iodotyrosines into diiodotyrosines. Initial Tg iodination produces monoiodotyrosine (MIT) and diiodotyrosine (DIT). Further iodination couples two DIT residues, both still in peptide linkage, to produce T<sub>4</sub>. When thyroid hormone is needed, Tg is internalized at the apical pole of thyrocytes, conveyed to endosomes and lysosomes and digested by proteases. After Tg digestion, T<sub>4</sub> and T<sub>3</sub> are released into the circulation. In addition, iodide is able to regulate thyroid activity by a mechanism called "autoregulation". Autoregulation in the thyroid refers to the regulation of iodine metabolism within the thyroid gland, independent of TSH. Wolff and Chaikoff reported that organic binding of iodide within the rat thyroid was blocked when the plasma iodide level achieved a critical threshold (Wolff and Chaikoff 1948). This inhibition defines the Wolff-Chaikoff effect. Afterwards, the researchers demonstrated that this inhibitory effect of excess iodide was transient, lasting from 26–50 hours, and that the thyroid escaped from or adapted to prolonged iodide excess, resuming near-normal hormone synthesis (Wolff, Chaikoff, and et al. 1949). The mechanism responsible for the acute Wolff-Chaikoff effect is not completely understood. Raben et al. demonstrated that the critical parameter of the Wolff-Chaikoff effect was the amount of intrathyroidal iodide but not plasma concentration (Raben 1949). In addition, Wolff-Chaikoff effect has been postulated to be caused by organic iodocompounds formed within the thyroid (Dugrillon 1996).

Studies on iodine metabolism were carried out in rats and demonstrated that adaptation to long-term iodide exposure was caused by a decrease in iodine transport, thereby reducing the intrathyroid iodine concentration below the critical inhibitory levels, therefore, allowing iodination to resume (Braverman and Ingbar 1963). Eng et al. suggested that iodide administration decreases both NIS mRNA and protein expression by a mechanism that is likely to be, at least in part, transcriptional. Their findings support the hypothesis that the escape from the acute Wolff-Chaikoff effect is caused by a decrease in NIS, with a resultant decreased iodide transport into the thyroid (Eng et al. 2001). Even though the control of NIS mRNA abundance by iodide excess is mainly associated with a transcriptional effect, there is evidence suggesting that it might occur at a posttranscriptional level. In fact, Serrano-Nascimento et al. showed for the first time that NIS mRNA abundance and poly(A) tail length were significantly reduced in all periods of iodide treatment, suggesting that iodide is able to regulate in a complex manner thyroid cell metabolism (Serrano-Nascimento, Calil-Silveira, and Nunes 2010).

## 2.6 Disturbs in iodine-intake are closely related to thyroid disorders

If the autoregulatory mechanism is not sufficient to keep thyroid hormone production normal, a further enhancement of thyroid activity will occur by an hypothalamic-pituitary feedback and other mechanisms that tend to stabilize the production of active thyroid hormone (Obregon, Escobar del Rey, and Morreale de Escobar 2005). These mechanisms are

able to guarantee that even small supplies of iodine will be sufficient to keep the thyroid function at a level 5.

Most recently, the situation has inverted: many patients are exposed to large quantities of iodide present in proprietary medications, food, and x-ray contrast media. Furthermore, there are data indicating an excess of iodine intake, probably related to salty snacks and industrialized food largely ingested in many occidental countries, especially by young people and children (Medeiros-Neto 2009).

Despite the widespread use of excess iodide in many places, a few apparently normal subjects with normal thyroid function prior to iodide exposure develop thyroid dysfunction, as previously reviewed (Markou et al. 2001). Many studies indicate that even a small change in the level of iodine intake by a population will lead to a reduced frequency of some thyroid disorders, including goiter, follicular cancer and thyroid nodules; however, other types of disease, such as autoimmune thyroiditis and papillary carcinomas, will become more common (Knobel and Medeiros-Neto 2007; Ward et al. 2007). Despite the evidence provided by previous studies, it is still controversial whether completely normal subjects may develop thyroid function disturbances when exposed to large doses of iodide, since there are some evidences that increased iodine intake is not associated with thyroid dysfunction (Markou et al. 2001; Nauman and Wolff 1993)

The only population in the world in which excessive intake of iodine had been described as a main cause of disease lived in some coastal areas of the Japanese island Hokkaido (Suzuki et al. 1965). The excessive iodine intake in this population was attributed to the daily use of iodine-rich seaweed for consumption. Iodide-induced goiter occurred in approximately 10% in the coastal regions of Hokkaido, a northern island of Japan (Higuchi 1964; Suzuki et al. 1965). Goiter has now disappeared after the restriction of intake of kelp products in the affected areas (Tajiri et al. 1986; Yoshinari et al. 1983). However, Kono et al. reported that the prevalence of nonautoimmune hypothyroidism was still 9.7% in coastal areas of the Hokkaido Islands, and it was proportional to iodide intake, whereas the incidence of nonautoimmune thyrotoxicosis was not related to iodide consumption (Konno et al. 1994). In addition, they also reported that when the iodine intake was restricted, the increased serum TSH concentrations were normalized in patients with negative antithyroid antibodies, but remained elevated in those with positive antibodies (Konno et al. 1993). This result suggests that both iodine intake and immunological features interact in the physiopathogeny of hypothyroidism.

In fact, histological examination of the thyroid of patients with iodine-induced hypothyroidism revealed extensive lymphocytic infiltration in half of the examined thyroids (Mizukami et al. 1993). Various findings were observed in other specimens, including hyperplasia in the follicles with papillary foldings, columnar or cuboidal changes in cells with clear and vesicular cytoplasm and markedly reduced colloid. Most of these findings may be reversed after iodine withdrawal (Mizukami et al. 1993). *In vivo* studies showed that while excess iodine is not necessary for the induction of thyroiditis, the ingestion of excess iodine works to exacerbate autoimmune thyroiditis in this genetically predisposed population (Burek and Talor 2009). Clearly, excess iodine intake has many roles in thyroid dysfunction, and one of these roles is to increase thyroglobulin immunogenicity (Papanastasiou et al. 2007), while another is to increase adhesion molecules on the thyrocyte itself (Sharma et al. 2005; Sharma et al. 2008).

Dietary iodine intake is among the possible environmental influences on the incidence and prevalence of thyroid disease in general and thyroid cancer in particular (Wartofsky

2010). Worldwide, incidence rates of thyroid cancer may vary widely, with the causes potentially related to inherent racial or ethnic, and geographical or environmental differences, including iodine excess or deficiency, and possible radiation exposure (Ain 1995). Increasing papillary thyroid cancer has also been related to increased dietary iodine intake (Dijkstra et al. 2007).

Cardis et al. indicated that iodine deficiency increases the risk of  $^{131}\text{I}$ -related thyroid cancer (Cardis et al. 2005). In addition, they indicated that the use of a dietary iodine supplement containing potassium iodide can reduce the risk of  $^{131}\text{I}$ -related thyroid cancer (Cardis et al. 2005). These authors reported that there would be two mechanisms by which dietary iodine supplementation could be related to the incidence of thyroid cancer after exposure to radioiodines. First, stable iodine given shortly before, during, or immediately after exposure reduces the radioactive iodine uptake by the thyroid and, therefore, reduces the radiation dose to the thyroid (Nauman and Wolff 1993; Zanzonico and Becker 2000). Second, long-term dietary iodine supplementation reduces the size of the thyroid in iodine-deficient areas, and a reduction in thyroid growth, particularly in children, would be expected to be associated with reduced incidence of cancer (Cardis et al. 2005).

In conclusion, iodine is very important for thyroid function and also plays a critical role on the pathogenesis of most thyroid disorders. Even though a body of evidence points iodide as one of the most important thyroid disruptors, it cannot explain, by itself, the increase in thyroid diseases that we have been experiencing in recent years. As stated long before on the above historic report of travellers in Brazil, multiple factors might be related to thyroid diseases, and we remain far from completely understanding how they work.

## 2.7 Reproductive factors – estrogen

In addition to their key role on the physiologic control of female development and reproduction, and the coordination of many other tissues and organ systems to support reproduction, estrogens are also associated with the development of cancers in estrogen-responsive tissues such as breast, uterus, and pituitary, and to thyroid diseases (Jeng and Watson 2011).

Thyroid diseases occur with a marked higher frequency in women than in men. Papillary thyroid carcinoma usually appears during the reproductive age (30-49 years old), in a ratio of three to five females to one male and has the highest incidence in premenopausal women (Ito and Miyauchi 2009; Pinchera 2007; Yeung and Serpell 2008). Thyroid autoimmune diseases are also five to 10 times more common in women than in men (Vanderpump et al. 1995).

Innumerable studies have demonstrated that the human thyroid gland expresses a low level of estrogen receptors (Ceresini et al. 2006; Kawabata et al. 2003; Money et al. 1989). Estrogen receptors expression seems to increase with proliferation of thyroid glandular tissue, both in cases of benign hyperplasia and, in certain instances, of thyroid malignancies as well (Ceresini et al. 2006; Kawabata et al. 2003; Money et al. 1989). Estrogen receptors are presented, in general, as two different isoforms: Estrogen receptors -alpha and Estrogen receptors -beta. It has been hypothesized that an imbalance in the expression of these two isoforms might play a role in the proliferation of thyroid lesions in the setting of estrogen exposure. This molecular interplay has been put forth as a key factor in the prevalence of thyroid carcinoma in young female patients (Ceresini et al. 2006; Zeng et al. 2007; Lee et al. 2005; Inoue et al. 1993).

Treatment with oral estrogen results in a marked increase in thyroxine-binding globulin (Tahboub and Arafah 2009), leading to an increase in the total serum capacity for thyroid

hormone. In normal subjects, this results in an increase in total T4 but only in a transient drop in free T4. However, in hypothyroid patients receiving thyroxine, a persistent elevation of TSH is observed and dose adjustments are necessary. This effect is not observed with transdermal estrogen therapy, as the absence of liver passage of the drug results in a much weaker effect on thyroxine binding globulin synthesis (Tahboub and Arafah 2009).

Soy protein and soybean isoflavones have been associated with reduced T4 absorption (important for hypothyroid infants on soy formula), interference with thyroid hormone action (Messina and Redmond 2006), and increased thyroid autoimmune disease. The usual intake of dietary soy in those with normal thyroid function has no consistent adverse effects (Doerge and Sheehan 2002). Doerge and Sheehan studied isoflavone interference with thyroid hormone action *in vitro* (Doerge and Sheehan 2002).

Chemicals with estrogen-mimicking activities were first documented as causing endocrine disruption in the 1950s. Starting the 1990s, additional mechanisms were demonstrated to be involved, encompassing androgen antagonism and disruption of thyroid hormone transport and action, as well as activities mediated by retinoid and peroxisome proliferator-activated receptors, steroidogenic enzymes and neurotransmitter receptors (Diamanti-Kandarakis et al. 2009). Environmental estrogens are manufactured products that contaminate food, water or air, which humans and animals are exposed to (Foster 2008; Sonnenschein and Soto 1998; Tapiero, Ba, and Tew 2002). Many environmental estrogens readily accumulate in the environment and in animal tissues and have been shown to affect both reproductive and nonreproductive organs/systems in animals and humans (Han et al. 2004; Hossaini et al. 2001; Moon et al. 2007; Tsai 2006). Although the genomic effects of these environmental estrogens have been studied extensively and found to be weak (causing government regulatory agencies to consider them "safe"), until recently little, it was known about their nongenomic effects of estrogens (Jeng and Watson 2011). A prominent characteristic that has greatly contributed to the confusion over their toxicity is the nonmonotonic concentration dependence of their nongenomic responses (Palanza, Parmigiani, and vom Saal 2001; Vandenberg et al. 2006; Watson, Jeng, and Kochukov 2010). Environmental estrogens are suspected of affecting a wide variety of functions by interfering with the actions of physiologic estrogens, but sufficiently sensitive techniques for quantitative documentation of these disruptions have generally been lacking (Jeng and Watson 2011).

In summary, estrogens are thought to be related to thyroid hormone deregulation, although the specific mechanisms involved in their action remain unclear.

## **2.8 Cigarette smoking**

Tobacco consumption has multiple effects both on thyroid function and thyroid morphology. These include minor and probably physiologically unimportant alterations in thyroid hormones (Christensen et al. 1984; Hegedus et al. 1985; Karakaya et al. 1987; Lio et al. 1989; Petersen et al. 1991; Hegedus et al. 1992; Melander et al. 1981; Eden et al. 1984; Sepkovic, Haley, and Wynder 1984; Nystrom et al. 1993) and thyrotropin concentrations (Christensen et al. 1984; Hegedus et al. 1985; Karakaya et al. 1987; Lio et al. 1989; Petersen et al. 1991; Hegedus et al. 1992; Melander et al. 1981; Eden et al. 1984; Sepkovic, Haley, and Wynder 1984), central (thyroid) and peripheral antithyroid actions (Muller et al. 1995; Utiger 1995), thyroid gland stimulation or goitrogenic effects (Christensen et al. 1984; Hegedus et al. 1985; Lio et al. 1989; Ericsson and Lindgarde 1991), carcinogenic properties (McTiernan, Weiss, and Daling 1984; Ron et al. 1987; Kolonel et al. 1990; Preston-Martin et al. 1993; Shore et al. 1993; Hallquist et al. 1994; Galanti et al. 1996; Rossing et al. 2000; Hallquist et al. 1993;

Kreiger and Parkes 2000) and also an increase in the susceptibility to Graves' disease (Bartalena et al. 1995; Bufalo et al. 2008; Prummel and Wiersinga 1993) and, in particular, in the incidence and clinical severity of thyroid-associated ophthalmopathy (Bartalena, Pinchera, and Marcocci 2000).

## 2.9 Graves' disease and ophthalmopathy

There are innumerable environmental factors that can contribute to the development of Graves' disease (at least 20 – 30%), which certainly include smoking, probable stress, iodine and selenium intake, several drugs, irradiation, pollutants, viral and bacterial infections, allergy, pregnancy, and postpartum (Tanda et al. 2009). Cigarette smoking is one of the most studied environmental factor and has been demonstrated to increase the risk of Graves' disease (Bartalena et al. 1995; Bufalo et al. 2008; Prummel and Wiersinga 1993; Vestergaard et al. 2002) and decrease the chance of remission of hyperthyroidism, following antithyroid drug treatment (Glinoe, de Nayer, and Bex 2001; Kimball et al. 2002).

Graves' ophthalmopathy (Heufelder 1998; Krassas and Heufelder 2001) is more frequent and severe, and less responsive to medical treatment in smokers than in non-smokers (Prummel and Wiersinga 1993; Bartalena et al. 1989; Bartalena et al. 1998). The first possible connection between cigarette smoking and Graves' ophthalmopathy was made by Hagg and Asplund who showed that 10 out of 12 patients were current smokers, a figure much higher than that observed in Graves' patients without ophthalmopathy (46%) or in control subjects (31%). In addition, the prevalence of heavy smokers was higher in patients with more severe ophthalmopathy (Hagg and Asplund 1987). In a cross-sectional study reviewing the smoking habits of patients with different thyroid diseases, Bartalena et al (Bartalena et al. 1989) found a 64% prevalence of smokers among patients with Graves' ophthalmopathy, a percentage much higher than that of Graves' disease without eye involvement (48%), and 30% in patients with nontoxic goiter, toxic nodular goiter and Hashimoto's thyroiditis. Finally, Tellez et al showed that the prevalence of eye disease correlated not only with smoking but also with ethnicity. The percentage of smokers was higher in Caucasian than in Asian patients (42% and 7.7%, respectively). This suggests that the genetic background and/or geographic location may influence the prevalence of Graves' ophthalmopathy. Furthermore, this study demonstrated that the effect of smoking was dose-related with a higher proportion of patients developing eye involvement with increasing cigarette consumption (Tellez, Cooper, and Edmonds 1992).

Smoking probably affects cytokine production and effect on the orbit. In this regard, smoke extracts have been shown to synergistically interact with interleukin-1 to increase adipocyte differentiation of orbital preadipocytes in culture (Cawood et al. 2006) and to stimulate glycosaminoglycan production (Cawood et al. 2007). It is well accepted that cytokines play an important role in the pathogenesis of Graves' ophthalmopathy (Krassas and Heufelder 2001; Heufelder 1995; Krassas et al. 2001). Cigarette smoking may affect this process because smoking-induced hypoxia in the retrobulbar tissue has been shown to increase the release of cytokines from orbital fibroblasts and endothelial cells (Metcalf and Weetman 1994) and thereby may enhance the expression of adhesion molecules (Heufelder and Bahn 1992). Based on this evidence, refrain from smoking should be strongly recommended to patients with Graves' disease and Graves' ophthalmopathy (Bartalena et al. 2008). In an observational study, smoking cessation was associated with Graves' patients with a decreased risk to develop exophthalmos and diplopia (Pfeilschifter and Ziegler 1996). Smoking seems to influence not only the occurrence and the course of Graves'

ophthalmopathy, but also the response to various therapeutic interventions, such as orbital radiotherapy and glucocorticoid administration (Bartalena et al. 1998), reported that in patients submitted to orbital radiotherapy and high-dose systemic glucocorticoid treatment for severe Graves' ophthalmopathy; a favorable response to treatment was observed in 61 of 65 non-smokers (94%) but only in 58 of 85 smokers (68%).

### **2.10 Smoking and autoimmune thyroid diseases (AITD)**

Hegedus et al (Hegedus et al. 1992) found no differences in thyroid-stimulating immunoglobulins (TSIs) among smoking and non-smoking healthy individuals.

Smoking is an independent risk factor for the development of both Graves' disease and Hashimoto's thyroiditis (Prummel and Wiersinga 1993; Vestergaard et al. 2002).

However, the effect of smoking on Hashimoto's disease seems to be the opposite of its effect on Graves' ophthalmopathy. A relationship between smoking and an increased risk of subsequent hypothyroidism has been demonstrated, possibly associated with an antithyroid effect of thiocyanate generated on tobacco smokers (Fukata, Kuma, and Sugawara 1996). Smoking also increased the risk of later development of hypothyroidism in a follow-up study of the general population (Nystrom et al. 1993). Furthermore, smoking seems to impair the effects of thyroid hormones, aggravating the effects of hypothyroidism in a dose-dependent manner (Muller et al. 1995). A study that evaluated the prevalence of eye signs in patients with Hashimoto's thyroiditis showed that there was no significant relationship between cigarette smoking and either eye abnormalities or calsequestrin or collagen XIII antibodies in these patients. The eye signs correlated only modestly with calsequestrin and collagen XIII antibodies. In contrast to patients with Graves' disease, where cigarette smoking is shown to be a major risk factor for ophthalmopathy (Bartalena and Tanda 2009; Lim et al. 2008; Nassar et al. 2009), there was no relationship between smoking status and eye abnormalities in patients with Hashimoto's thyroiditis (Tjiang et al. 2010).

Muller et al. found that among women with subclinical hypothyroidism, smokers had higher serum TSH levels, higher serum ratio of T3 to free T4, and also higher cholesterol and low-density lipoprotein cholesterol concentrations than non-smokers (Muller et al. 1995). In contrast, patients with overt hypothyroidism, either smokers or non-smokers, had similar serum TSH and thyroid hormone levels (Muller et al. 1995). These results do not indicate that smoking causes hypothyroidism, but that it may increase the severity of the effects of hypothyroidism (Utiger 1995).

Conversely, a decreased prevalence of TPO-Ab in smokers compared with non-smokers was reported among women of the Amsterdam autoimmune thyroid disorders cohort (Strieder, Prummel et al. 2003). The OR of current smoking for the presence of TPO-Ab was 0.69 (95% CI, 0.48–0.99). Data from the third National Health and Nutrition Examination Survey (NHANES III) (Belin et al. 2004) indicated that fewer smokers (11%; 95% CI, 10–13) had TPO-Ab and/or Tg-Ab compared with non-smokers (18%; 95% CI, 17–19). The relationship persisted when analyzing the presence of one antibody independently of the status of the other antibody. A Danish population study (Pedersen et al. 2008) found that smoking was negatively associated with the presence of thyroid antibodies, with the strongest association between smoking and Tg-Ab (OR, 0.5–0.6). More recently, Grigoris et al performed a prospective study that demonstrated that the discontinuation of smoking increased the risk of *de novo* occurrence of serum TPO-Ab and/or Tg-Ab in subjects susceptible for developing autoimmune thyroid disorders. The OR of smoking for developing TPO-Ab and/or Tg-Ab were 0.62 [95% confidence interval (CI), 0.37–1.04] one year before seroconversion, and 0.59

(95% CI, 0.35–0.99) at seroconversion. When only conversion to TPO-Ab is considered, the OR of smoking were 0.58 (95%CI, 0.31–1.09) one year before seroconversion and 0.54 (95%CI, 0.29–1.02) at the time of seroconversion (Effraimidis, Tijssen, and Wiersinga 2009). These studies showed good evidence that smoking decreases the prevalence (Strieder, Prummel et al. 2003; Belin et al. 2004; Pedersen et al. 2008) and incidence (Effraimidis, Tijssen, and Wiersinga 2009) of antibodies TPO and Tg, raising an important issue on the physiopathology of such opposite effects of smoking on autoimmunity.

### 2.11 Smoking and goiter

Differing results have been published concerning the association between tobacco smoking and goiter. Some authors found no association between smoking habits and goiter prevalence (Prummel and Wiersinga 1993; Bartalena et al. 1989; Petersen et al. 1991) or thyroid volume (Gomez et al. 2000; Olbricht and Hoff 1988; Berghout et al. 1987). Others have found a positive association between smoking habits and goiter prevalence (Hegedus et al. 1985; Ericsson and Lindgarde 1991; Christensen et al. 1984; Brix et al. 2000) or thyroid volume (Nygaard et al. 1993; Barrere et al. 2000; Hegedus et al. 1985; Georgiadis et al. 1997). It has been suggested that these discrepancies could be caused by differences in iodine status, as the association seems to be stronger in iodine-deficient areas (Bartalena et al. 1995; Bertelsen and Hegedus 1994). In addition, the association between smoking and thyroid volumes is stronger in areas with iodine deficiency. Serum thyroglobulin is also positively associated with smoking habits, and multiple nodules are found more often in smokers than in non-smokers (Knudsen et al. 2002). All these effects are most likely mediated by thiocyanate acting as a competitive inhibitor of iodine uptake and organification (Knudsen et al. 2002).

### 2.12 Thiocyanate

Thiocyanate, a perchlorate-like goitrogen, acts by preventing iodine trapping (Karakaya et al. 1987). It is generated from cigarette smoke as a detoxifying product of cyanide (Karakaya et al. 1987). The levels of the thiocyanate in the blood are related to the amount of cigarette smoke inhalation (Karakaya et al. 1987).

The competitive inhibition of iodide transport by thiocyanate may be important for understanding the action of thiocyanate *in vivo*. In fact, thiocyanate concentrations obtained by smoking competitively inhibit iodide transportation into the thyroid gland *in vitro* study on porcine thyroid follicles (Fukayama et al. 1992). Thiocyanate also acts independently of the TSH concentration, inhibiting iodide organification and increasing iodide efflux from the cells (Fukayama et al. 1992). Iodine deficiency may enhance the antithyroid action of thiocyanate and iodide excess may diminish this effect. Thereby, thiocyanate may be responsible for the goitrogenic effect of cigarette smoking seen at least in iodine deficient areas (Hegedus et al. 1985). This mechanism may, in part, explain the diverging results and the regional differences in the goitrogenic effect of cigarette smoking.

Although nicotine content in cigarette smoking does not influence iodide turnover (Fukayama et al. 1992), it might cause sympathetic activation, which could increase thyroid secretion (Melander et al. 1981; Bertelsen and Hegedus 1994). Alternatively, nicotine or other component(s) of tobacco smoking, such as benzopyrene, may have direct thyroid-stimulatory actions or stimulating effects on hepatic oxidative metabolism, which in turn may stimulate hepatic conversion of T4 to T3 (Jusko 1979).

It is well known that many factors are involved in the regulation of thyroid volume. TSH levels, iodine concentration, the presence of thyroid autoimmunity and several goitrogens



are generally accepted factors but others, such as sex, age, body weight or personal habits (e.g. cigarette smoking, alcohol abuse) are controversial in regard to their effect on thyroid volume (Pontikides and Krassas 2002).

### 2.13 Thyroid cancer

During the last two decades, several studies have reported a reduced risk of thyroid cancer, mainly in women, although not always statistically significant, in association with cigarette smoking (McTiernan, Weiss, and Daling 1984; Ron et al. 1987; Kolonel et al. 1990; Preston-Martin et al. 1993; Shore et al. 1993; Hallquist et al. 1994; Galanti et al. 1996; Rossing et al. 2000), in the two major histological groups (papillary and follicular cancers) from all geographic regions (Mack et al. 2003).

Kreiger and Parkers recently explored the association of cigarette smoking with thyroid cancer in both sexes in a case-control study (Kreiger and Parkes 2000). Reduced risk was observed for ever/never cigarette smokers of both sexes with an odds ratio of 0.71 for females (95% confidence interval (CI) = 0.60-0.83) and 0.77 (95% CI=0.58-1.02) for males.

Since thyroid cancer occurs more frequently in women than in men, hormonal factors may also be involved in its pathogenesis. The mechanism (s) by which cigarette smoking may be protective for thyroid cancer probably involves the lowering of endogenous levels of TSH [1, 8, 13, 42]. It has been suggested that increased levels of TSH or elevated levels of other thyroid stimulators, as occurs during pregnancy, are associated with an increased risk of thyroid cancer (Ron et al. 1987). Female preponderance of thyroid cancer has led to the hypothesis that estrogen metabolism may play a role in its pathogenesis. In fact, it has been suggested by some studies that smoking may have an anti-estrogen effect (Baron, La Vecchia, and Levi 1990). However, there is no evidence that smoking has similar effect(s) on androgen metabolism (Dai et al. 1988).

A highly significant correlation has been demonstrated between thiocyanate levels in the mother and cord blood, a finding indicating that thiocyanate freely crosses the placenta (Othman et al. 1990). An inverse correlation was found between thiocyanate levels in cord blood, birth weight, significantly decreased serum TSH levels and increased T4 levels in newborn babies of smoking mothers when compared with non-smoking ones (Othman et al. 1990). Thiocyanate has a biological half-life of 1-2 weeks (Gasparoni et al. 1998). Therefore, measurements in plasma may be used to detect the degree of exposure to smoking from days to weeks after the last consumption. Gasparoni et al (Gasparoni et al. 1998) found that infants whose mothers and fathers smoked had higher cord serum thyroglobulin and thiocyanate concentrations in comparison with infants whose parents did not smoke. However, the infants had no other evidence of thyroid abnormalities, such as increased T4, T3 or decreased TSH concentrations (Meberg and Marstein 1986).

Our group demonstrated an inverse association of germline cytochrome P4501A1 (CYP1A1) inheritance and smoking with the risk of thyroid nodules and papillary carcinomas, helping to explain the reduced risk of differentiated thyroid cancer in tobacco consumers observed in epidemiologic studies (Bufalo et al. 2006).

### 2.14 Infections

Infections have been implicated in the pathogenesis of several autoimmune, endocrine and nonendocrine diseases (Tomer and Davies 1993). Furthermore, they have long been invoked as an underlying etiology or trigger factor for the induction of autoimmune thyroid

diseases, such as subacute thyroiditis and autoimmune thyroiditis. They are also suspected to be involved in carcinogenesis and in the onset of thyroid lymphomas (Desailloud et al. 2009). Both viral and bacterial infections might represent a risk factor for autoimmune thyroiditis development in genetically predisposed individuals (Desailloud and Hober 2009; Lehmann et al. 2008; Thomas et al. 2008; Tozzoli et al. 2008; Tomer and Davies 1993).

It is possible that multiple exposures to infections are necessary to train our immune system to perform well, but some infections are able to break the tolerance of susceptible individuals and allow autoimmune diseases to develop (Davies 2008). Specific infections could be a triggering factor to disease initiation by liberating antigens (via cell destruction or apoptosis), by forming altered antigens or causing molecular mimicry, by cytokine and chemokine secretion, or by inducing aberrant human leukocyte antigen (HLA)-DR expression and toll-like receptor activation (Harii et al. 2005). Clearance of cell debris can lead to abnormal antigen presentation, especially that of previously sequestered antigens (Mackay, Leskovsek, and Rose 2008). Infection of a rat cell line with reovirus types 1 and 3 induces thyroid cell major histocompatibility complex class II, which may allow presentation of thyroid antigen to the immune system and participate in the initiation of autoimmune thyroid disease (Neufeld, Platzer, and Davies 1989).

Paradoxically, infections may both enhance autoimmune thyroiditis and also act as a protection against autoimmune thyroiditis. There is growing acceptance of a hygiene hypothesis which implies that the immune system is educated by multiple exposures to different infections, allowing better controlled autoimmune responses (Bach 2005; Bloomfield et al. 2006). Thus, improved living standards and decreased exposure to infections are associated with an increased risk of autoimmune disease and lower socioeconomic groups have a reduced prevalence of thyroid autoantibodies (Bach 2005; Kondrashova et al. 2008). A recent study carried out in two adjacent regions of Finland (good socioeconomic and hygiene conditions) and Russia (lower socioeconomic and hygiene conditions), with likely similar genetic background, showed that the presence of chronic autoimmune thyroiditis (and subclinical hypothyroidism) among schoolchildren was much higher in the Finnish population than in the Russian population (Kondrashova et al. 2008).

The best-studied infection in autoimmune thyroiditis is that with *Yersinia enterocolitica*. Humoral and cellular immunity against *Yersinia enterocolitica* in patients with autoimmune thyroid diseases has been demonstrated (Bech et al. 1974; Lidman et al. 1974), and Wenzel et al (Wenzel et al. 1988; Wenzel et al. 2003) have found antibodies against virulent plasmid-encoded proteins of *Yersinia enterocolitica* in a large proportion of patients with autoimmune diseases. A potential mechanism could be molecular mimicry (Albert and Inman 1999). In a recent Dutch study, an increased prevalence of antibodies to enteropathogenic *Yersinia enterocolitica* virulence proteins was found in relatives of autoimmune diseases patients, but this was unrelated to the higher prevalence of TPO antibodies in these subjects (Strieder, Wenzel et al. 2003). According to this hypothesis, the generated *Yersinia*-specific immune response cross-reacts with thyroid-specific components to cause tissue damage and disease (Albert and Inman 1999).

Epstein-Barr virus seems to play a role in the natural killer/T-cell lymphoma subtype of the orbit, as has long been described for Burkitt lymphoma. Bacteria seem to induce reactive lymphoid proliferation, while viruses directly infect the lymphoid cells, affecting the cell cycle and suppressing apoptosis, with subsequent malignant transformation. In general, proteins leading to cell cycle progression, like retinoblastoma protein, are elevated, and proteins inhibiting cell cycle progression, like p16 and p21, are absent or unable to function

normally. Inactivation of p53 by mutation of its DNA, which leads to elevation of defective p53 protein and inhibition of apoptosis, allows oncogenic by-chance mutations to become effective (Auw-Hadrich, Gobel, and Illerhaus 2010). Although apoptosis could help to control the reproduction of abnormal cells and, therefore, play a role in the protection of host cells against viral infection and antiviral immune response, there is also some evidence that it can also trigger autoimmunity. Virally induced apoptosis is considered to be at least partially responsible for the various associated pathologies (Young, Dawson, and Eliopoulos 1997). There is increasing evidence that apoptosis could play a role in the development of autoimmune thyroid diseases. Apoptosis induction may result in thyrocyte destruction and lymphocytic infiltration of the thyroid (Wang and Baker 2007)

Human herpesviruses are ubiquitous with widespread tissue tropism and have been found in the thyroid, which can be a reservoir of latent (Nahmias, Lee, and Beckman-Nahmias 1990; Staras et al. 2008). Herpesvirus infection might be involved in the pathophysiology of several chronic autoimmune inflammatory processes (Posnett 2008; Scotet et al. 1999). In fact, herpesviruses were demonstrated in thyroid tissues with both nodular and autoimmune diseases. Thomas et al isolated herpesviruses from 72.22% of thyroid tissue blocks of patients with Graves' disease and Hashimoto's thyroiditis (Thomas et al. 2008). Krueger et al showed that herpesviruses types 6 and 7 are highly prevalent in the healthy population with a cumulative probability for seropositivity of almost 100% in young age (Krueger et al. 1998). In addition, herpesviruses may use the thyroid gland and the gastrointestinal system as reservoirs (Kim et al. 2010). Like other infectious agents, herpes infection potentially increases the efficiency of T cell activation by enhanced expression of costimulatory molecules, upregulation of HLA expression on specialized antigen-presenting cells, and attraction of dendritic cells from peripheral to secondary lymphoid tissues (Leite et al. 2010). Herpesviruses human antiviral response includes two reciprocal cellular programs: cell survival with the production of protective cytokines and other cytokines that could lead to apoptosis for the elimination of infected cells; by that mechanism, virus would be involved in the initiation and progression of autoimmune thyroid disease. Continued activation of innate immunity against herpesviruses infection can also potentiate immune disorders. Using a subset of 280 Graves' disease patients carefully paired with a control group of 284 individuals, we demonstrated that patients with 72TP53 Pro/Pro variants (inherited diminished *TP53* apoptotic function) had five times more chance to develop Graves' disease and almost three times more chance to be infected by human herpesviruses type 7. This infection was associated with an increase of three times the susceptibility to Graves' disease (Leite et al. 2010). We suggested that human herpesviruses type 7 may take advantage of the poorer apoptotic ability of 72TP53 variants to trigger the autoimmune process that leads to Graves' disease. Thus, the inheritance of less efficient *TP53* genes would increase the risk of Graves' disease development and also favor human herpesviruses type 7 infection and perpetuation, which in turn may initiate and perpetuate the autoimmune process in Graves' disease (Leite et al. 2010).

Viruses are one of the high-risk factors closely related to human cancers. Many widespread chronic diseases, previously thought to be due to metabolic imbalances or genetic modifications, are increasingly linked to infectious events.

It is estimated that viruses are a contributory cause of 20% of all human cancers (Dimmock and Primrose 1994). Viruses, such as specific types of human papillomaviruses, Epstein-Barr virus, and human herpesviruses type 8, have been emerging as major causal factors of some human cancers (Tsai et al. 2005). Viral infection significantly affects the tumor

microenvironment, induces angiogenesis, and favors the development of metastases (Tsuji et al. 2008). There are several indicators of a possible role for viruses in thyroid cancer. Activation of virus-inducible signaling pathways, such as Toll-like receptor (TLR) signaling, has been shown in papillary thyroid carcinomas (McCall et al. 2007). Parvovirus B19 has been detected in thyroid cancer samples (Wang et al. 2008) and Epstein–Barr virus protein expression was demonstrated in poorly differentiated thyroid cancer (Shimakage et al. 2003). *In vitro* studies showed expression of herpesviruses entry mediator and nectin-1 in thyroid cancer cell lines (Huang et al. 2007).

Recent evidences also demonstrated that viruses could be directly involved in regulation of the epithelial-to-mesenchymal transition and contribute to the development of metastases. It has been shown that Epstein–Barr virus proteins can control the expression of Twist (Horikawa et al. 2007) and the development of metastases by regulating the metastasis suppressor *Nm23* (Kaul et al. 2009).

In humans, herpes simplex virus DNA was detected in thyroid samples from patients with autoimmune disorders (Thomas et al. 2008). Herpes simplex virus replication in epithelial cells is associated with activation of the RAS/MEK/MAPK pathway, which might be important to thyroid carcinogenesis (Smith et al. 2000), since these genes are commonly activated in thyroid cancer. Jensen et al showed that herpes simplex virus can be frequently detected in thyroid cancers, suggesting that during tumor progression, thyroid tumor cells acquire increased susceptibility to herpes simplex virus due to increased expression of nectin-1 and activation of mitogenic signaling (Jensen et al. 2010).

A viral disease is the result of an interaction between virus and host, in which the genetic background plays a role (Desailloud and Hober 2009). Further studies are needed to clarify the pathogenic mechanisms implicated in viruses-induced thyroid disease, aiming to propose new therapeutic strategies.

## 2.15 Thyroid disruptor action and human reaction

Endocrine disruptors act by physiological mechanisms replacing the hormones in our organism, blocking their natural action, or altering (increasing or decreasing) the original amount of hormones, thereby causing important endocrine function disturbances (Santamarta 2001). Most endocrine disruptor agents reduce circulating thyroid hormone levels or impair thyroid hormone action, and some may influence the pituitary and thyrotropin secretion, or even be partial thyroid hormone receptor agonists (Brent 2010). However, the thyroid is able to compensate their effect by increasing serum TSH and continue to produce a normal amount of thyroid hormone despite disruption (Brent, Braverman, and Zoeller 2007). Conversely, most carcinogens do not produce their biological effects per se, but require metabolic activation before they can interact with cellular macromolecules (Cascorbi 2006). Many compounds are converted to reactive electrophilic metabolites by oxidative (phase I) enzymes such as cytochrome P-450, which presumably most important effect is to activate the majority of carcinogens. Nevertheless, phase II enzymes usually act as inactivating enzymes catalyzing the conjugations of carcinogenic substance (Hein 2002). Phase II group of enzymes includes glutathione S-transferase, UDP-glucuronosyltransferase and N-acetyltransferase systems (Hein 2002).

We demonstrated that the inheritance of a series of these enzyme systems is involved in the risk for thyroid cancer development and in thyroid autoimmune disease susceptibility (Bufalo et al. 2006; Bufalo et al. 2008; Granja et al. 2005). Identifying a risk profile for thyroid diseases may help delineate polygenic models of susceptibility and prognosis. Such models

are particularly interesting, considering the elevated prevalence of thyroid diseases in the population, and may help select individuals for specific preventive interventions and determine which patients are most likely to benefit from specific measures.

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## **Part 2**

# **Pregnancy and Endocrine Disease**



# Thyroid in Pregnancy

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## 1. Introduction

Many changes in the functioning of the thyroid gland occur during pregnancy and some diseases of the thyroid gland can affect both the pregnant woman and the fetus. (Casey et al., 2006; Lazarus & Premawardhana, 2005; Poppe et al., 2007). Hypothyroidism is the most serious disorder of those occurring during pregnancy, and it might go unnoticed as some 'nonspecific' problem. Pregnant women with subclinical hypothyroidism seem to escape early clinical detection (Lazarus, 2002). While the hyperfunction during pregnancy usually manifests itself by clinical symptoms or a relapse of a previously cured disease (mostly Graves - Basedow) (Abalovich et al., 2007), lowered functioning is much more dangerous because of its non-specific symptoms. During the 1<sup>st</sup> trimester, the fetus is completely dependent upon thyroxin produced by the mother (Smallridge & Landerson, 2001). Even a small unnoticed malfunction of the thyroid gland, which doesn't have to endanger the course of the pregnancy, can affect the psychomotor development of the child (Morreale de Escobar et al., 2004; Mitchell & Klein, 2004)). Some women with subclinical hypothyroidism are absolutely asymptomatic and there is no reliance on the clinical image, while diagnostic of thyroid dysfunction (Klein et al., 2001).

Malfunction of the thyroid gland during pregnancy is long-term, and still not a sufficiently solved problem (Lazarus, 2002). On many pages of scientific literature and specialist literature there are still new arguments to systematically screen pregnant women for thyroid dysfunction and asymptomatic chronic thyroiditis in order to give such women the appropriate treatment (Surks et al., 2004). Results of surface population screenings are slightly varied, depending upon on level of medical care and approach to prevention, geographical conditions, supplementing with iodine, and other circumstances (including used diagnostic criteria) (Vaidya et al., 2007). Evaluating thyroidal function during pregnancy is difficult, considering the other differing influences of pregnancy.

Guidelines for management of thyroid dysfunction during pregnancy and postpartum (Abalovich et al. 2007) recommend not universal but only case finding screening. The first aim of the study was to assess the value of this recommendation. The other aim was to introduce an estimation of thyroid dysfunction during pregnancy, selection of suitable biochemical markers and determination of reference intervals for these markers in pregnancy.

## 2. Investigation of thyroidal dysfunction in pregnancy

In cooperation with General insurance company in Czech Republic was in 2009 started a pilot project for universal screening of thyroid dysfunction among women in the first

trimester of pregnancy. A pilot project was performed during 2009-2010 in 13 regions, (only 4 regions in 2010) of the Czech Republic. The data from 3577 pregnant women with sufficient iodine intake were available. The blood for TSH (thyroid stimulating hormone), TPO Ab (antibodies to thyroid peroxidase) and FT4 (free thyroxine) estimation was collected in 9-11 week of pregnancy. All participating pregnant women gave informed written consent with this subsequent investigation.

## **2.1 Conditions for screening**

First question is what we want to find? Which disorders are mostly indicated in pregnancy and what is really danger for pregnant women. Former studies resolve problems, which laboratory parameters are the best for investigation, the sampling of blood from pregnant women timing and also applicability of commonly used reference intervals for each parameter (Lazarus & Premawardhana, 2005; Poppe et al., 2007). Iodine supplementation is also usually asked (Glinoe et al., 1995).

### **2.1.1 Thyroid disorders in pregnant women**

Evaluating thyroidal function during pregnancy is difficult, considering the other differing influences of pregnancy (Dayan et al., 2002; Lazarus, 2002). New thyroid nodules should be aggressively investigated during pregnancy because of a high incidence of malignancy. Radioactive Iodine is contraindicated in pregnancy. Nursing mothers who have radioactive iodine uptake scans should pump and discard their milk for 48-72 hours after the test.

#### **2.1.1.1 Hypothyroidism**

Hypothyroidism is the most serious disorder of those occurring during pregnancy, and it might go unnoticed as some 'nonspecific' problem. Pregnant women with subclinical hypothyroidism seem to escape early clinical detection. The implications are staggering when one considers that there is a significant increase in intrauterine deaths, spontaneous abortions, premature births, and pre-eclampsia; also the development of the fetus, such as major malformations and loss of IQ (Haddow et al., Pop et al., 2003). It has been clearly proven that even slight (subclinical) hypothyroidism affects not only the course of pregnancy, but (especially later-on) the neuropsychological development of the child. Symptoms of hypothyroidism (fatigue, lowered performance, sleepiness, psychological lability) can also accompany the physiological pregnancy; some women with subclinical hypothyroidism are absolutely asymptomatic and there is no reliance on the clinical image, while diagnostic of functional failure. Untreated patients with hypothyroidism rarely conceive and carry a pregnancy. Treated hypothyroidism usually has no associated pregnancy complications. Patients will require increased levothyroxine doses during their pregnancies. Monitoring of thyroid function tests each trimester and at other clinically indicated times is recommended (Dashe 2005).

Prenatal vitamins can decrease the absorption of levothyroxine.

#### **2.1.1.2 Hyperthyroidism**

The hyperfunction during pregnancy usually manifests itself by clinical symptoms or a relapse of a previously cured disease. 95% of hyperthyroidism in pregnancy is secondary to Graves - Basedow disease. A good pregnancy outcome can be expected in patients with good control. Untreated hyperthyroidism is associated with decreased fertility, an increased rate of miscarriage, intrauterine growth retardation (IUGR), premature labor, and perinatal



mortality (Poppe et al., 2007). Poorly controlled thyrotoxicosis is associated with thyroid storm especially at labor and delivery. Beta blockers and propylthiouracil (PTU) can be safely used in pregnancy and in nursing mothers. PTU crosses the placenta but does not usually cause fetal hypothyroidism and goiter unless used in high doses. Treatment goals favor mild hyperthyroidism over hypothyroidism. Like other immune mediated diseases in pregnancy, Grave's disease tends to improve in the third trimester. Exacerbations may occur in the first trimester and postpartum. Neonatal and fetal thyrotoxicosis may occur because of transplacental passage of thyroid stimulating antibodies.

### **2.1.1.3 Postpartum thyroiditis**

Postpartum thyroiditis is a destructive autoimmune thyroiditis that begins with a period of hyperthyroidism followed by a period of hypothyroidism (Negro et al., 2007). The gland is often enlarged. There is usually complete recovery but a chance of recurrence in subsequent pregnancies exists. 80-85% of patients will have positive antithyroid antibodies. A radioactive iodine uptake scan can differentiate postpartum thyroiditis from an exacerbation of Graves- Basedow disease.

TPO Ab antibodies are markers of autoimmune process in the thyroid gland, their determination is diagnostically and prognostically important. Presence of TPO Ab during pregnancy also alerts to the danger of development of postpartum thyroiditis (Nicholson et al., 2006; Dosiou et al., 2008); about 50% of TPO Ab positive women have some thyroid dysfunction after delivery (Premawardhana et al., 2004; Nicholson et al., 2006), so it is necessary to follow-up these women. Postpartum thyroiditis is in an important consideration in women with postpartum depression.

### **2.1.1.4 Hyperemesis gravidarum**

Hyperemesis is associated with abnormal thyroid function tests in a significant number of cases. Hyperthyroidism may be the cause of hyperemesis or hyperemesis may be the cause of the hyperthyroidism (Goodwin et al., 1992).

## **2.1.2 Iodine supplementation in pregnancy**

The iodine requirement during pregnancy is sharply elevated because of an increase in maternal thyroxine production to maintain maternal euthyroidism and to transfer thyroid hormone to the fetus, iodine needs to be transferred to the fetus for fetal thyroid hormone production in later gestation and a probable increase in renal iodine clearance. The recommended dietary allowance for nonpregnant, nonlactating women aged  $\geq 14$  year is 150  $\mu\text{g}/\text{d}$ , for pregnant women is it 220  $\mu\text{g}/\text{d}$  (Zimmermann & Delange, 2004). In Czech Republic has been iodized salt in regular use since the 1950s, a good level of iodine supplementation can be expected also on Zamrazil study (Zamrazil, 2004). Women in this study were in addition supplemented by 100-150  $\mu\text{g}$  of iodide daily.

## **2.1.3 Timing of the blood taking**

The study group consists of 3 577 asymptomatic pregnant women (in their 9<sup>th</sup> - 11<sup>th</sup> week of pregnancy, 99% Caucasian) who were undergoing their first trimester prenatal screening. In the pilot project were used laboratories, which were able to investigate serum for the first trimester screening (investigation of PAPP-A and free  $\beta$  hCG) and simultaneously in the same sample determine TSH (thyroid stimulating hormone), TPO Ab (antibodies to thyroid peroxidase) and FT4 (free thyroxine).

### 2.1.4 Selection of laboratory parameters

The thyroid gland increases slightly in size during pregnancy.

Determining TSH in the serum is a basic searching procedure in the diagnosis of function of the thyroid gland in the general population. Its regulation is based on feedback, however during pregnancy there are also other mechanisms taking place. Suppressed serum TSH concentration during gestation follow hyperthyroidism as well as hyperemesis gravidarum or high hCG levels. Lower serum TSH in pregnancy is influenced by the thyrotropic activity of elevated circulating human chorionic gonadotropin concentrations, mainly in the first trimester (Abalovich 2007; Dashe 2005).

The comparison two groups of women, on pregnant and the other nonpregnant shows shift levels of TSH in pregnancy. On Fig.1 is possible to see only low levels of TSH.

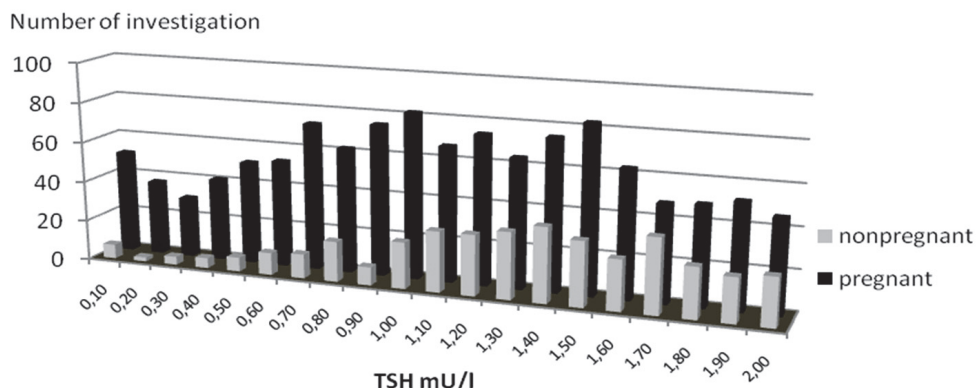


Fig. 1. Comparison TSH in group of pregnant (first trimester of pregnancy) and nonpregnant women in part with low TSH levels

In the group of pregnant women, with suppressed TSH, the average level of hCG was almost double ( $M=95.6$  mg/ml), in comparison with the group with TSH in the reference interval ( $M=68.9$  mg/ml) or with TSH  $>3.67$  mU/l ( $M=62.1$  mg/ml). Differences between the normal and raised TSH groups in hCG levels were not significant at  $p<0.050$ . The other authors confirm that sub-normal serum TSH levels in the first trimester are coincident with rising hCG levels (Surks et al., 2004).

On Fig.2 is possible to see, how high is hCG in women with low TSH. (Springer et al., 2009) By using the classical reference interval for serum TSH one might misdiagnose as healthy those women who already have a slight TSH elevation and, conversely, one might suspect hyperthyroidism in normal women who have a lowered serum TSH value.

Determining FT4 is by watching the amount of biologically active hormone which is available to the organism of a pregnant woman (as well as the fetus), and is not affected by the concentration of binding proteins. Its concentration during pregnancy is partly effected by inflow of iodine and the duration of the pregnancy. Free thyroxine levels remain within the normal range during pregnancy (though total thyroxine levels are increased secondary to increased TBG). Some consider it even more informative than TSH during pregnancy (Lazarus, 2002). During the 1<sup>st</sup> trimester, the fetus is completely dependent upon thyroxin produced by the mother. Even a small unnoticed malfunction of the thyroid gland, which doesn't have to endanger the course of the pregnancy, can affect the psychomotor

development of the child. Early maternal hypothyroxinemia alters histogenesis and cerebral cortex cytoarchitecture of the progeny.

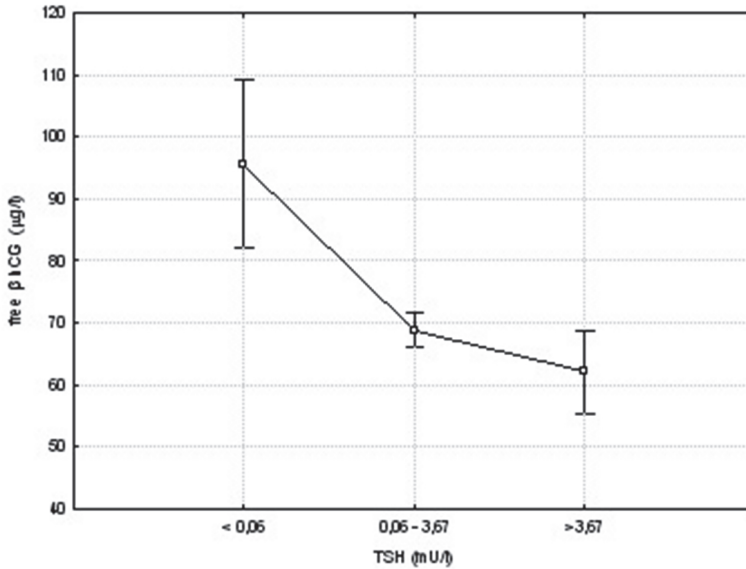


Fig. 2. Free  $\beta$  hCG in groups with increasing TSH levels

Anti-TPO antibodies (TPO Ab) are markers of autoimmune process in the thyroid gland, their determination is diagnostically and prognostically important. Presence of TPO Ab during pregnancy also alerts to the danger of development of postpartum thyroiditis; about 50% of TPO Ab positive women have some thyroid dysfunction after delivery, so it is necessary to follow-up these women. The relationship between TPO Ab and TSH is not definite, despite it being known that women with high level of TSH more frequently have positive TPO Ab.

### 2.1.5 Reference intervals

Reference intervals for different methods and manufacturer's may vary, they have been established using pools of nonpregnant normal sera and with different antibodies. Such reference ranges are not valid during pregnancy. A reference interval is the range of values of a test result for a defined population. In older references the reference interval is often designated as the reference range.

The main problem in setting of reference intervals for used laboratory markers is in case of using different immunoanalytic assays. Several factors can affect the setting of reference intervals, in particular manufacturer's methodology, euthyroid definition and iodine status. Alterations in thyroid hormone concentrations during pregnancy differed at different stage of gestation and to those of a non-pregnant state. If the non-pregnant TSH reference range was applied to pregnant women whose serum TSH concentration was within the first trimester specific reference range would be misclassified as having subclinical hyperthyroidism, or those with a TSH concentration above the first trimester specific upper reference limit would not be identified.

Determining the reference range suitable for the 1<sup>st</sup> trimester of pregnancy is possible by using the suggestion of the National Academy of Clinical Biochemistry (NACB) on selected samples from the population, or as 95<sup>th</sup> percentile from the cohort of women (Demers & Spencer, 2003).

Evaluating thyroidal function during pregnancy is difficult, considering the other differing influences of pregnancy (Dayan et al., 2002). TSH regulation is based on feedback, however during pregnancy there are also other mechanisms taking place - mainly there is suppression of TSH, presumably due to a thyroid-stimulating activity of hCG early in pregnancy when hCG levels are the highest. (Goodwin et al., 1992).

#### 2.1.5.1 Determination of own reference intervals

TSH, TPO Ab, and FT4 were assayed by ADVIA® Centaur™ (Siemens), with chemiluminometric detection. TSH was determined by sandwich immunoanalysis with direct chemiluminometric technology; for TPO Ab and FT4 competitive immunoanalysis with direct chemiluminometric technology was used.

Reproducibility of this method is expressed by the interassay variability. For TSH, it is 5 - 7% for levels of 0.43 - 15.00 mU/l; for TPO Ab it is 10% for the level of 70 kU/l and 7% for the level of 170 kU/l. Interassay variability for FT4 is 7 - 9% for levels 10.1 - 33.0 pmol/l.

For mostly used systems were possible to use comparable TSH reference intervals, that were determined on large group of pregnant women (Springer et al., 2009). For evaluation of the reference interval, a selected group was created in accordance with the recommendations of the NACB. From the group of pregnant women (5,520), all those with a history of thyroid disease were excluded; TPO Ab > 60 kU/l (the manufacturer's cut-off) and free  $\beta$  hCG higher than triple of the median (Mdn=56.6  $\mu$ g/l), in view of the suppression of TSH by a high level of hCG, were also excluded. TSH do not follow a normal distribution, data have to be normalized using log transformation. The reference interval - 95<sup>th</sup> percentile - was than determined using this log transformed data. The TSH reference interval for ADVIA:Centaur Siemens was determined to be 0.06 - 3.67 mU/l.

FT4 levels fit a Gaussian distribution, so reference intervals were derived using nonparametric analyses such as the 95<sup>th</sup> percentile. The calculated reference interval (9.55 - 23.0 pmol/l) was almost identical to the manufacturer's (9.8 - 23.1 pmol/l) for all populations.

Differences in TPO Ab manufacturer's reference interval are not comparable from 0.5 to 100 kU/l (Haddow et al., 2004; Hollowell et al., 2002; Negro et al., 2006). Reference intervals for thyroid antibody tests should be by the NACB recommendation; established from young male subjects, free from any history of thyroid disease or predisposition for any autoimmune disease (Demers & Spencer, 2003). Establishment of decision values for thyroid antibodies in the healthy population is difficult and results are method dependent (Jensen et al., 2006). The pregnancy positivity cut-off for TPO Ab was calculated at the 95<sup>th</sup> percentiles. From all of the women, women with a known history of thyroid disease were separated, as well as those with TSH lower than 0.1 and higher than 4.0 mU/l. This interval had been used earlier for the evaluation of positive results in pregnancy; it was deduced from both specialized literature and own experience. The positivity of TPO Ab in nonpregnant individuals is about 11% (Hollowell et al., 2002); in the pregnant population it is very similar. Negro mentioned 11.7% TPO Ab positive pregnant women (Negro et al., 2006); Dossioui selected groups by age and the positivity was 10.4 and 12.6 for ages 25 and 35 years, respectively (Dossioui et al., 2008). When was used the reference interval recommended by the producer of reagents (> 60 kU/l),

in study group was 22.1% positivity. If was used the 90<sup>th</sup> percentile (143 kU/l) as the cut-off for the group of pregnant women, was the positivity 11.2%.

## 2.2 Results

The pilot project for universal screening was performed during 2009-2010 in 13 regions of the Czech Republic with the financial support of the General Insurance Company. Cooperation in 10 regions was good or suitable; 3 regions cooperated poorly and their data was not used in the first year of pilot project. In the second year were used only four laboratories for project. Thyroid examination was offered to women in the 9-11th week of pregnancy. The women with any positivity were offered immediate to endocrinological examination. Blood tests (TSH, FT4 and TPO Ab) were carried out in 3577 asymptomatic pregnant women.

On Fig. 3 is showed the map of Czech Republic with selected regions. The number under town name is number of implement investigations in the pilot project, the red one are excluded regions, green number included investigations also from second part of pilot project in 2010.



Fig. 3. The map of Czech Republic with cooperating regions. Under name of the town are number of investigations, green number indicate laboratories worked on second part of levels. The red marked regions were excluded from statistic.

There were used different analytical systems for analysis: the radio-immunoanalysis in five laboratories, chemilumino-immunoanalysis in six and electrochemilumino-immunoanalysis in two of them. Specific reference intervals for TSH, FT4, and positivity cut-off for TPO Ab during pregnancy were applied in all laboratories.

In all 3577 women investigated in pilot project were found 679 (19.0%) pregnant women with some positivity.

### 2.2.1 TSH

A raised concentration of TSH was found in 7.63% of women; and a suppression of TSH was found in 3.05% of women. The prevalence of hypertyreosis in pregnant women is 1.7%, and 0.4% of these women had an elevated serum FT4 level. This is similar to that reported for non-

pregnant individuals. Low level of TSH with high FT4 level were only in 0.48%, which is in concordance with previous hypotheses, as well as with Haddow study (Haddow at all., 2004). Many authors have determined the prevalence of hypothyreosis (overt and subclinical) in pregnancy and it is estimated to be 0.3 - 0.5% for overt hypothyroidism and 2 - 3% for subclinical hypothyroidism (Haddow at all., 2004). In this study there were 7.63% of pregnant women with TSH over determined own reference interval; it is evident, that no all higher TSH means hypothyreosis. Also selection of pregnant women maybe was influenced by gynaecologists, which prefer high-risk women for investigation in pilot project.. On Fig. 4 is schema with positivity in TSH levels:

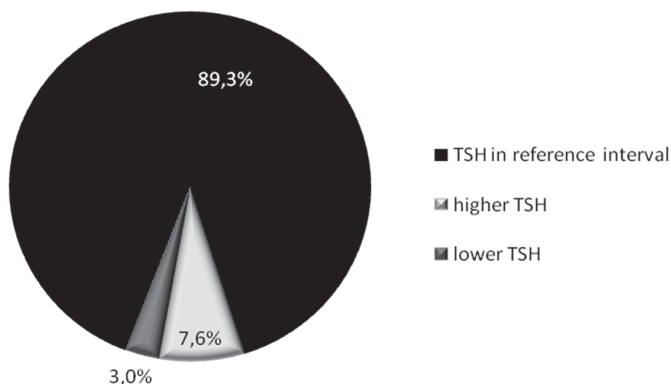


Fig. 4. Distribution of TSH levels in study group.

### 2.2.2 FT4

A raised concentration of FT4 was found in 0.48% of women; and a suppression of FT4 was found in 3.44% of women. On Fig. 5 is showed distribution of FT4 in the study group of pregnant women.

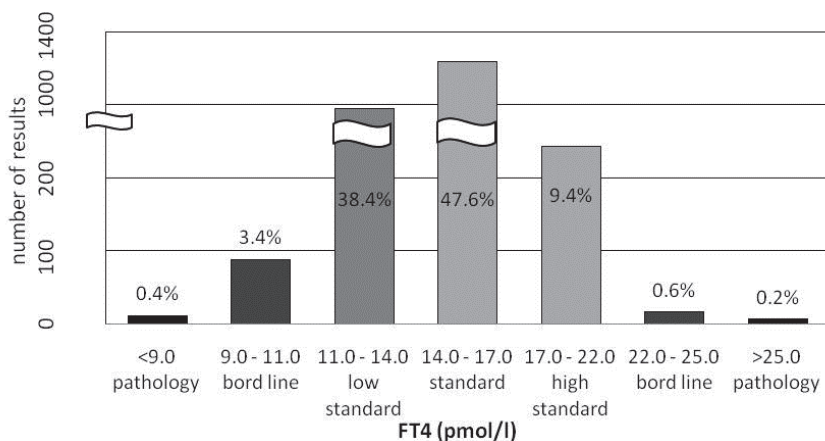


Fig. 5. Distribution of FT4 in the study cohort.

Differences of FT4 in euthyroid women with suppressed, normal and elevated TSH were found and are showed on Fig. 6.

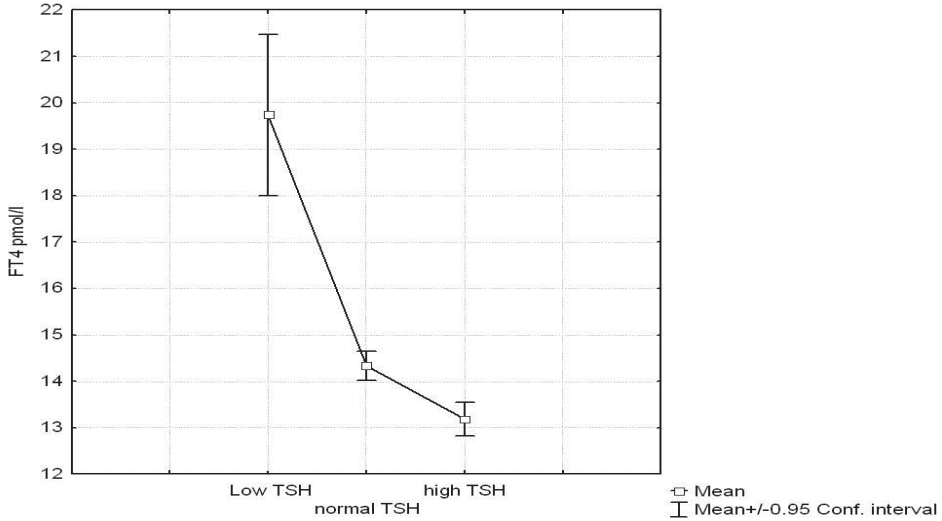


Fig. 6. FT4 in euthyroid women with suppressed, normal and elevated TSH

**2.2.3 TPO Ab**

8.78% of pregnant women were found TPO Ab positive and there were also 5.4% of women, which had only TPO Ab positivity without another differences in TSH or FT4 levels. This distribution of results for whole study group is on Fig. 7 showed.

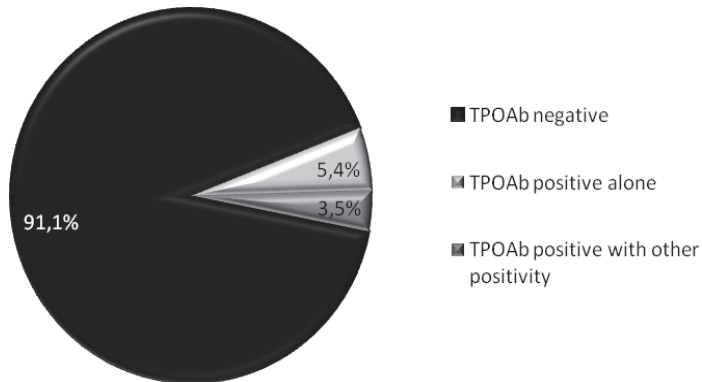


Fig. 7. TPO Ab in whole study group

**2.3 General or only high-risk pregnant women testing?**

Presently available information that supports the hypothesis that an inappropriate first trimester surge in maternal FT4, whatever the circulating TSH, would interfere with the

development of the cerebral cortex, even if maternal euthyroidism is maintained by normal circulating T3. There is at present increasing consensus (Morreale et al., 2004) that maternal hypothyroidism, both clinical and subclinical, requires early detection and prompt treatment, because of its important negative effects for the woman, the pregnancy and the child. There also exists a positive association between the presence of thyroid antibodies and pregnancy loss with postpartum thyroiditis.

The most practical approach is to screen all pregnant women for hypothyroidism as early in pregnancy as possible (or before conception). In the case of the mother, screening would reset in early diagnosis and treatment of subclinical hypothyroidism. Unfortunately, pregnant women with subclinical hypothyroidism seem to escape early clinical detection. In Mitchel study (Mitchel & Klein, 2004), 58% of the hypothyroid women were unaware of their disorder, and it took a median of 5 years from the time of the pregnancy for the clinical diagnosis to be made.

Vaidya study shows that targeted thyroid function testing of only high-risk pregnant women would miss nearly one-third of women with overt/subclinical hypothyroidism during early pregnancy (Vaidya et al., 2007). In Czech Republic, case finding screening is able to disclose less than 20% of asymptomatic mild or deep hypothyroidism or women with positive TPO Ab in pregnancy (Springer et al., 2009).

#### 2.4 Relationship between TPO Ab and TSH, resp. FT4

The relationship between TPO Ab and TSH is not definite, despite it being known that women with high level of TSH more frequently have positive TPO Ab. In study group, divided by serum TSH concentration, were 44.1% TPO Ab positive (in part), with TSH >3.67 mU/l and 10.1% or 9.15% in the group with TSH < 0.06 mU/l or TSH in the reference interval, as is showed on Fig. 8.

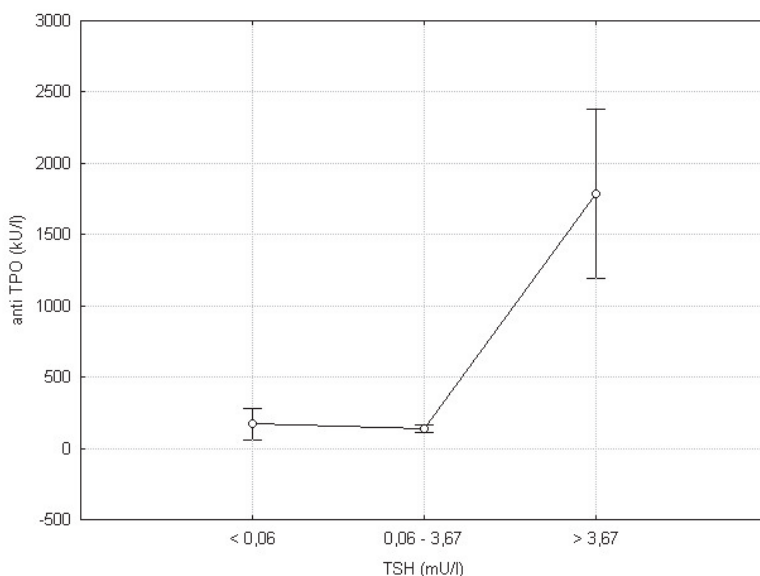


Fig. 8. TPO Ab in groups with different TSH level



Glinoer (Glinoer et al., 1995) also documented somewhat higher TSH levels among the sub-population of women with elevated antibody levels and these findings are confirmed in the present study.

Serum concentrations of FT4 were lower in TPO Ab positive as compared to TPO Ab negative women as is showed on Fig. 9.

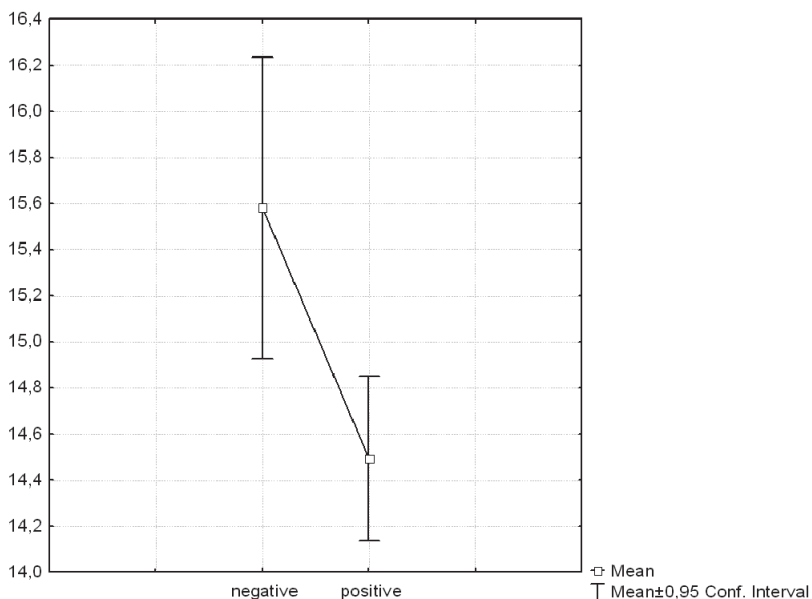


Fig. 9. Serum concentrations of FT4 in TPO Ab positive and TPO Ab negative women

### 2.5 Cost effectiveness of the thyroid failure screening

An answer to the question of screening cost-effectiveness of thyroid function in pregnancy was already presented by Dosiou (Dosiou et al., 2008). In this study is not defined cost-efficiency, but it is unquestioned fact that early diagnosis of thyroid disorder is cost-effective and beneficial for both mother and child.

### 3. Conclusion

The importance of maternal thyroxine for the development of the fetus brain early in pregnancy has received increasing acceptance. It has more recently become evident that maternal hypothyroxinemia results in the birth of children with decreased mental and psychomotor development.

This project proved the usefulness of universal screening of thyroid disease in pregnancy. The occurrence of pathological results in laboratory tests was 679/3577. Determination of the specific reference intervals for TSH, FT4, and TPO Ab in pregnancy is one of the basic requirements when implementing the general examination. Cooperation with gynaecologists differed, the main stumbling block was the willingness of gynaecologists to inform pregnant women about the project.

In Czech Republic, case finding screening is able to disclose less than 20% of asymptomatic mild or deep hypothyroidism or women with positive TPO Ab in pregnancy. Investigation of combination of TSH and TPO Ab is necessary. Maternal hypothyroxinemia appears to be a much more frequent cause of deficits in the progeny than congenital hypothyroidism, for which we have successful neonatal thyroid screening programs. This study maybe will help define the impact of universal screening (TSH, FT4, TPO Ab) on the health care system. The introduction of general screening of thyroid failure in pregnancy needs to be emphasized in public education; moreover, interdisciplinary cooperation of gynaecologist, endocrinologist and general practitioner, not to mention midwives should be improved. The other analysis would be more clearly identify the causal relationships between mild thyroid hormone deficiency and thyroid autoimmunity, on the one hand, and fetal neurological development on the other. In the meantime, physicians and obstetricians must use their own judgment about the optimal management for their individual patients.

#### 4. Acknowledgment

The authors are grateful to the General Insurance Company of the Czech Republic for founding of the pilot project. The special thank belongs to all cooperating laboratories for help with blood sampling and perfekt resolving all administrative problems. Last but not least thank technicians from the Institute of Clinical Biochemistry and Laboratory Diagnostics Charles University, Prague, Czech Republic for their support in setting of reference intervals for pregnancy.

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# Adrenal Disease and Pregnancy

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## 1. Introduction

During pregnancy, maternal-endocrine regulation, as well as all their physiology, undergoes profound adaptive changes in the framework of a functional organization structured in three interrelated compartments: the mother, placenta and fetus. Many of these changes are initially induced by estradiol and progesterone produced by the corpus luteum, combined with chorionic gonadotropin. As the pregnancy progresses, steroid and peptide hormones produced by the fetoplacental unit, took over. As a result of these adaptive phenomena is achieved adequate nutritional support of the fetus, uterine quiescence is maintained during childbirth, and breastfeeding is finally possible (Schindler, 2005).

## 2. Cushing's syndrome

During normal pregnancy, serum cortisol increase gradually from the second quarter, keeping the circadian rhythm. Part of the increase in serum cortisol is due to increased estrógens and secondarily to increased cortisol binding protein (CBG), although serum free cortisol, urine and saliva can be elevated up to 2-3 times. The plasma concentration of ACTH is usually normal, although during pregnancy can be reduced or increased. There is a gradual increase in late pregnancy and during delivery (Lindsay & Nieman, 2005). The placenta during gestation can produce CRH which is released into the maternal circulation, although this may have implications in the regulation of ACTH and cortisol secretion are not well known.

Cushing's syndrome is uncommon during pregnancy, because hypercortisolism produces anovulation and infertility by altering androgenos and gonadotropin.

The frequency of ACTH-independent cases is increased in pregnant as compared to non pregnant individuals. Of the approximately 136 reported cases, approximately 60% had ACTH independent Cushing's Syndrome: 44% adenoma y 11% carcinoma and the remainder a mix of primary pigmented nodular adrenal disease, ACTH independent hyperplasia and ectopic ACTH secretion. Five pregnant women with the ectopic ACTH syndrome have been reported (Guilhaume et al., 1992).

The fetus is partially protected from the hypercortisolemia because placental 11-beta-hydroxysteroid dehydrogenase converts 85% of maternal cortisol to biologically inactive cortisone. However, untreated Cushing's syndrome has been associated with spontaneous abortion (25%), premature delivery (50%) and rarely, neonatal adrenal insufficiency (Aron et al., 1990).

Maternal complications also can occur. These include hypertension in nearly 70%, gestational diabetes in 25%, myopathy, opportunistic infections, fractures, preeclampsia and rarely heart failure. Two maternal deaths have been reported (Vilar et al., 2007).

### 2.1 Diagnosis

The presence of Cushing's Syndrome during pregnancy may be masked since some of the symptoms and signs of this disorder (weight gain, hypertension, striae) can also occur in normal pregnant women. Biochemical changes during normal pregnancy can interfere with the diagnosis:

The normal pregnancy rises in ACTH and cortisol levels. The hypercortisolism of pregnancy continues to exhibit a normal circadian rhythm, though with a higher nighttime nadir; loss of diurnal rhythm is characteristic of all forms of Cushing syndrome. Salivary cortisol measurements may assist in determining this lack of diurnal response, but normal midnight levels have no been standardized for pregnancy. Urinary free cortisol levels greater than 3 times the upper limit of normal may be interpreted as indicating Cushing's syndrome in the second and third trimester. There are limited or no data using antibody based assays or mass spectrometry. Normal pregnancy is also associated with inadequate suppression of ACTH and cortisol during the overnight dexamethasone suppression test (Carr et al., 1981). ACTH levels may not reliably distinguish between pituitary and adrenal etiologies, as the levels may be normal or high in all form of Cushing syndrome, likely from placental ACTH production. When plasma ACTH levels are suppressed, preferably as measured using a two-site immunometric assay, no further biochemical testing is needed.

The high dose dexamethasone suppression test will cause >80% suppression of serum cortisol in normal pregnancy. Patient with adrenal Cushing may be identified with this test as they don't suppress, but the test may misclassify those with Cushing disease, as three of seven cases failed to suppress in small series (Lindsay et al., 2005).

### 2.2 Differential diagnosis

Because of the persistent elevations of ACTH in pregnancy, an ACTH suppressed is not always found in independent causes of Cushing syndrome. Thus, while its measurement may be useful, lack of suppressed value does not exclude an ACTH independent cause.

There are limited data on the response to dexamethasone, 8 mg, of women with adrenal causes of Cushing's syndrome and pituitary adenomas. In general, those with an adrenal etiology do not suppress, while those with Cushing disease do suppress serum cortisol. (Barasch et al., 1988).

Because adrenal form of Cushing's syndrome are common in pregnancy, it is reasonable to perform an adrenal ultrasound to look for a mass, as well as an ACTH level and dexamethasone suppression test as an initial evaluation.

CRH stimulation test has been performed rarely in pregnant patients. Magnetic resonance (MRI) cannot be performed in the first trimester because of concerns about potential adverse fetal effects; during the remainder of gestation it is not commonly given with gadolinium contrast.

### 2.3 Treatment

Adrenal surgery has been performed at the 16 to 21 weeks of pregnancy without complicating the pregnancy (Buescher, 1996). It can be performed using a laparoscopic

approach. There is no clear evidence that early treatment is more beneficial than later treatment, this is likely because treatment is generally begun late. Early second trimester surgical treatment would be optimal. For women who do not want surgery or are diagnosed later, maternal hypercortisolism can be controlled with metyrapone. Metyrapone has side effects including hypertension and preeclampsia. (Blanco et al., 2006). Ketoconazole therapy is associated with intrauterine growth retardation, but no malformation or neonatal adrenal insufficiency (Berwaerts et al., 1999). Aminoglutethamide may cause fetal masculinization and mitotane is teratogenic; both should be avoided.

Transphenoidal surgery and pituitary radiation have both been used without complicating the pregnancy (Casson et al., 1987).

Treating the Cushing's syndrome does not reduce the frequency of intrauterine growth restriction (about 30%) or premature birth (about 65%), but it does appear to prevent stillborn deliveries (about 10% with treatment) (Buescher, 1996).

### 3. Adrenal insufficiency

The adrenal insufficiency (AI) is rare, but early diagnosis is very important to improve maternal-fetal treatment. The primary AI or Addison's disease involves adrenal cortex atrophy with response insensitivity to ACTH and angiotensin 2, which causes a deficiency of glucocorticoids and aldosterone. The secondary and tertiary AI due to lack of ACTH or CRH are secondary to hypothalamic-pituitary diseases or chronic administration of exogenous corticosteroids. The secondary and tertiary AI are not associated with mineralocorticoid deficiency (Ambrosi et al., 2003).

The prevalence of primary adrenal insufficiency in pregnancy is unknown, with a series from Norway suggesting an incidence of 1 in 3000 births from 1976 to 1987. The most common etiology for primary adrenal insufficiency is autoimmune adrenalitis, which may be associated with autoimmune polyglandular syndrome. Primary adrenal insufficiency from infections, bilateral metastatic disease, hemorrhage or infarction is uncommon. Secondary adrenal insufficiency, from pituitary neoplasm or glucocorticoid suppression of the hypothalamic-pituitary-adrenal axis is more common (Stechova, 2004).

Gestation in patients with AI should be considered high-risk pregnancy (Lindsay & Nieman, 2005). Maternal mortality is rare today, after the introduction of hydrocortisone in 1950, as well as improved diagnosis and early treatment. When AI is not diagnosed during pregnancy, may not have negative effects to mother or fetus, indicating the transplacental passage of maternal fetal glucocorticoids. In these cases, disease appears in the postpartum. When the disease is previously known, it is necessary to adjust the dose of corticosteroids in order to avoid a default adrenal crisis defect or undesirable effects of excess treatment as hypertension and preeclampsia.

The prevalence of fetal mortality is unknown, although cases have been reported about intrauterine death, most undiagnosed in pregnant women. Intrauterine growth retardation and low birth weight are the most frequent effects in untreated mothers. The concomitance between AI and other autoimmune diseases (DM, lupus, anticardiolipin antibodies) increases maternal-fetal morbidity.

#### 3.1 Diagnosis

Most cases are diagnosed before pregnancy.

Adrenal insufficiency is associated with laboratory findings of hyponatremia, hyperkalemia, hypoglycemia, eosinophilia and lymphocytosis. Hyperkalemia may be absent, because of the pregnancy increase in the rennin angiotensin system (Gradden et al., 2001). Early morning plasma cortisol levels of  $<3.0$  mcg/dl confirms AI, while a cortisol  $>19$  mcg/dl in the first or early second trimester excludes the diagnosis in a clinically stable patient. Plasma cortisol levels may fall in the normal "nonpregnant" range due to increase in CBG concentrations in the second and third trimesters, but will not be appropriately elevated for the stage of pregnancy. Appropriate pregnancy-specific cutoffs for diagnosis with the standard cosyntropin test using 250 mcg dose have not been established. Plasma cortisol levels were 60% to 80% above nonpregnant responses in normal pregnant women tested in the second and third trimesters in one series (Nolten et al., 1978).

McKenna et al (McKenna et al., 2000) examined the 1 mcg low dose cosyntropin test for diagnosis of secondary adrenal insufficiency in women at 24-34 weeks gestational age, and found high sensitivity of diagnosis using a cutoff of 30 mcg/dl. Accuracy of dosing is more difficult with this than with the standard cosyntropin test. The cosyntropin test is less sensitive to detect early secondary or tertiary forms of adrenal insufficiency. Cortisol and ACTH responses to CRH are blunted in pregnancy, making the CRH stimulation test unreliable for differentiating secondary and tertiary adrenal insufficiency in pregnancy. With primary AI, ACTH levels will be elevated and a level above 100 pg/ml is consistent with the diagnosis. However, ACTH will not be low with secondary forms because of the placental production of this hormone, which is nevertheless insufficient to maintain normal maternal adrenal function. ACTH values fluctuate widely, and a single value is insufficient for diagnosis. Adrenal antibodies may assist in confirming idiopathic adrenal insufficiency, as approximately 90% of patients will have 21 hydroxylase antibodies and 30% will have antibodies to 17-hydroxylase and side-chain cleavage enzymes. Aldosterone to renin ratios are low with elevated plasma rennin activity in patient with mineralocorticoid deficiency from adrenal atrophy (Symonds & Craven, 1978).

### **3.2 Treatment**

Patients with AI should be managed during pregnancy and childbirth by a multidisciplinary team that includes endocrinologist, obstetrician and an expert neurosurgeon.

#### **3.2.1 Glucocorticoids**

The aim is to achieve a physiological replacement dose for optimal maternal and fetal monitoring.

The most important period is the first trimester, because the symptoms may go unnoticed by the emesis of pregnancy and childbirth stress. Hydrocortisone is the glucocorticoid of choice at doses of 12-15 mg/m<sup>2</sup> in 2 divided doses at breakfast and 1 / 3 at lunch. It is rare to increase the dose during pregnancy.

Women must be educated on the need for treatment with parenteral hydrocortisone in cases of nausea, vomiting or intercurrent systemic disease. Prednisone or prednisolone should be avoided because Hydrocortisone is more physiological.

#### **3.2.2 Mineralocorticoids**

They are only required in primary AI. The most widely used on the single dose is fluorocortisona 9 $\alpha$  (0.05 to 0.2 mg/day). The dose is stable during pregnancy, and it can be reduced in the third trimester if there are edemas or hypertension.



### 3.2.3 Adrenal crisis

It may appear at any time if the AI is not diagnosed, but it is more often during the delivery, when there is infection, preeclampsia or hemorrhage. Treatment should be started with hydrocortisone intravenous (bolus of 100 or 200mg and then 50-100 mg every 6-8 hours). In women with hypoglycemia, it is necessary to administer dextrose.

After stabilization, return to oral treatment. It is not necessary to administer Mineralocorticoids in the acute phase. During delivery, the dose of glucocorticoids should be doubled at onset of labor or to administer 50 mg of intravenous hydrocortisone. In case of caesarean, it is necessary to start treatment with hydrocortisone, 100 mg IV every 8 hours. The oral treatment must be restarted within 48 hours.

After delivery, all women must maintain treatment with corticosteroids and with dosis of mineralocorticoids similar to the prepregnancy period.

It is not necessary to follow hypothalamic-pituitary axis in children of mothers with AI if they have been well treated during pregnancy. It can be necessary in mothers who have received suprphysiological doses.

The replacement therapy can continue during lactation. It is excreted 0.5% per liter of milk of the absorbed dose (Sidhu & Hawkins, 1981).

## 4. Primary hyperaldosteronism

Primary hyperaldosteronism rarely has been reported in pregnancy and is most often caused by an adrenal adenoma (Okawa et al., 2002). There are reports of glucocorticoid remediable hyperaldosteronism in pregnancy (Wyckoff et al., 2000). The elevated aldosterone levels found in patients are similar to those in normal pregnant women, but the plasma rennin activity is suppressed. Moderate to severe hypertension is seen in 85%, proteinuria in 52% and hypokalemia in 55%, and symptoms may include headache, malaise, and muscle cramps. Placental abruption and preterm delivery are risks. Progesterone has an antimineralocorticoid effect at the renal tubules, and the hypertension and hypokalemia may ameliorate during pregnancy (Matsumoto et al., 2000).

The physiologic rise in aldosterone during pregnancy overlaps the levels seen in primary aldosteronism, making diagnosis difficult. Suppressed rennin in the setting of hyperaldosteronism, is diagnostic. Salt loading test may be used to diagnose hyperaldosteronism, but there are potential fetal risks and no normative data. If baseline and suppression testing are equivocal, or radiological scanning does not suggest unilateral disease, patients may be treated medically until delivery to allow more definitive investigations. Spironolactone, the usual nonpregnant therapy, is contraindicated in pregnancy as it cross the placenta and is a potent antiandrogenic which can cause ambiguous genitalia in a male fetus. There is no published experience with the use during pregnancy of eplerenone, the new aldosterone receptor antagonist. Surgical therapy may be delayed until postpartum if hypertension can be controlled with agents safe in pregnancy, such as amiloride, methyldopa, labetalol, and calcium channel blockers. Potassium supplementation may be required, but as noted above, the hypokalemia may ameliorate in pregnancy because of the antikaliuretic effect of progesterone. Both hypertension and hypokalemia may exacerbate postpartum due to removal of the progesterone effect (Nursan et al., 2009).

## 5. Pheochromocytoma

Pheochromocytoma is a rare cause of hypertension during pregnancy, with clinical features similar to those in the general population (Keely, 1998). The prevalence is estimated at 1 in

54000 pregnancies (Botchan et al, 1995). As the uterus enlarges and an actively moving fetus compresses the neoplasm, maternal complications such as severe hypertension, hemorrhage into the neoplasm, hemodynamic collapse, myocardial infarction, cardiac arrhythmias, congestive heart failure, and cerebral hemorrhage may occur. Extra-adrenal tumours which occur in 10%, such as in the organ of Zuckerkandl at the aortic bifurcation, are particularly prone to hypertensive episodes with changes in position, uterine contractions, fetal movement, and Valsalva maneuvers. Unrecognized pheochromocytoma is associated with a maternal mortality rate of 50% at induction of anesthesia or during labor (Lau et al., 1996). There is a minimal placental transfer of catecholamines likely due to high placental concentrations of catechol-O-methyltransferase and monoamine oxidase. Adverse fetal effects such hypoxia are a result of catecholamine-induced uteroplacental vasoconstriction and placental insufficiency, and of maternal hypertension, hypotension, or vascular collapse (Saarikoski, 1974).

### 5.1 Diagnosis

Diagnosis of pheochromocytoma requires a high index of suspicion. Preconception screening of families known to have MEN 2 with RET proto-oncogene is essential. The diagnosis should be considered in pregnant women with severe or paroxysmal hypertension, particularly in the first half of pregnancy or in association with orthostatic hypotension or episodic symptoms of pallor, anxiety, headaches, palpitations, chest pain, or diaphoresis. Symptoms may occur or worsen during pregnancy because of the increased vascularity of the tumour and mechanical factors such as pressure from the expanding uterus or fetal movement (Harper et al, 1989). As in nonpregnant women, the diagnosis is usually based upon the results of 24-hour urinary fractionated metanephrines and catecholamines and plasma fractionated metanephrines. If possible, methyl dopa and labetalol should be discontinued prior to the investigation as these agents may interfere with the quantification of the catecholamines. MRI is the imaging test of choice in the pregnant women. Stimulation test and <sup>123</sup>I-MIBG scintigraphy are not considered safe for pregnant women.

### 5.2 Treatment

Medical therapy should be initiated with alpha adrenergic blockade (usually phenoxybenzamine) (Stenström & Swolin, 1985).

It is started at a dose of 10 mg twice daily, with titration until the hypertension is controlled. Placental transfer of phenoxybenzamine occurs, but is generally safe (Sant'eiro et al., 1996). Beta blockade is reserved for treating maternal tachycardia of arrhythmias which persist after full alpha blockade and volume repletion.

Beta blockers may be associated with fetal bradycardia and with intrauterine growth retardation, when used early in pregnancy (Chatterjee & Parekh, 1985). All of these potential fetal risks are small compared to the risk of fetal wastage from unblocked high maternal levels of catecholamines. Hypertensive emergencies should be treated with phentolamine or nitroprusside, although the latter should be limited because of fetal cyanide toxicity.

The timing of surgical excision of the neoplasm is controversial. In the first half of pregnancy, surgical excision may proceed once adequate alpha-blockade is established, although there is a higher risk of miscarriage with first trimester surgery. In the early second trimester, abortion is less likely and the size of the uterus will not make excision difficult. If

the pheochromocytoma is not recognized until the second half of gestation, increasing uterine size makes surgical exploration difficult (Sarathi et al., 2010).

Successful laparoscopic excision of a pheochromocytoma in the second trimester of pregnancy has been described (Finkenstedt et al, 1999).

Other options include combined cesarean delivery and tumour resection or delivery followed by tumour resection at a later date. Delivery is generally delayed until the fetus reaches sufficient maturity to reduce postpartum morbidity, providing successful medical management exists. Although successful vaginal delivery has been reported, it has been associated with higher rates of maternal mortality than caesarean section (Schenker & Granat, 1982).

## 6. Congenital Adrenal Hyperplasia (CAH)

It occurs in a family of monogenic inherited enzymatic defects of adrenal steroid biosynthesis, with manifestations secondary to an accumulation of precursors proximal to the enzymatic deficiency. The most common form of CAH is 21-hydroxylase deficiency, seen in more than 90% of the CAH cases in pregnancy (Forest, 2004).

Classic, severe 21-hydroxylase deficiency is associated with ambiguous genitalia, and inadequate vaginal introitus, and progressive postnatal virilization including precocious adrenarche, advanced somatic development, central precocious puberty, menstrual irregularity, a reduced fertility rate, and possible salt wasting (White & Speiser, 2000).

The spontaneous abortion rate is twice that in the normal population, and congenital anomalies are more frequent. Conception requires adequate glucocorticoid therapy, which then continues at stable rates during gestation, except at labor and delivery. Nonclassical 21-hydroxylase deficiency patients present with pubertal and postpubertal hirsutism and menstrual irregularity, and may have improved fertility with glucocorticoid therapy (Krone et al., 2001).

ACTH stimulation testing to measure 17-OH progesterone demonstrates overlap between heterozygotes for CAH and the normal population. Ideally CYP21 genotyping should be performed. Virilization is not seen in the female fetus with non classical 21-hydroxylase deficiency, but occurs in a fetus with classic 21-hydroxylase unless fetal adrenal androgen production is adequately suppressed.

Dexamethasone most readily crosses the placenta as it is not bound to CBG and is not metabolized at a dose of 20 mcg/kg maternal body weight per day to a maximum of 1.5 mg daily in 3 divided doses beginning at recognition of pregnancy before the 9<sup>th</sup> week of gestation, though lower doses are recommended by some (Coleman MA, Honour, 2004). Maternal plasma and/or urinary estriol levels reflect fetal adrenal synthesis and are monitored to assess efficacy. Maternal cortisol and DHEA-S levels will represent maternal adrenal suppression.

As only 25% of female fetuses are affected in a family with CAH, it is important to discontinue therapy as soon as possible in the male fetus and unaffected female fetus.

Chorionic villus sampling at 9-11 weeks gestation may be used for gender determination and direct ADN analysis for the 21-hydroxylase gene CYP21.

Side effects of dexamethasone therapy are potentially significant, including excessive weight gain, severe striae with scarring, edema, irritability, gestational diabetes mellitus, hypertension, and gastrointestinal intolerance. In affected pregnancies, dexamethasone may

be lowered to 0.75 to 1 mg/day in the second half of pregnancy to decrease maternal side effects while avoiding fetal virilisation (Pang et al, 1990).

## 7. Conclusion

Pregnancy is a stage of life very important. It is important to recognize and to treat adrenal disease during pregnancy to avoid complications in the mother and fetus.

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## **Part 3**

### **Effects of Androgens**





# Cardiovascular Effects of Androgens

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## 1. Introduction

Androgens are the male sex hormones responsible for development of the male reproductive system. Testosterone is the main androgen, however, other circulating androgens also exist; these are dehydroepiandrosterone, androstenedione and androstenediol. In some tissues, testosterone can be converted to dihydrotestosterone by action of 5 $\alpha$ -reductase. Testosterone is also found in females, but at much lower levels than males; it can be converted into estradiol by the enzyme aromatase. Testosterone has 19 carbon atoms, and is produced from cholesterol, mainly by Leydig cells in the testes. Androgens are responsible for primary and secondary sexual characters in men, and also for the development of skeletal muscle mass and strength, erythropoiesis and bone density, amongst other functions.

The divergent effects that androgens have between sexes can be explained by differences in concentration, metabolism and receptor expressions. Male sex hormones are also known to fluctuate along the day and throughout life. Prior to puberty, testosterone level is usually low in males. However, after puberty, testosterone level increases and reaches its peak around the age of 20-25 in men. As aging occurs, testosterone levels decline.

Testosterone and its derivatives are well known for their androgenic properties and anabolic effects. These hormones direct the differentiation of organs and tissues towards the adoption of male phenotypes. Thus far, the effects of androgens on the cardiovascular system remain incompletely understood. Some studies point to increased cardiovascular risk associated with a high circulating androgen level, related to responses that lead to changes in blood pressure, ion channel activity and cardiomyocyte hypertrophy. However, other studies support a long-term cardio-protective role for these steroid hormones. Despite these differences, increasing clinical evidence supports consideration for transient use of testosterone derivatives to improve cardiovascular function.

## 2. Mechanism of androgen action

Androgens exert most their effects through the direct binding to specific intracellular receptors acting as transcriptional activators (Beato, 1989). Intracellular androgen receptors have been described in neonatal and adult cardiomyocytes (Hickson et al., 1984; Marsh et al., 1998). Genomic responses to testosterone are mediated through the intracellular androgen receptor, which is a 110-kDa protein with domains for androgen binding, nuclear localization, DNA binding, and transactivation (TD). The conserved domain structure has three major functional regions: an NH-terminal transactivation domain, a centrally located

DNA binding domain (DBD), and a COOH-terminal hormone-binding domain (HBD) (**Figure 1**). The COOH-terminus contains an additional activation domain and a hinge region connecting the HBD and the DBD. Upon ligand binding, the nuclear receptors translocate to the nucleus where they dimerize and bind to regulatory DNA sequences on target genes and activate transcription (Simental et al., 1992). Several co-regulatory proteins that bind and regulate the activity of receptors have been identified. These include both co-activators that positively regulate transcriptional effects of intracellular receptors after ligand binding and co-repressors that negatively regulate receptor activity. These effects are slow, with a latency period before onset, but they are also long lasting, remaining active for hours after hormone stimulation.

In addition to this transcriptional or genomic mode of action, increasing evidence suggests that androgens can exert rapid, non-genomic effects. The time course of these responses is not compatible with the classic genomic mechanism for the action of steroids, since they have a rapid onset without an apparent latency period. Common to these early effects is a fast increase in intracellular  $\text{Ca}^{2+}$  and activation of  $\text{Ca}^{2+}$ -dependent pathways and second messenger cascades (Estrada et al., 2000, 2003). Second messenger induction by nongenomic steroid action is insensitive to inhibitors of either transcription or translation. Little is known about these non-genomic effects in cardiac cells. However, these responses involve the generation of different patterns of  $\text{Ca}^{2+}$  signals and also in the activation of complementary  $\text{Ca}^{2+}$ -dependent pathways. Thus, in addition to the classical mechanism of steroid action in cardiac cells, androgens activate additional specific signal transduction pathways (Altamirano et al., 2009; Vicencio et al., 2006). An interesting hypothesis is that these second messenger cascades may ultimately serve to modulate the transcriptional activity of the intracellular androgen receptor and its associated global response. This idea of an integrated mechanism for androgen action will be discussed.

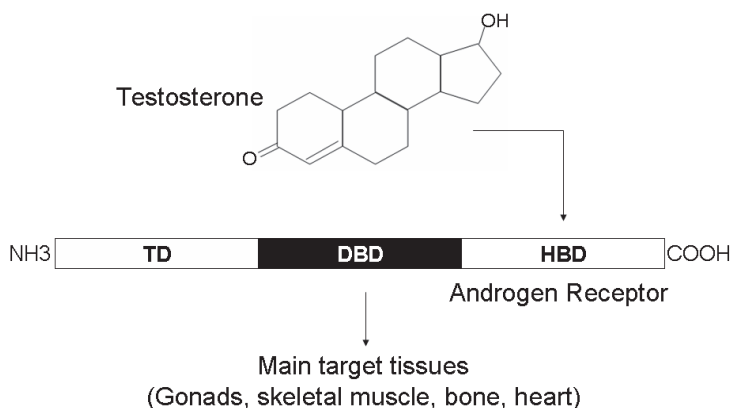


Fig. 1. Schematic representation of testosterone and the main domains of intracellular androgen receptor.

### 3. Cardiovascular effects of androgens

Androgens influence cardiac function by acting directly on the heart or by affecting the vascular system (English et al., 2001; Hayward et al., 2001; Jones et al., 2003; Kienitz &

Quinkler, 2008). In humans and experimental animals, anabolic steroids have been associated with an increased risk of coronary artery disease by adversely affecting the plasma lipid and lipoprotein profile, producing thrombosis and cardiac hypertrophy (Liu et al., 2003; Sullivan et al., 1998). In particular, use of elevated doses of testosterone or its synthetic cognates has been related to cardiac hypertrophy, ventricular remodelling, cardiomyopathy, myocardial infarction, and sudden cardiac death. On the other hand, with a normal steroid level, androgen actions are necessary for a range of developmental and biological processes, including maintaining the health of cardiomyocytes. Androgens might also produce additional hemodynamic effects by relaxing vascular bed, reducing after-load and rapidly increasing cardiac contractility, resulting in increased cardiac output (Liu et al., 2003). This dual androgen effect is not clear yet. Research in the field of androgens and androgen receptors signaling pathways will provide considerable understanding of the physiological and pathological roles of these hormones. These effects could be related to different concentrations of these hormones as well as to differential activation of signal transduction pathways.

Both beneficial and pathological effects of androgens are observed clinically. Patients with chronic heart failure suffer considerable morbidity as well as early mortality. They exhibit altered structure and function of cardiac and skeletal muscle and excessive activation of catabolic hormones and inflammatory cytokines (Malkin et al., 2004). Men with chronic heart failure have relatively low androgen levels, which may contribute to the pathophysiological process.

### **3.1 Role of androgens in hypertension**

Hypertension is a major risk factor for developing cardiovascular disease, atherosclerosis and sudden cardiac death. Hypertension is more prevalent and occurs earlier in men than in women. Blood pressure is elevated from the onset of puberty in males, when they manifest the effects and differences of sex steroids. These differences in blood pressure between men and women are maintained until 60 years of age. Epidemiological data indicate that systolic blood pressure in men under the age of 60 years is 6-7 mm Hg higher than in women, and diastolic pressure is higher by 3-5 mm Hg (Stamler et al., 1976). In women over the age of 60, show gradual increases of blood pressure over a period of 5 to 20 years, until hypertension is highly prevalent in women as in men. Estrogen reduction after menopause is partially responsible for this effect and experimental data indicate that the change in the estrogen/androgen ratio seems to be the cause (Akkad et al., 1997; Pripp et al., 1999).

Epidemiological studies show that androgens are important determinants of sex differences in blood pressure. An elevated level of anabolic steroids, found in anabolic abuse by high performance athletes has been associated with hypertension, cardiac remodelling and myocardial ischemia (Kienitz & Quinkler, 2008). This suggests a direct role in the development of hypertension. However, clinical evidence shows beneficial effects of testosterone on hypertension. Observational studies in humans indicate that there is an inverse relationship between systolic pressure and the plasma level of testosterone in men. There is also higher incidence of hypertension in individuals with reduced free circulating androgen (Fogari et al., 2005). The beneficial effect of testosterone replacement has also been well documented. Accordingly, in randomized, double-blind, case-control clinical studies hormone administration was related to the reduction of vascular tone (Malkin et al., 2006). In other study, administration of testosterone significantly reduced ST segment depression

in patients with stable angina and improved post-exercise myocardial perfusion in patients with occlusive coronary artery disease (Jaffe, 1977).

Furthermore, experimental evidences provide controversial information. In some animal models potential role for androgens in the pathogenesis of hypertension have been observed. Mice hypertension models show that castration and subsequent testosterone supplementation at high doses produce the onset of hypertension (Dubey et al., 2002). This effect is mediated by androgen receptors, as shown by the inhibitory effect of flutamide, an antagonist of the intracellular androgen receptor. However, others *in vitro* studies indicate a direct vasodilator effect of androgens by the activation of the nitric oxide (NO) pathway mediated by the endothelium, activation of voltage-dependent potassium channels and blockage of voltage and receptor-operated calcium channels (Jones et al., 2003, 2004).

Thus, androgens appear to be involved in the regulation of vascular tone. Aging induces a progressive reduction in the plasma testosterone levels along the years, which begins to be deleterious as aging occurs; while physiological stress produces acute diminution of androgen levels. These conditions are determinants for clinical manifestation of hypertension. Therefore, testosterone supplementation in men with low levels of androgens could be beneficial to restore altered blood pressure (Handelsman & Liu, 2005).

### **3.2 Androgens in endothelial function, atherosclerosis and heart perfusion**

Numerous clinical studies show that men exhibit higher susceptibility to atherosclerosis than pre-menopausal women. The available information indicate that the evolution of atherosclerosis is faster in males – independent of dyslipidemia or evidence of endothelial damage – than females (Kaushik et al, 2010.). However, a negative correlation between testosterone plasma level and cardiovascular disease in men has been observed (Iliescu & Reckelhoff, 2006; Thijs et al., 2003). Testosterone deficiency affects approximately 30% of men under 30 years and as aging occurs, testosterone level decline even more, a male condition known as andropause (Handelsman & Liu, 2005). Low androgen concentrations are strongly associated with increase in cardiovascular risks including atherogenic lipid profile, insulin resistance, obesity and prothrombotic profile (Jones et al., 2005; Kapoor et al., 2007). However, other studies indicate that testosterone increases cholesterol uptake by macrophages promoting its transformation into foam cells, which are involved in the formation of atheroma lesions (McCrohon et al., 2000). Testosterone also induces apoptosis of endothelial cells *in vitro* and promotes proliferation and migration of vascular smooth muscle cells, which are involved in the development of atherosclerotic lesions (Kaushik et al., 2010).

This appears to explain the faster progression of atherosclerosis occurring in men than women. However, the amount of clinical evidence suggests protective effects of androgens during atherosclerosis. For example, high to normal range of testosterone concentrations exhibit preventive role in coronary artery disease. In fact, patients with coronary artery disease show significantly lower testosterone level than patients without coronary artery disease (English et al., 2000). Animal models show similar results (Alexandersen et al., 1999). At cellular level, the mechanism for these testosterone effects involve inhibition of macrophage migration, which decrease the expression of adhesion molecules and pro-inflammatory cytokines such as TNF $\alpha$  (Malkin et al., 2004).

In addition, androgens produce direct and rapid vasodilatory effects in human coronary disease and stable angina, increasing the coronary flow between 12 and 17% (Webb et al., 1999). This has not been free of controversy, because in isolated vessels the chronic

treatment with testosterone produce increase in the vasoconstrictor responses to adrenergic agonists, while reduce the response to nitric oxide (Iliescu & Reckelhoff, 2006; Malkin et al., 2006). In animal models, testosterone produces a similar effect, independent of androgen receptor. Vasodilatation occurs by inhibition of plasma membrane calcium channels and activation of potassium channel (Ding & Stallone, 2001). However, this effect is acute and only produced in response to high doses of testosterone, so this does not appear to be an appropriate strategy for anti-ischemic treatment. But this could explain the positive effects on peripheral vascular resistance in response to acute administration of testosterone in men with heart failure, stable angina or in arteries isolated from healthy men with heart failure. Thus, current data indicate that androgens, particularly testosterone, do not have a permissive role in the evolution of atherosclerotic lesions at the cellular level. However, testosterone diminution as in aging is associated with accelerated atherosclerosis. This occurs by increase in the expression of adhesion molecules and pro-inflammatory cytokines. Moreover, this also could be stimulated by high exogenous androgen supplementation.

### 3.3 Effects of testosterone on cardiac excitability

Gender differences are well-established in the generation of ventricular arrhythmias associated with a prolonged QT interval. This interval is used to estimate the electrocardiographic duration of ventricular action potential. Thus, women exhibit greatest risk of ventricular arrhythmias such as "*torsade de pointes*", a type of highly fatal polymorphic ventricular tachycardia. This clinical condition is worse in women compared with men, particularly in response to drugs that prolong the QT interval (Rautaharju et al., 2006). This has been associated with sex differences in the rate of ventricular repolarization being slower in adult women. These differences are manifested from puberty, becoming the QT interval increasingly longer until women reach adulthood. In men, once they start to drop in plasma testosterone levels gradually prolong the QT interval similar to women older than 60 (Zhou et al., 1992). Studies in experimental animals have established that testosterone levels are negatively correlated with the duration of the action potential (Pham et al., 2001) and other researchers reported that dihydrotestosterone administration in castrated male rabbits shortened QT interval (Liu et al., 2003).

This information indicates that testosterone is a regulator of sex differences in the action potential duration, ventricular repolarization and propensity to ventricular arrhythmias. In isolated cardiomyocytes has been established that testosterone increases the expression of  $\beta$ -adrenergic receptors, L-type calcium channel and sodium-calcium exchanger (Golden et al., 2004). Moreover, testosterone increases by 30% the density of repolarizing potassium currents, which is due to changes in both gating kinetics of the delayed rectifier potassium currents (I<sub>kr</sub>) and shortening the duration of the QT interval (Liu et al., 2003)

Testosterone has been shown to interact directly with the NO system. Activation of this pathway induces shortening in the duration of the action potential by activating the slow component of delayed rectifier potassium current (I<sub>ks</sub>) and inhibiting L-type calcium currents (I<sub>ca,L</sub>) (Bai et al., 2004, 2005).

## 4. Androgen-mediated cardiac hypertrophy

The most striking evidence of cardiac effect of androgens is the case of highly conditioned athletes, which have died by sudden cardiac death. *Post mortem* examinations indicated anatomical abnormalities in the heart, known as hypertrophic cardiomyopathy (Maron et

al., 1978, 1980). Thus, it was assumed that elevated testosterone administration induces cardiac hypertrophy.

Cardiac hypertrophy is an adaptive mechanism to enhance cardiac output in response to cardiovascular challenges. Cardiac hypertrophy is produced in response to several pathophysiological conditions, such as mechanical stretching or neuro-hormonal deregulation. This process is characterized by increases in cardiomyocyte size and protein synthesis, as well as by the re-expression of various fetal genes (Frey & Olson, 2003; Izumo et al., 1987). At the cellular level, cardiomyocytes depend on the activation of several signalling pathways related to cell growth, which are crucial for the development of cardiac hypertrophy. These pathways involve both androgen receptors as well as intracellular signalling pathways.

#### **4.1 Androgen receptor in hypertrophy**

Cellular effects of testosterone depend on activation of androgen receptor, which is localized in cytoplasm and acts as a transcriptional factor when it binds testosterone. Marsh et al. have shown that androgens produce cardiac hypertrophy by a direct, receptor-specific mechanism (Marsh et al., 1998). They have also revealed that androgens regulate functional expression of an L-type calcium channel in isolated rat ventricular cardiomyocytes, leading to a modulation of cardiac performance in males. Li et al. reported that either castration or administration of flutamide, an androgen receptor antagonist, markedly attenuated cardiac hypertrophy and fibrosis in guanylyl cyclase-A knock-out male mice (Li et al., 2004). In baroreceptor-denervated rats, left ventricular hypertrophy is gender-dependent and elevated testosterone stimulates cardiac hypertrophy (Cabral et al., 1988a, 1988b). Moreover, *in vitro* studies provide evidence that androgens induce hypertrophic growth in cultured cardiomyocytes, suggesting that the growth promoting effect is direct (Marsh et al., 1998). The hypertrophic effects of testosterone are associated with increased protein synthesis mediated by the androgen receptor and specific nuclear coactivators related to cell growth (Hickson et al., 1984).

#### **4.2 Intracellular signaling pathways**

Pro-hypertrophy stimuli activate diverse intracellular signaling pathways. Activation of these pathways by androgens could lead to long-term cellular effects by activation of co-transcriptional modulators. Induction of multiple signaling pathways in cardiac hypertrophy has been described as follows:

##### **Ca<sup>2+</sup>/Calcineurin/NFAT pathway**

Calcium ion (Ca<sup>2+</sup>) is one of the most diverse and important intracellular second messenger as well as a key element in the excitation-contraction coupling of cardiac muscle. Ca<sup>2+</sup> has been related to cardiac hypertrophy, because of its ability to promote the activation of the protein phosphatase calcineurin through establishment of Ca<sup>2+</sup>/calmodulin complex (Heineke & Molkentin, 2006). Calcineurin promotes the translocation of the nuclear factor of activated T cells (NFAT) from cytoplasm to nucleus. NFAT family proteins are responsible for the expression of c-fos, c-jun and c-myc, early fetal genes; which are expressed during fetal development. These are silenced in adult stages and then re-expressed during cardiac hypertrophy, reason why they are considered as hypertrophic markers (Frey & Olson, 2002, 2003; Heineke & Molkentin, 2006).

##### **PI3K/Akt/GSK3-β pathway**

Another cellular pathway involved in cardiac hypertrophy is PI3-K (Phosphoinositide 3-kinase)/Akt proteins. This signalling pathway has been related to cell survival and

proliferation in almost all cell types. However, the up-regulation of the pathway by several stimuli induces cardiac hypertrophy. One of the most common downstream targets of Akt is the protein kinase GSK3- $\beta$  (Glycogen Synthase Kinase 3- $\beta$ ) (Antos et al., 2002). Activated GSK3- $\beta$  phosphorylates several members of NFAT family, which promotes their translocation from nucleus to cytoplasm. Akt phosphorylates and inhibits GSK3- $\beta$ , which increases the residence of NFAT in the nucleus. Also, Akt has the ability to phosphorylate the protein mTOR (mammalian target of rapamycin), another downstream target of PI3K/Akt pathway. In cardiac cells, protein synthesis is highly regulated by mTOR, which stimulates protein translation and ribosome biosynthesis (Proud, 2004). mTOR lies upstream of critical translation regulators such as the 40S ribosomal protein S6 kinase 1 (S6K1) and the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). Activation of the mTOR pathway is a critical step to induce cardiac hypertrophy *in vitro* (Altamirano et al., 2009; Takano et al., 1996) and *in vivo* (Shioi et al., 2003).

### G-protein-coupled receptors pathway

Plasma membrane G-protein G-protein-coupled receptors (GPCR) are crucial for cardiac function and hypertrophic growth. Both Gas and G $\alpha$ q are sufficient to produce cardiac hypertrophy. Gas induces the synthesis of cAMP and PKA activation, which could induce hypertrophy. G $\alpha$ q promotes the activation of the protein phospholipase C (PLC) and IP<sub>3</sub> production, which induces intracellular Ca<sup>+2</sup> oscillations and hence, the activation of different proteins responsible of cardiac hypertrophy, such as calcineurin (Heineke & Molkentin, 2006).

These signalling pathways are related to the hypertrophic growth of cardiomyocytes. It has been described that testosterone induces intracellular Ca<sup>+2</sup> increase through a nongenomic action mechanism, as we discussed earlier in this chapter. Studies in cultured cardiomyocytes show that through a nongenomic mechanism, testosterone is implicated in the activation of a membrane receptor coupled to a G $\alpha$ q protein, thus resulting in the production of IP<sub>3</sub> and Ca<sup>+2</sup> release from endoplasmic reticulum (Vicencio et al., 2006). Then, calcium oscillations induce the activation of the mitogen-activated protein kinase ERK 1/2, which in turns phosphorylates mTOR, promoting hypertrophic cardiac growth (Altamirano et al., 2009).

Aside from classical action mechanism for testosterone, non-classical effects also are implicated in cardiac hypertrophy development has been described. The establishment of testosterone-androgen receptor complex acts as a transcriptional factor for the expression of different genes and proteins necessary for protein synthesis, energy production and cell growth, which are crucial for hypertrophic growth.

### 4.3 Cardiac remodelling

Cardiac remodelling involves changes in the structure, mass and function of the heart, mainly due to increase in the number of myofibrils or cardiomyocyte hypertrophy. Proliferation of cardiac fibroblasts and consequent increase in the extracellular matrix is called cardiac fibrosis. The balance between synthesis and degradation of different collagen types determine the content of extracellular matrix proteins. Cardiac fibrosis increases the stiffness of the heart chambers, which produce high end diastolic pressure. If this event is persistent, leads to progressive and irreversible cardiac damage and heart failure. Controversial information exists about the role of sex hormones on cardiac remodelling during cardiac hypertrophy, including fibrosis. However, these differences are manifested

as gender differences in cardiac remodelling. Heart size of men is bigger than women, even when is corrected for body weight. Men also have higher incidence of fibrosis; however direct effects of androgens are not clear. The increase in cardiac mass is higher in men from puberty and has been shown that estrogens have preventive effects on cardiac hypertrophy (Weinberg et al., 1999). In castrated mice, administration of androgen receptor antagonists does not abolish the sex differences in heart size or cardiac fibrosis (Li et al., 2004), suggesting that androgens are not the only determinants for these differences. Negative consequences of fibrosis are mediated mainly by local effect of the renin-angiotensin system. Angiotensin II regulates differentiation and proliferation of ventricular fibroblasts and synthesis of extracellular matrix, which consist mainly of collagen type I and III (Krenning et al., 2010). The synthesis of collagen type I and III by fibroblasts is stimulated by TGF- $\beta$ 1 and inhibited by androgens (Ikeda et al., 2005). Cardiac fibroblasts express androgen receptor, which suggest a role for androgens during cardiac remodelling. Experimental evidence in androgen receptor knockout (ARKO) mice show reduced ventricular volume and wall thickness, but no differences in blood pressure or heart rate as compared with normal animals (Ikeda et al., 2005). It has also been demonstrated that dihydrotestosterone increase atrial natriuretic peptide (ANP) secretion from neonatal rat cardiomyocytes (Marsh et al., 1998). Furthermore, ARKO mice show a significant reduction in ANP gene transcription, resulting in increased cardiac fibrosis. On the other hand, testosterone has been shown to increase the amount of collagen measured around coronary blood vessels contributing to fibrosis in male hypertensive rats independent of the renin-angiotensin system. Cavasin et al. (2006) have determined that high testosterone levels enhance acute myocardial inflammation, adversely affecting myocardial healing and early remodelling (Cavasin et al., 2006). Even when the evidence is still controversial, androgens show a protective role on cardiac remodelling and protect the heart from maladaptive fibrosis, preventing the deleterious effects of increased pre- and after-load induced by angiotensin II.

## 5. Cardiac metabolism

Normal heart requires a continuous supply of energy to maintain muscle contraction as well as hemodynamic adaptation, survival, growth and metabolism. Neuro-hormonal activation is an important input for cardiovascular response to metabolic demands. Although initially adaptive, neuro-hormonal activation together with metabolic alterations has deleterious effects on cardiovascular system.

In the heart muscle, ATP production is crucial for all cellular and contractile process. In the adult heart, ATP comes mainly from the  $\beta$ -oxidation of fatty acids, an oxygen dependent process. The 10 - 40% remaining come from glucose degradation through glycolysis, an oxygen independent process (Neglia et al., 2007; Stanley et al., 2005).

$\beta$ -oxidation depends upon fatty acids uptake, which occurs through different fatty acid translocases located in the plasma membrane, like CD36 protein. Fatty acids must be transported to the mitochondria for  $\beta$ -oxidation, which occurs through the enzyme carinitine palmitoyl transferase-1 (CPT-1). Once inside mitochondria, fatty acids are oxidized to produce acetyl-CoA, which will be incorporated into Krebs cycle for NADH, FADH<sub>2</sub> and CO<sub>2</sub> production. These intermediates provide oxidative power during the oxidative phosphorylation in mitochondria, where ATP is finally produced by ATP synthase complex V.



On the other hand, glycolysis requires glucose uptake, which occurs through the glucose transporters 1 and 4 (GLUT1 and GLUT4), the main glucose transporters in cardiac cells. Once inside the cell, the enzymes hexokinase and phosphofructokinase, the glycolysis pacemaker enzyme, lead the conversion of glucose to pyruvate. Through this process 2 ATP molecules are generated from each glucose molecule incorporated.

However, the heart muscle is able to modify the ratio between  $\beta$ -oxidation and glycolysis in response to pro-hypertrophic agents (Stanley et al., 2005). For example, cardiac hypertrophy due to pressure overload is accompanied of a reduction in  $\beta$ -oxidation and an increase in the glycolytic rate as a compensatory mechanism that improves ATP production in the heart muscle (van der Vusse et al., 2000, 2002). In contrast, individuals with uncompensated diabetes usually develop a different cardiac hypertrophy, known as diabetic cardiomyopathy. Since diabetic patients are not able to metabolize glucose, diabetic cardiomyopathy is characterized by an increase in the fatty acids assimilation by cardiac cells, which carry cardiac lipotoxicity (van der Vusse et al., 2000).

During heart failure, cardiac muscle is not able to produce enough ATP for cardiac contraction, resulting in poor contractile performance. It is common to observe heart failure in pathologies associated to bad glucose handling for ATP production, as insulin resistance and diabetes. Metabolic syndrome, which comprehends both pathologies, has been correlated with low testosterone blood levels (Saad & Gooren, 2009, 2011). It has been recently postulated that decrease in testosterone is a link between metabolic syndrome and heart failure. Clinical trials have demonstrated that the administration of testosterone at physiological concentrations improved insulin sensitivity, reduced glycaemia and central obesity, and heart failure progression in men suffering metabolic syndrome (Cook & Romashkan, 2011). In addition to clinical trials, there are new studies related to testosterone effects on glucose metabolism. It has been described that testosterone is necessary to maintenance insulin and glucose concentration in blood. Recent reports have shown an important role for testosterone in the mobilization of glucose transporter GLUT4 to the plasma membrane in skeletal muscle, liver and fat tissue (Muthusamy et al., 2007, 2009).

In spite, elevated testosterone administration in humans is usually associated with the development of cardiac hypertrophy and heart failure; there are several studies that examine the relationship between the testosterone supplementation and heart failure with non positive correlation between them. Nowadays, several clinical trials lead to propose that testosterone supplementation at physiological doses could be a treatment for men with metabolic syndrome and heart failure (Pugh et al., 2000). The effect of testosterone as a metabolic modulator could improve insulin sensitivity and glucose uptake in the heart, which could lead to an increase in ATP production when cardiac output increases. However, evidences about the metabolic properties of testosterone on heart muscle come from observational studies. Research on the molecular mechanisms implicated in the metabolic testosterone actions is required.

## 6. Integrated model for androgen action

Androgens diffuse across plasma membrane due to their lipophilic nature. Once inside the cell, they are recognized by the cytosolic androgen receptor, which forms a complex with its ligand. This complex works as a transcription factor, which translocates to cell nucleus, binds to DNA and promotes the activation of several genes (Beato, 1989). This is called the

classic or genomic androgen response pathway. On the other hand, it has been reported that androgens, exert a rapid, non-genomic effect mediated by a putative plasma membrane receptor. Its activation implicates participation of kinases, intracellular calcium oscillations and transcription factor activation, among other fast events.

Cardiomyocytes are terminally differentiated cells. Cardiac hypertrophy is characterized by an increase in cell size and protein synthesis and reactivation of a fetal gene program. Emerging evidence indicate that androgen action involves a crosstalk between genomic and nongenomic mechanisms. These could imply a two-step process, where both non-genomic and genomic effects occur sequentially (**Figure 2**). First, fast effects would be generated at the plasma membrane level with second messenger participation. Second, there would be gene activation; the pathways used by each cell type could provide specificity to the signal through a cooperative mechanism, which can act in a concerted form.

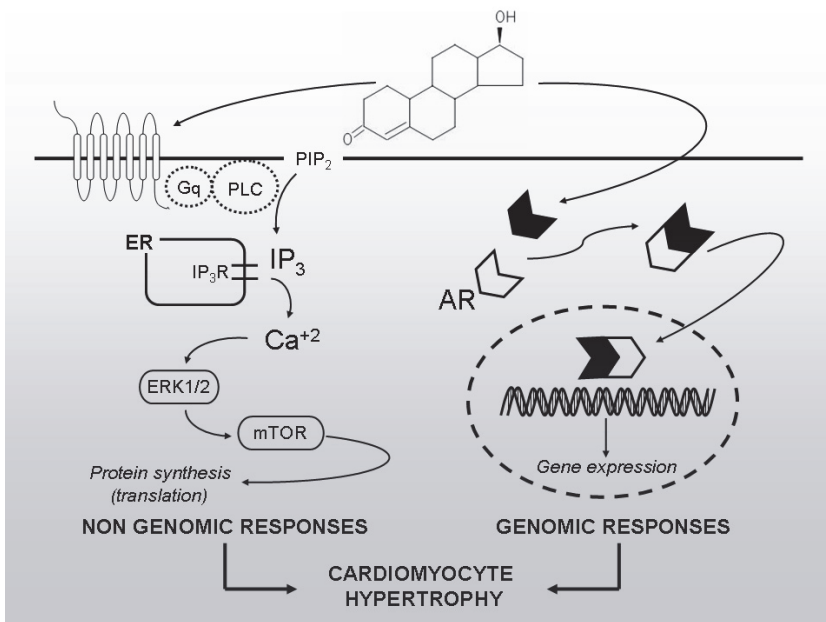


Fig. 2. Integrated mechanism for testosterone effects in cardiomyocytes. In cardiomyocytes, testosterone activates plasma membrane androgen receptors coupled to heterotrimeric Gq protein. G $\beta\gamma$  dimers activate phospholipase C (PLC), which locally produces inositol IP<sub>3</sub> thus activating IP<sub>3</sub> receptors (IP<sub>3</sub>R). The consequent Ca<sup>2+</sup> increase leads to the activation of several pathways, including the MEK/ERK/mTOR axis. These responses combined induce a series of genomic changes that lead to increased gene expression and increased protein synthesis, ultimately leading to cardiac hypertrophy.

While studying these hypertrophy effects on cardiomyocytes, our laboratory determined that testosterone uses a cooperative mechanism between the genomic and nongenomic pathways to produce the hypertrophic growth of cardiac cells. Thus, with normal levels of androgens the interactions among signaling pathways lead to the maintained health of cardiac cells, whereas high doses recruit additional signals which are hypertrophic. As

depicted in Figure 2, testosterone activates a plasma membrane G-protein coupled receptor, a signaling pathway that results in a nongenomic mechanism. Activation of this pathway facilitates the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C, thereby generating diacylglycerol and IP<sub>3</sub>. IP<sub>3</sub> mediates opening of IP<sub>3</sub> receptors in the sarcoplasmic reticulum, which in turn produce rapid (1-5 min) Ca<sup>2+</sup> release (Vicencio et al., 2006). Similar effects of testosterone have been identified in T-cells, skeletal muscle and neuroblastoma cells (Benten et al., 1999; Estrada et al., 2003, 2006). In cardiomyocytes, nongenomic signals activate the extracellular signal-regulated kinase (ERK) and the mTOR pathways. Activation of Ca<sup>2+</sup>/ERK/mTOR axis is independent of androgen receptor. The anabolic properties of testosterone indicate that this hormone regulate multiple genes involved in growth and metabolism. Traditionally, ligand-dependent androgen receptors act on DNA within the nucleus and in this compartment there is cross-talk among the nuclear receptors and cytosolic signaling. We have determined that testosterone induce a hypertrophic pattern in cardiomyocytes, evaluated as increase in the expression of fetal proteins, cardiomyocyte size, and protein synthesis. Hypertrophy effects of testosterone were blocked by either inhibition of mTOR or androgen receptor (Altamirano et al., 2009). We propose that cell growth produced by androgens require both androgen receptor activity and translation control through mTOR signaling pathway. Thus, both mTOR pathway and androgen receptor could control protein synthesis by a coordinated mechanism, where mTOR regulates translation and the intracellular androgen receptor regulates gene expression.

## 7. Perspective

The role of sex steroid hormones in modulating cardiovascular function is of the highest importance, given that androgen deficiency is strongly associated with common medical conditions including metabolic syndrome, obesity, diabetes, hypertension and atherosclerosis. However, cardiovascular side effects of high doses of anabolic/androgen steroid reduce its actual therapeutic use. Research in this field is necessary to know the real cardiovascular effects of androgens and to elaborate future optimal therapeutic replacement protocols.

## 8. Acknowledgment

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# Testosterone Therapy and the Heart

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## 1. Introduction

Testosterone (T) therapy has been offered to men who have low T levels, who are symptomatic with classical androgen deficiency syndromes, to induce and maintain secondary sex characteristics and to improve muscle mass and strength, bone mineral density, their sexual function, and sense of well-being. Hypogonadism is a clinical syndrome that is caused by inability of the testis to produce physiological levels of T in men. The age-related reduction in T levels is a result of defects in both hypothalamic-pituitary and testicular function. A diagnosis of androgen deficiency should be made only when low serum T and consistent symptoms and signs are encountered in men. It is not recommended to offer T therapy to all older men with low T level. A decision should be made on an individualized basis for older men with clinically significant symptoms of androgen deficiency and consistently low T level. Hyperandrogenemia in women and hypoandrogenemia in men are associated with increased risk of coronary artery disease. In addition, these states are associated with insulin resistance, visceral obesity, elevated triglycerides, low high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol and plasminogen activator inhibitor (PAI-1). These gender differences and confounders make the exact role of endogenous androgens in atherosclerosis unclear. In contrast, exogenous androgens exert both apparently beneficial and deleterious effects on cardiovascular risk factors by reducing serum levels of PAI-1 (apparently deleterious), HDL-C, insulin, leptin, lipoprotein(a), visceral fat mass (apparently beneficial), and fibrinogen in women as well as men. Androgen-induced reduction in circulating HDL-C levels should not reflexively be considered to be pro-atherogenic, because it may show increased reverse cholesterol transport instead. Short-term use of supraphysiological doses of exogenous T can decrease the severity and frequency of angina pectoris and reverse the electrocardiographic signs of myocardial ischaemia; yet long-term effects have not been determined. Nonetheless, the effects of pharmacological doses of androgens particularly on flow-mediated dilatation and arterial compliance must be interpreted with caution, since in addition at physiological concentrations detrimental effects, neutral, and beneficial effects on vascular reactivity can be observed. Testosterone induces an anti-atherogenic effect by facilitating efflux of cellular cholesterol to HDL and induces pro-atherogenic effects on macrophage function by increasing the uptake of modified lipoproteins.

## 2. Endogenous T and CAD morbidity/mortality

In half of cross-sectional studies [Wu and Eckardstein 2003; Alexandersen et al 1996], serum levels of T were lower in patients with CAD than healthy controls. The other half of the

studies demonstrated no difference in T levels when cases and controls are compared. In none of the studies, high levels of T were seen in patients with CAD. In all the studies, an inverse association was found between CAD and levels of free T [Wu and Eckardstein 2003; Alexandersen et al 1996]. None of six longitudinal studies revealed any significant association between the future risk of CAD events and serum levels of T in men [Wu and Eckardstein, 2003; Alexandersen et al., 1996].

European Prospective Investigation into Cancer in Norfolk Study demonstrated that serum levels of T at baseline were inversely associated with cardiovascular mortality and all-cause mortality in men aged 40-79 with no history of cancer or cardiovascular disease (Khaw et al., 2007). This risk association persisted after correction for traditional cardiovascular risk factors, indicating that the benefit may involve additional pathways. Also, other studies have showed the association between low testosterone and cardiovascular disease and increased mortality (English et al., 2000; Shores et al., 2006). Thus, increasing research on the clinical implications of physiological decline in androgens with aging has led to rekindled interest in the potentially cardioprotective role of T. Abuse of T is associated with increased risk of cardiovascular disease and sudden death, yet higher T serum levels within the physiological range have been associated with a more favourable cardio-metabolic risk profile. In contrast, low T serum levels have been associated with elevated triglyceride and low high-density-lipoprotein-cholesterol levels, glucose intolerance, diabetes, and central obesity (Kapoor et al., 2005; Tong et al., 2005; Wu and Eckardstein, 2003). This is in part because of the increased triglyceride and lipoprotein lipase uptake by adipocytes, causing central obesity and insulin resistance. The central obesity leads to increased peripheral conversion of T to estradiol by aromatase activity, further worsening this vicious cycle. Decreased serum levels of T are associated with a pro-inflammatory state with raised levels of inflammatory mediators like TNF- $\alpha$  and IL-1 $\beta$ , which can be improved with T substitution (Malkin et al., 2004).

Besides its pro-atherogenic effects by altered lipid profile and insulin resistance, T has also direct effects on the vasculature. T causes coronary vasodilation and improves chronic stable angina in men (English et al., 2000). Androgen receptors have been found in the myocardium and may exert some of the effects of T on the cardiac remodeling and renin-angiotensin-aldosterone system. T and other anabolic hormone deficiencies are associated with the severity of heart failure and increased mortality (Jankowska et al., 2006). T augments expression of heat shock protein 70 in cardiac myocytes, through which it may play an important role in preconditioning and protection against cardiac ischemia (Liu et al., 2006).

Studies that demonstrated an association between low serum T levels and raised cardiovascular mortality in general excluded patients at high risk for cardiovascular disease or those with established cardiovascular disease. This association whether applies to subjects with established cardiovascular disease has not been defined. In a study of 930 men with angiographically proven coronary disease that were followed up for a mean duration of 7 years, a high prevalence of low T at baseline ranging from 17 to 24% was seen. Decreased serum T levels as defined by baseline serum T levels of <2.6 nmol/l were an independent predictor of vascular and all-cause mortality (Malkin et al., 2010). In a recent study, Ruige et al. (Ruige et al., 2010) have reviewed the literature on serum T levels and overall mortality up to 2009. They demonstrated an excess mortality in elderly men (>70 years of age) who have a reduced T, which has been especially marked in studies since 2007. It is important to consider the limitations of observational studies in showing the associations between coronary artery disease (CAD) and serum levels of endogenous androgens. These

studies have had extremely variable CAD endpoints (e.g., ultrasound- or postmortem- based diagnosis, unspecified cardiac events, morbid conditions such as myocardial infarction [MI] and angina, mortality, and the performance of angiography), heterogeneous study populations, and variable selection criteria. For instance, most CAD patients have been taking medications and have changed their lifestyle, factors which could affect them compared to controls. In some studies, allocation of poorly matched controls may be problematic in analysis. Among patients, the time interval from MI to sampling changed from 3 months to several years and has not been always standardized for diurnal rhythm of hormone levels. Also, in most of the studies, no adjustment has been made for confounding factors. For instance, the diagnoses of hyperandrogenemia in women and hypoandrogenemia in men were confounded by accompanying concurrent metabolic disorders, such as insulin resistance, obesity, impaired fibrinolysis, and dyslipidemia, which are frequently seen in chronic diseases, including CAD and lower serum levels of T (Eckardstein & Wu, 2003).

### 3. Exogenous T in cardiovascular disease

The long-term effects of exogenous T on incidence of coronary events have not been determined. However, few small studies demonstrated that therapeutic doses of T decreased the severity and frequency of angina pectoris and reversed electrocardiographic signs of myocardial ischemia. Webb et al. [Webb et al 1999] demonstrated that a single intravenous (IV) bolus of 2.3 mg T prolonged time to 1-mm ST segment depression by 66 seconds in 14 men with low plasma T levels and CAD. The plasma levels of T increased from 5.2 to 117 nmol/l, suggesting that this is a pharmacological action on the coronary vessels. These acute, direct pharmacological effects of T have been further investigated during coronary angiography. Administration of T over a 3-min period into the coronary arteries of 13 men with the diagnosis of CAD during coronary angiography at supra-physiological doses of 8 fmol/l, but not at the physiological doses of 8 nmol/l, cause coronary vessel diameter and blood flow to increase significantly at all four doses of T tested. These results have been also confirmed by a similar study of 14 men with CAD [Rosano ET 1999].

T has been employed at either pharmacological doses to exert selective effects on haematopoietic marrow, muscle, bone, or other salutary effects in men with various chronic diseases (Handelsman, 2005; Liu, 2004) or at physiological doses as androgen replacement therapy for men with disorders of the hypothalamo-pituitary-testicular axis (Handelsman, 2005; Zitzmann & Nieschlag, 2000). Randomized placebo-controlled studies demonstrated that T employed to patients with chronic heart failure decreases systemic vascular resistance and improves both overall exercise capacity and cardiac output (Malkin et al., 2006; Pugh et al., 2003, 2004). Yet, effects on objective echocardiographic findings were little, suggesting that improvements could have been indirect through enhanced exercise endurance resulting from skeletal muscle or psychological effects. The effects of androgens on undamaged and damaged myocardium might be variable. One randomized placebo-controlled study demonstrated no deleterious effects on cardiac structure or function in 16 athletes after 8 weeks of 200 mg nandrolone decanoate injections (Hartgens et al., 2003). Likewise, the effects of testosterone have not been tested to determine whether androgen activation by aromatization to estradiol and/or amplification by 5 $\alpha$  reduction influences effects of androgens on cardiac function. Routine echocardiographic evaluation used in the above studies may not have sufficient sensitivity to determine subtle changes of myocardial function seen with drugs including androgens.

Androgens might exert cardiac effects through indirect mechanisms like in blood vessel disorders such as atherosclerosis, increased erythropoiesis, hyperviscosity, haematocrit, and hypertension. In addition, androgens might exert direct effects on cardiac muscle and its function by the androgen receptor expressed in cardiac muscle. In vitro studies of nonhuman cardiac myocytes demonstrated that T can decrease peak shortening times and action potential duration (hence altering repolarization) (Kimura et al., 1993). The direct effects of androgens on cardiac muscle have been less investigated (Sader et al., 2001). They are mostly based on case reports of cardiomyopathy in some androgen abusers on postmortem examinations (Sullivan et al., 1998). Some studies of anabolic steroid abuse indicate that androgen abuse in athletes may cause left ventricular hypertrophy, which may persist afterwards. Yet, several important limitations should be mentioned such as the lack of randomization, the confounding role of strength training, and self-selection. A study of testosterone and nandrolone (Chung et al., 2007) employed for 4 weeks demonstrated no adverse effects of androgens on non-invasive measures of cardiac function, which is consistent with a previous study using nandrolone for 8 weeks (Hartgens et al., 2003). Despite these promising findings during short-term use, longer studies would be necessary to show the maintenance of such a safety profile for long term. Retrospective data of over 2000 elite athletes from the East German national sports doping program, who were taking high dose synthetic androgens and were regularly monitored medically showed no cardiovascular complications. However, hepatic and other complications were frequently reported (Franke & Berendonk, 1997).

Previous work suggests that T may relieve symptoms in patients with angina pectoris and resolves post-exercise ST-segment depression in patients suffering from angina (Jaffe, 1977). Jaffe et al. (Jaffe, 1977) demonstrated that intramuscular T injection for 4 to 8 weeks in 50 men, who had ST-segment depression after exercise, improved post-exercise ST-segment depression, compared with placebo. This work did not study mechanisms by which T reduced ST-segment depression. Although early studies suggested beneficial effects of T in patients with angina, these studies were not placebo-controlled, were not randomized and the diagnosis of coronary artery disease was not angiographically established. Furthermore, the study by Jaffe (Jaffe, 1977) investigated the effect of T only on post-exercise ST-segment depression. It did not provide any information on hemodynamic parameters and the time course of ST-segment depression. Furthermore, not all previous studies in patients with angina pectoris have used pure T and therefore have not investigated the effect of T by itself on cardiovascular physiology. Yue et al. (Yue et al., 1995) demonstrated that T exerts endothelium independent dilatation of isolated rabbit aorta and coronary artery. The authors indicated that this may depend on an effect of T on potassium channels and potassium conductance but not on ATP-sensitive potassium channels, and they demonstrated that this effect is neither mediated by a classic hormonal receptor nor is sex-dependent (Yue et al., 1995). Experimental evidence indicates that T may have a potential beneficial effect on myocardial ischemia by playing a role in the regulation of coronary artery tone.

Mechanisms of action proposed for the effects of T were vasodilation of coronary arteries or their collaterals and an increase in hemoglobin levels which improves oxygen-carrying capacity of blood. Yet, the vasoactive properties of T have not been reported until recently.

The time elapsed to 1-mm ST-segment depression was reported to be prolonged after intravenous infusion of T. This indicates an acute anti-ischemic effect of T. The increase in heart rate- blood pressure product and heart rate shown either at 1-mm ST-segment depression or at peak exercise may suggest a direct vasodilator effect of T on coronary circulation. However, heart rate and blood pressure at rest were similar before and after T

employment. This suggests that a peripheral effect of T may not determine its anti-ischemic effect. The prolongation of time to 1-mm ST-segment depression observed is similar to that observed in women after acute employment of 17 $\beta$  estradiol, suggesting a similar effect of sex hormones in men and women (Rosano et al., 1993). Collins et al. (Collins et al., 1995) indeed demonstrated that intracoronary infusion of 17 $\beta$ -estradiol restores endothelium dependent vasodilatation in women but not in men, indicating a specific role of sex hormones on the cardiovascular system. The anti-ischemic effect of T reported might explain the cardio-protective effect of T substitution in men with hypotestosteronemia. In the present study, mostly the patients with lower testosterone benefited from testosterone employment (Rosano et al., 1999).

A recent study has shown that ventricular functions are normal in men with idiopathic hypogonadotropic hypogonadism (IHH) and substitutive therapy has no effects on ventricular functions, however it may increase peak pulmonary artery pressure (PAP) in small amounts (Yaylali et al., 2010). This finding is in line with previous studies demonstrating normal left ventricular functions in IHH men (Baysan et al., 2007; Celik et al., 2001; Hartgens et al., 2003). In addition, substitutive therapy had no effects on RV systolic or diastolic functions. This study found no evidence of clinically significant effects of the use of substitutive therapy on ventricular functions in the short term. However, substitutive therapy may increase PAP in small amounts which may be related to its vasoactive properties in the pulmonary vasculature. The younger normal even testosterone deplete heart is a totally different state than the diseased atherosclerotic or failing older heart. In this respect previous studies examined the effects of exogenous testosterone on the hearts of older men which were already affected by atherosclerosis or syndrome of heart failure. In contrast, this study investigated the ventricular functions in much younger hearts with no evidence of atherosclerosis or heart failure before and after substitutive therapy.

It has been shown that 12 weeks of treatment with T in men with CHF was well tolerated, safe, and led to significant improvements in symptoms and physical capacity. Larger clinical trials are needed to evaluate the mechanism of action of T, beneficial effect and confirmation of its safety in selected patients (Lellamo et al., 2010).

#### **4. Effects of T on cardiovascular risk factors**

Several cross-sectional population studies have demonstrated statistically significant correlations between various cardiovascular risk factors and plasma levels of T, however the correlations were opposite in women and men. The plasma T levels in men were frequently found to have positive correlations with serum levels of HDL -C and have inverse correlations with plasma levels of low-density lipoprotein cholesterol (LDL-C), triglycerides, fibrinogen, total cholesterol, and PAI-1 (Hergenc et al., 1999; Tchernof et al., 1996; Tsai et al., 2000). Yet, serum levels of T were found to have even stronger inverse correlations with BMI, waist-hip ratio (WHR), waist circumference, amount of visceral fat, insulin, serum levels of leptin, and free fatty acids. The correlations of T with the cardiovascular-disease risk factors except visceral fat and insulin lost their statistical significance after adjustment for obesity and insulin resistance (Hergenc et al., 1999; Tchernof et al., 1996; Tsai et al., 2000). These findings suggest that a low serum level of T is a component of the metabolic syndrome in eugonadal men, which is a cluster of cardiovascular risk factors such as obesity, hypertriglyceridemia, glucose intolerance or overt type 2 diabetes mellitus, low HDL-C, and arterial hypertension. Metabolic syndrome is considered a procoagulatory and an anti-fibrinolytic state. And insulin resistance plays an important role in its etiology. Thus,

the frequent association of high T levels with a more favorable cardiovascular risk factor profile in men perhaps does not reflect direct regulatory effects of T on lipoprotein metabolism and the hemostatic system. Some studies demonstrated the loss of these associations when serum levels of free T were used to correlate with lipids and other cardiovascular-disease risk factors instead of serum levels of total T were used. In agreement with these findings, a reduced number of CAG repeats in the androgen receptor, which facilitates its binding to T, was associated with decreased levels of leptin, HDL-C, BMI, and low body fat mass (Zitzmann et al., 2001, 2003). One reason for the discrepancy between the associations of endogenous T and the biological effects with various cardiovascular risk factors is the inhibitory effect of insulin on the production of SHBG so that insulin resistance results in low levels of SHBG and low levels of total T as a result.

The association between increased risk of coronary artery disease and plasma testosterone levels has been attributed in part to the unfavorable effect of T on fibrinolysis and HDL cholesterol (Glueck et al., 1993; Jones et al., 1989; Sorva et al., 1988). Plasma levels of HDL cholesterol are higher in women than in men, and some studies indicate that T substitution in men causes a decrease in plasma levels of HDL cholesterol (Jones et al., 1989; Sorva et al., 1988). Glueck et al. (Glueck et al., 1993) demonstrated that T correlates with tissue plasminogen activator, the major stimulator of fibrinolysis positively, and with fibrinogen and plasminogen activator inhibitor inversely. However, recent studies have demonstrated that 2-month therapy with testosterone undecanoate had a beneficial effect on lipoprotein profiles in older men (Uyanik et al., 1997). Although there might be an effect of plasma T levels on prothrombotic state, yet there is no epidemiological evidence showing an association between coronary artery disease and high testosterone levels (Uyanik et al., 1997).

## 5. Effects of androgens on vascular reactivity

T can exert vasodilatation or vasoconstriction via endothelium-independent or endothelium-dependent mechanisms and by genomic or non-genomic modes of action. The variation in these findings appears to be due to differences in concomitant disease, species, gender, and, most importantly, the dosage of T given. The significant reduction in nitrate-induced (and hence endothelium-independent) dilatation of the brachial arteries in female-to-male transsexuals taking high-dose androgens indicate these possible adverse effects associated with use of T (McCredie et al., 1998). In another study, castrated patients with prostate cancer had a greater flow-induced (i.e., endothelium-dependent) dilatation of brachial arteries compared to controls; however, the degree of nitroglycerin induced endothelium dependent vasodilatation did not differ between the two groups (Herman et al., 1997). In a study of 110 healthy men, a positive association between endothelium-dependent as well as endothelial-independent vasodilatation and the numbers of CAG repeats in exon 1 of the androgen receptor gene was observed. Therefore, the greater the sensitivity to T, the less brachial arteries dilate in response to either administration of nitrate or increased blood flow (Zitzmann et al., 2001). In contrast to these studies, IV administration of T in acute interventional studies to male patients with CAD showed apparently beneficial vasodilatory effects of T. Likewise, most in vitro studies with animal vessels and in vivo studies in monkeys and dogs of both sexes indicate that T induces beneficial effects on vascular reactivity. Intracoronary injections of acetylcholine caused a significant endothelium-dependent vasodilatation in ovariectomized female cynomolgus monkeys treated with T for 2 years compared to untreated animals. In contrast, endothelium-independent vasodilatation occurred in the two groups normally (Adams et

al., 1995). In dogs, T induced vasodilatation of coronary arteries by both endothelium-dependent and -independent mechanisms (Chou et al., 1996; Costarella et al., 1996). In addition, the results of studies with isolated aortas and/or rings of coronary arteries from pigs, rats, and rabbits showed that T administration improved both endothelium-independent and/or endothelium-dependent vascular responsiveness in both sexes (Chou et al., 1996; Costarella et al., 1996; Yue et al., 1995). Yet, it must be noted that all these studies used supra-physiological doses of T in the micro-molar range. Teoh et al. (Teoh et al., 2000) demonstrated a direct vasodilatory effect of T on porcine coronary artery rings when given at micro-molar concentrations. But no direct effect was observed when T given at nanomolar concentrations. In contrast, physiological doses of T reduced the vasodilatory effects of calcium ionophores and bradykinin. Likewise, T impaired endothelium-dependent relaxation of aortic rings from rabbits that were either exposed to tobacco smoke or that were either made hypercholesterolemic. In addition, T inhibited the adenosine-mediated vasodilatation of rat coronary arteries (Ceballos et al., 1999; Farhat et al., 1995; Hutchison et al., 1997). The molecular and cellular mechanisms by which E2 and T regulate vascular tone are not well known. Evidence for and against endothelium-dependent or endothelium-independent mechanisms has been found. Some studies indicate endothelial nitric oxide involvement (Chou et al., 1996; Costarella et al., 1996; Geary et al., 2000). The nitric oxide synthase inhibitor L-NMMA inhibited T-induced vasodilatation in the aortas of rats, the coronary arteries of dogs, and the cerebral arteries of rats. Yet, another study demonstrated no effect of L-NMMA on T-induced vasodilatation of rabbit coronary arteries and aortas (Yue et al., 1995). Likewise, Hishikawa et al. (Hishikawa et al., 1995) demonstrated that E2 but not T stimulated *in vitro* expression of nitric oxide synthase in human aortic endothelial cells. It has been observed that T increases the response of coronary arteries to prostaglandin F<sub>2a</sub> (Farhat et al., 1995). Also, dihydro-T increases the density of thromboxane receptors in rats and guinea pigs (Masuda et al., 1995). Yet, results of some *in vitro* and *in vivo* animal studies suggest that pretreatment with the prostaglandin synthesis inhibitor indomethacin induced no effect on T-induced vasodilatation. The role of eicosanoids in mediating the actions of T on the arterial wall still remains controversial. It is unknown whether T regulates vasoreactivity by either non-genomic or genomic effects or both. Androgen receptor expression has been determined in rat endothelial cells and aortic smooth muscle cells. In human arterial cells expression of the androgen receptors has not been directly demonstrated. However, the association of endothelium-independent and endothelium-dependent vasoreactivity with the CAG repeat polymorphism in the androgen receptor suggests some indirect evidence for the expression of the androgen receptor on vascular smooth muscle cells and endothelial cells, respectively (Zitzmann et al., 2003). Also, steroid hormones can regulate vascular tone by modulation of cell membrane channels (e.g., voltage-dependent, ATP-sensitive, and calcium-activated potassium channels) and by non-genomic mechanisms that involve plasma membrane steroid receptors. Several observations indicate that T, especially at supra-physiological doses, modulates vascular tone via its secondary metabolites (e.g., E2) and/or non-genomic modes of action. Flutamide or cyproterone acetate, the androgen receptor antagonists did not reduce the effects of T on pig or rabbit coronary arteries (Teoh et al., 2000). The expression of aromatase in vascular endothelial cells (Harada et al., 1999) increases the possibility that T can be converted to E2 and T induces its vasoactive effects through stimulation of the estrogen receptors. Yet, neither the estrogen-receptor antagonist ICI 182,780 nor the aromatase inhibitor aminoglutethimide prevented the T-induced vasodilatation (Chou et al., 1996). Barium chloride decreased the T-induced

vasorelaxation of rabbit coronary arteries and aortas, indicating that T modulates the opening of potassium channels in vascular smooth muscle cells.

## 6. Conclusion

The clinical picture of male hypogonadism carries a significant morbidity. After a careful diagnostic evaluation to identify the underlying cause of hypogonadism, substitutive therapy may be instituted after examining benefit to risk ratio for an individual patient. The clinician has a choice among different T preparations, tailoring pharmacokinetics and route of employment to the patient. Long-term effects of substitutive therapy on the cardiovascular system still remain unclear. Published data mostly consists of small, observational studies with no clear definition of outcome. We still lack large randomized controlled studies investigating the relationship between substitutive therapy and cardiovascular risk, although androgen replacement therapy has been found to have beneficial effects on bone density, body composition, metabolism, and muscle strength in hypogonadal men. The effects of physiological doses of T on the lipid profile remain to be fully defined and the risk of not replacing T in the deficient men also remains unclear. Firstly, we might think that T and cardiovascular disease are clearly related, but the intricacies of the relationship are much more complex. As the role of androgens in cardiovascular system becomes clearer in the future, we may find out the mechanisms that play a role in the observed sex differences in the incidence of coronary artery disease in the middle-aged population.

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# Androgens and Vascular Function

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## 1. Introduction

It is widely recognized that vascular function is modulated by the endocrine system. So it is known that hormones as aldosterone, renin-angiotensin II system, thyroids hormones, oxytocin, ghrelin, vasopressin, etc exert action on the vascular tone (Axelband *et al.*, 2011; Nguyen & Touyz, 2011; Szmydynger-Chodobska *et al.*, 2011; Tesauro *et al.*, 2010;). Regarding sex hormones, epidemiological studies have demonstrated that there is a gender difference in the morbidity associated with hypertension and that there is an increased prevalence of cardiovascular diseases in postmenopausal women (Leung *et al.*, 2007; Teede, 2007). Simultaneously, androgens had been associated with an increased risk of cardiovascular disease, but recent studies have explored protective effects of androgens in males (Jones, 2010; Traish and Kypreos, 2011). For example, it has been demonstrated that men with coronary artery disease have decreased levels of testosterone, which were conversely correlated to the degree of coronary artery narrowing (Saad *et al.*, 2008). Likewise, lower testosterone, predicted incident stroke and transient ischemic attack in older men (Braga-Basaria, 2006). In general terms, it seems to be demonstrated that men with cardiovascular disease had lower levels of testosterone, and what is more important, new emerging evidence points to androgen deficiency more likely to be associated with cardiovascular diseases than gender per se (Traish & Kypreos, 2011). Testosterone deficiency alters carbohydrate, lipid and protein metabolism, this contributing to oxidative stress, endothelial dysfunction and increased production of pro-inflammatory factors, promoting alterations on vascular function. Among the alterations induced by testosterone deficiency are the loss of muscle mass and strength, increasing visceral fat mass, reduced libido, erectile dysfunction, increased osteoporosis, lethargy, lack of energy, and changes in mood. In addition, testosterone deficiency has been associated with increased risk of metabolic syndrome, type 2 diabetes, obesity, insulin resistance and atherosclerosis (Jones & Saad, 2009; Kapoor *et al.*, 2005).

Most of results obtained about that effects have been obtained from patients with prostate cancer subjected to androgen deprivation therapy. This therapy improves cancer related symptoms and quality of life (Bain, 2010), but shows side effects as sexual dysfunction, decreased lean body mass, decreased quality of life, osteoporosis, and detrimental changes in metabolic status (Basaria & Dobs, 2001; Chodak *et al.*, 2002; Smith *et al.*, 2002). Increased insulin levels and insulin resistance and increased prevalence of fasting hyperglycemia and hypertriglyceridemia have also been observed in men with prostate cancer treated with

androgen deprivation therapy (Braga-Bassaria *et al.*, 2006; Keating *et al.*, 2006). This type of therapy provides an invaluable method to correlate vascular alteration and sex hormone status. However, at mechanistic level the animal models are of valuable interest. In this sense our research group has been focused on analyzing the effects produced by the loss of gonadal function on vascular reactivity, as well as some of the underlying mechanisms.

Vascular tone is regulated by several mechanisms in which nitric oxide (NO) plays an important role. NO is formed through several NO synthases (NOS), i.e., endothelial NOS (eNOS), inducible (iNOS), and neuronal (nNOS). Independently from the source of NO, one of the major downstream events occurring after NO release is an increase in cGMP formation through soluble guanylate cyclase stimulation, and the subsequent activation of cGMP-dependent protein kinase (PKG) (Murad, 1997). PKG contributes to reduce the intracellular calcium concentration through a wide spectrum of PKG substrates, leading to vasodilation (Lincoln *et al.*, 2001; Munzel *et al.*, 2003). In addition, NO (Bolotina *et al.*, 1994) and cGMP (Ferrer *et al.*, 1995) can induce membrane hyperpolarization and subsequent relaxation.

Concerning NO, and independently from the NO-activated signalling pathway, it is important to take into account that vascular function of endothelial NO depends on its bioavailability, which is determined by the rate of NO production and by its scavenging by superoxide anion. Therefore, the elimination of superoxide anion within the vessel wall is fundamental and it is performed by superoxide dismutases (SODs) (Wolin, 2002). It is well understood that alterations of different steps along the NO pathway determine its effect on the vascular tone.

Vascular tone is also regulated by prostanoids originated by arachidonic acid metabolism through the cyclooxygenase (COX) pathway (Henrion *et al.*, 1997). One of the best studied prostanoids is thromboxane A<sub>2</sub> (TXA<sub>2</sub>) which has been implicated as mediator in diseases such as myocardial infarction and hypertension (FitzGerald *et al.*, 1987). Additionally, the role of COX derivatives other than TXA<sub>2</sub>, such as prostaglandin (PG) F<sub>2α</sub> and PGE<sub>2</sub>, or PGI<sub>2</sub> which can induce a vasoconstrictor or vasodilator response is the subject of numerous studies (Blanco-Rivero *et al.*, 2005; Félétou & Vanhoutte, 2006) since they could also participate in vascular dysfunction.

On the other hand, proteins kinases are important regulators of different cell signalling pathways. Among different proteins kinases, PKC merits special attention since it is able to regulate the activity of different enzymes such as NOS and COX-2 (Kim *et al.*, 2008; Shanmugam *et al.*, 2004). In turn, PKC can be activated by different reactive oxygen species. At this point, it is important to note that sex hormones possesses antioxidant properties, and the loss of gonadal function can induce oxidative stress and, in turn triggers modulatory actions in different cell signalling pathways that are working simultaneously to ensure the optimal response of the vessel to different stimuli.

For this, this chapter will review how the loss of gonadal function modifies the release and function of different mediators, such as reactive oxygen species, nitric oxide and prostanoids, and their functional involvement in the reactivity of aorta and mesenteric artery of the male rats. Future studies in the research field of androgens on cell signaling pathways will be commented, since they will be of important interest to implement therapeutic strategies that could improve vascular function.

## 2. Androgens and nitric oxide

The functional role of NO in vascular tone regulation has been widely reported (Furchgott & Zawadzki, 1980; Toda & Okamura, 2003; Vanhoutte 1996). NO is formed through several

NO synthases (NOS), i.e. endothelial NOS (eNOS), inducible (iNOS) and neuronal (nNOS) (Förstermann *et al.*, 1991). Most of the studies about the effects of androgens on vascular function have been focused on analyzing the interaction between androgens and endothelial NO (Tep-areenan *et al.*, 20043; Jones *et al.*, 2004). More specifically, most studies have been focused on analyzing the effects of androgenic derivatives on different aspects of the NO system, such as eNOS expression, NO release or NO vasodilator effect (Ceballos *et al.*, 1999; Hutchison *et al.*, 1997; Teoh *et al.*, 2000). However, few studies exist about the specific effect of endogenous male sex hormones on these aspects referred to endothelial or neuronal NO when they are simultaneously studied. Our data were obtained from aorta and mesenteric artery from intact and orchidectomized male Sprague-Dawley rats (6 months old), and seem not to be related to haemodynamic changes, since orchidectomy did not modify blood pressure ( $137 \pm 5.8$  mm Hg in control rats and of  $145 \pm 6.2$  mm Hg in orchidectomized rats;  $p > 0.05$ ). Moreover, the results on vascular function would have to be androgen-related, as confirmed by the decreased testosterone levels (control:  $2404 \pm 323$  pg/mL; orchidectomized:  $220 \pm 49$  pg/mL;  $n = 6$ ;  $p < 0.001$ ).

The reported effects of androgens on NO release are contradictory. Testosterone impairs relaxation and worsens endothelial dysfunction in male rabbits (Hutchison *et al.*, 1997). However, it has also been reported that testosterone or its derivatives increase eNOS (Liu & Dillon, 2002; Weiner *et al.*, 1994) and nNOS activity in the central nervous system of guinea pig (Weiner *et al.*, 1994) and mouse (Scordalakes *et al.*, 2002). Nevertheless, to our knowledge, we showed first evidence on the effect of endogenous male sex hormones on the nNOS/eNOS expression and nNOS-/eNOS-derived NO release in vascular tissues (Martin *et al.*, 2005; Blanco-Rivero *et al.*, 2007).

## 2.1 Neuronal nitric oxide

The analysis of the expression of nNOS, by Western blot, showed that it was higher in segments from control rats than from orchidectomized rats (Martín *et al.*, 2005). The NO release was quantified by measuring nitrite production and also the fluorescence emitted by DAF-2, and the results showed that electrical field stimulation EFS-induced NO release was similar in segments from both control and orchidectomized rats. The measured NO release seems to come from nerve endings, since preincubation with tetrodotoxin abolished the nitrite release in endothelial-denuded segments from both groups of rats. This result indicates that male sex hormones apparently do not modulate nNOS-derived NO release, in contrast to our observations with female sex hormones in which we found that the loss of these hormones provoked by ovariectomy increased nNOS-derived NO release (Minoves *et al.*, 2002).

To study the involvement of neuronal or endothelial NO on vasomotor responses, EFS induced contractile responses in endothelium-denuded mesenteric segments were analyzed by using vascular reactivity technique (Nielsen & Owman, 1971). The contractile response induced by EFS were practically abolished by tetrodotoxin and markedly reduced by phentolamine, the respective blockers for nerve impulse propagation and  $\alpha$ -adrenoceptors in arteries from both control and orchidectomized rats. Therefore, these responses appear to be mediated by noradrenaline (NA) release from adrenergic nerve terminals and the subsequent activation of  $\alpha$ -adrenoceptors in both experimental hormonal conditions, as has been described in other rat strains (Ferrer *et al.*, 2000; 2001).

The present results show that vasoconstrictor response to KCl and exogenous NA are diminished in segments from orchidectomized male rats, which is in agreement with most studies demonstrating that testosterone treatment enhanced the action of several contractile

agents (Baker *et al.*, 1978; Calderone *et al.*, 2002; Greenberg *et al.*, 1974). However, the responses induced by EFS was similar in both groups of rats Fig. 1 which would suggest that EFS increased the release of a vasoconstrictor factor, that could be the NA release from adrenergic endings in segments from orchidectomized animals.

Concerning this point, most studies have been performed in the central nervous system and, conflicting results exist. Thus, a decrease (Guan & Dluzen, 1991; Holmquist *et al.*, 1994), an increase (Shan & Dluzen, 2002; Siddiqui & Shah, 1997) and no modification (Agostini *et al.*, 1981; Chen *et al.*, 1999) of NA release have been reported in orchidectomized male animals. In a later study we demonstrated that the NA release was not modified by orchidectomy (Blanco-Rivero *et al.*, 2006).

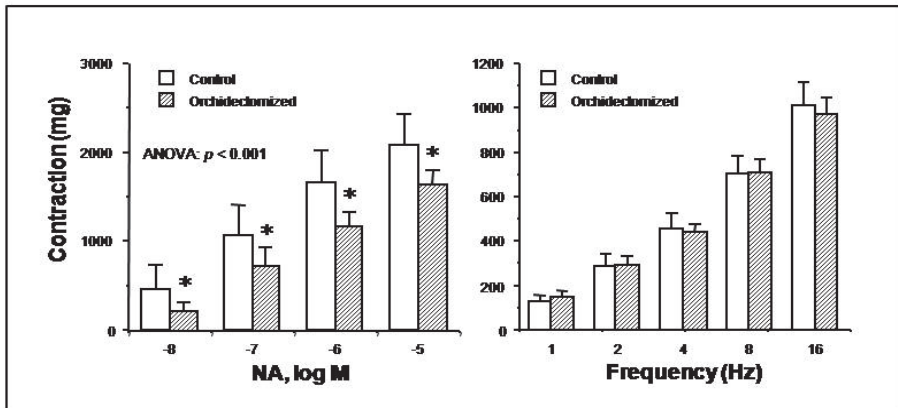


Fig. 1. Contractile response induced by cumulative concentration-response curves to noradrenaline (NA) and by frequency-response curves in denuded mesenteric arteries from control and orchidectomized rats. Results (means  $\pm$  SEM) are expressed in milligram. Number of animals: 25-30. \* $p < 0.01$  compared with control rats (from Martín *et al.*, 2005).

Since sex hormone modulation of the calcitonin gene related peptide (CGRP) system has been described (Sun *et al.*, 2001), and that the CGRP, the essential neurotransmitter in sensory nerves (Kawasaki *et al.*, 1988), had been proposed to play a role in vascular tone regulation, the participation of sensory innervation in the vasomotor response to EFS was assessed by the use of capsaicin, which selectively depletes the sensory nerves of the neurotransmitter (Li & Duckles, 1992). Capsaicin did not have a significant effect on the vasomotor response to either EFS or exogenous NA in mesenteric arteries from control and orchidectomized rats (data not shown), indicating that sensory innervation does not modulate the vasomotor response to EFS in our experimental conditions. Sun *et al.*, (2001) analyzed how orchidectomy modulates CGRP release, while we analyzed the functional involvement of the sensory innervation in the response to EFS without separately studying CGRP release and/or response.

The NOS inhibitor L-NAME increased the vasoconstrictor response to EFS in segments from both control and orchidectomized rats. The fact that the endothelium was removed and that AMT, an inhibitor of iNOS (Ferrer *et al.*, 2000; Ishikawa & Quock, 2003; Tracey *et al.*, 1995), did not modify the response induced by EFS in segments from both rat groups, reinforced the neuronal origin for the NO release.



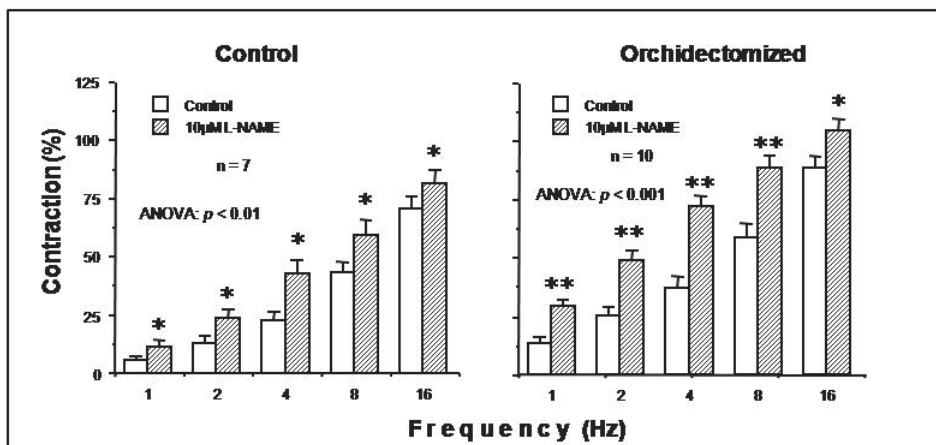


Fig. 2. Effect of L-NAME on the frequency-response curves performed in mesenteric arteries from control and orchidectomized rats. Results are expressed as a percentage of a previous tone with 75 mM KCl. n, number of animals; \* $p < 0.05$ ; \*\* $p < 0.01$  compared with control rats (from Martín *et al.*, 2005).

The greater response to EFS in the presence of L-NAME in segments from orchidectomized animals compared with the response obtained in segments from control rats Fig. 2 indicated differences in steps downstream from neuronal NO release, that will be considered later.

The reported effects of androgens on NO release are contradictory. Testosterone impairs relaxation and worsens endothelial dysfunction in male rabbits (Hutchison *et al.*, 1997). However, it has also been reported that testosterone or its derivatives increase eNOS (Liu & Dillon, 2002; Weiner *et al.*, 1994) and nNOS activity in the central nervous system of guinea pig (Weiner *et al.*, 1994) and mouse (Scordalakes *et al.*, 2002). Nevertheless, to our knowledge, there is no experimental evidence on the effect of male sex hormones on the nNOS expression and nNOS-derived NO release in vascular tissues. Therefore, we analysed the expression of nNOS by western blot, and found it was higher in segments from control rats than from orchidectomized rats. We also quantified the NO release induced by EFS in segments from both groups of rats by measuring nitrite production and the fluorescence emitted by DAF-2, and the results showed that the EFS-induced NO release was similar in segments from both control and orchidectomized rats.

## 2.2 Endothelial nitric oxide

The eNOS expression in aorta and mesenteric artery showed that not was modified by orchidectomy (Blanco-Rivero *et al.*, 2007; Martorell *et al.*, 2008), in contrast to that observed in endothelial-denuded mesenteric artery (Martín *et al.*, 2005), which indicates that endogenous male sex hormones act in a different manner depending on the target protein. Additionally, these results also indicate that effects induced by endogenous hormones are quite different from those induced by exogenous hormones, since it has been reported that androgenic derivatives can increase (Simoncini *et al.*, 2003), decrease (Chatrath *et al.*, 2003) or not affect (McNeill *et al.*, 1999) eNOS expression.

Regarding eNOS activity, androgen-induced increase (Liu & Dillon, 2002; Simoncini *et al.*, 2003) and decrease (Mukherjee *et al.*, 2001) have both been reported. Others researchers have

demonstrated that androgens increased (Orshal & Khalil, 2004; Wynne & Khalil, 2003) and decreased (Ba *et al.*, 2001; Gonzales *et al.*, 2004) the vasodilator effect of endothelial NO. In our experimental rat model, we have found that the release of endothelial NO was not modified by orchidectomy, either in mesenteric artery (Blanco-Rivero *et al.*, 2007) or aorta (Blanco-Rivero *et al.*, 2006). However, orchidectomy reduced the endothelium-dependent vasodilator response induced by Acetylcholine (ACh) in mesenteric artery while increased in rat aorta (Fig. 3).

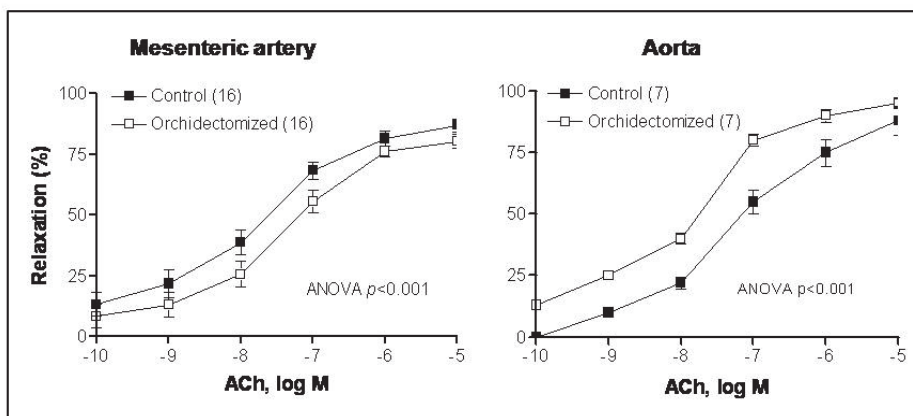


Fig. 3. Concentration-response curve to acetylcholine (ACh) in mesenteric artery and aortic segments from control and orchidectomized rats. Results are expressed as a percentage of a previous tone with noradrenaline. Number of animals is indicated in parentheses (from Blanco-Rivero *et al.*, 2007; Ferrer *et al.*, 1999).

These results could indicate that orchidectomy, depending on the vessel, may alter the sensitivity to NO and/or the release of factors other than NO, that will be revised in the next sections.

### 3. Androgens and production of reactive oxygen species

Reactive oxygen species are involved in metabolising NO (Gryglewski *et al.* 1986, Ferrer *et al.* 2000, 2001) and it has been reported that can induce oxidative processes associated with cardiovascular disorders (Harrison 1994; Munzel *et al.*, 1997). Among all the reactive oxygen species, superoxide anion plays a critical role since it is a source of many other reactive oxygen intermediates (Beckman & Koppenol, 1996). Therefore, both production and removal of superoxide anion are important contributors to the maintenance of appropriated levels of this oxygen species. Since several studies have demonstrated androgens antioxidants properties of androgens (Békési *et al.*, 2000; Yorek *et al.*, 2002), the effect of the orchidectomy on the production of superoxide anion was analyzed, by measuring the lucigenin chemiluminescence (control =  $117 \pm 43$  U/mg/min, n= 5; orchidectomized=  $546 \pm 58$  U/mg/min, n= 5;  $p < 0.001$ ). A similar result was obtained with acetylcholine-induced superoxide anion in aortas from the same animals (control:  $34.7 \pm 5.6$  U/mg/ min, n= 4; orchidectomized:  $132.8 \pm 3.2$ , n= 4;  $p < 0.001$ ). These results agree with previous studies showing the antioxidants properties of androgens (Békési *et al.*, 2000; Yorek *et al.*, 2002). The elimination of superoxide anion within the vessels is fundamental since this oxygen specie can reduce the NO bioavailability.

Within the vessel wall, SODs transform superoxide anion to hydrogen peroxide (Oury *et al.*, 1996, Price *et al.*, 2000, Muzykantov, 2001). Although three SOD isoforms have been identified: cytosolic Cu/ZnSOD, mitochondrial MnSOD, and extracellular ecSOD –which is also Cu/Zn-dependent (Strehlow *et al.*, 2003), we have focused on analyzing the participation of Cu/ZnSOD since it is the predominant isoform in peripheral vessels (Namgaladze *et al.*, 2005), and this enzyme therefore plays a crucial role in the pathogenesis of vascular dysfunction (Wolin, 2002). We analyzed the expression and activity of SODs because there was no information about modulation of these enzymes by endogenous male sex hormones. We found that both the expression and activity of Cu/ZnSOD were increased in aortic segments from orchidectomized rats (Fig. 4). These results were in line with other studies that described an increased expression and/or activity of SOD in cardiovascular pathologies, in which superoxide anion overproduction exists (Kobayashi *et al.*, 2002, Tanaka *et al.*, 2005).

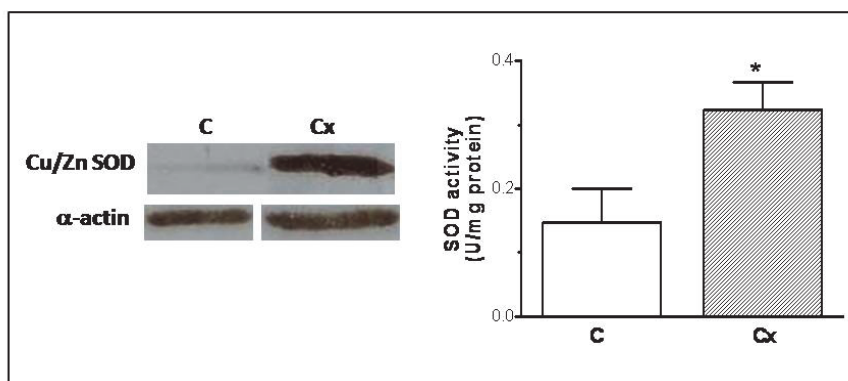


Fig. 4. Representative western blot for Cu/ZnSOD and  $\alpha$ -actin expression and SOD activity in aortic segments from control (C) and from orchidectomized (Cx) rats (from Blanco-Rivero *et al.*, 2006).

In addition, these results indicated that the increases in both expression and activity of Cu/ZnSOD could be a compensatory mechanism to eliminate the elevated superoxide anion formation induced by orchidectomy in male rats. On the other hand, additional mechanisms may exist to try to maintain vasodilator function. One of these mechanisms could be the activation of calcium-dependent potassium channels by superoxide anion (Ferrer *et al.*, 1999). In that report, we confirmed that the calcium dependent potassium channels ( $K_{Ca}$ ) activation was specifically induced by superoxide anion, since the NO synthesis was inhibited.

However, the vascular response depends on the vascular bed analyzed. For example, in mesenteric artery from orchidectomized rats, the vascular ROS-induced relaxation was due to the production of peroxynitrite and hydrogen peroxide  $H_2O_2$ . The increased formation of peroxynitrite in arteries from orchidectomized rats was studied by immunohistochemical localization and was reinforced by functional analysis on vasodilator response induced by sodium nitroprusside (SNP):

The presence of SOD increased the response to SNP in segments from orchidectomized rats which could be mediated through the decreased peroxynitrite formation by removing superoxide anion, and the simultaneous increased of  $H_2O_2$  formation, which exert

vasodilator action (Martín *et al.*, 2005; Rubanyi & Vanhoutte, 1986; Wei *et al.*, 1996). The simultaneous incubation of arteries with SOD and catalase reversed the vasodilator response to SNP, indicating that the  $H_2O_2$  synthesized in the presence of SOD, would participate in that vasodilator effect, when the formation of peroxynitrite is inhibited (Fig. 5).

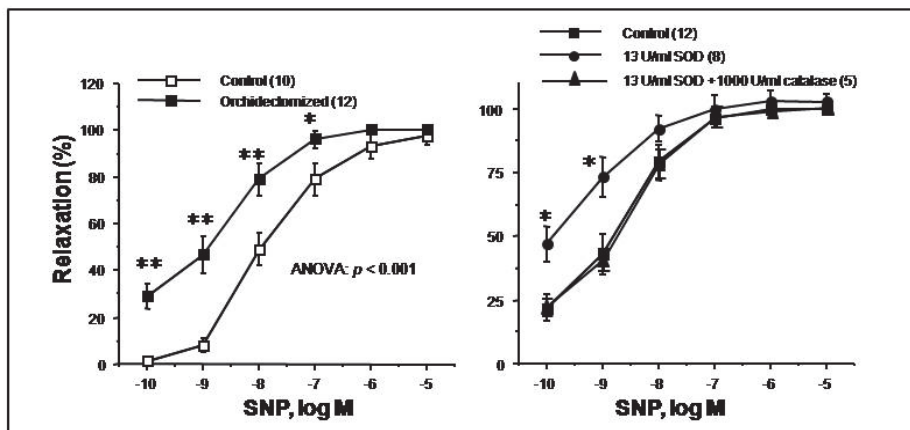


Fig. 5. Effect of orchidectomy, and effect of superoxide dismutase (SOD) and SOD plus catalase on the concentration-response curves to sodium nitroprusside (SNP) in mesenteric artery segments from orchidectomized rats. Results are expressed as a percentage of the inhibition of contraction induced by noradrenaline. Number of animals is indicated in parentheses.  $*p < 0.01$  compared with control (from Martín *et al.*, 2005).

At this point, it has been discussed that the loss of male sex hormones did not modify:

(1) the nNOS-derived NO release induced by EFS although it did increase the NO metabolism through superoxide anion and peroxynitrite generation; however, the functional role of the nNOS-derived NO release was more pronounced in arteries from orchidectomized animals, due to products generated from the NO metabolism, such as peroxynitrite and hydrogen peroxide, that seem to be able to compensate for the loss of NO bioavailability, probably through their direct vasodilator effect,

(2) the eNOS-derived NO release induced by Ach in both aorta and mesenteric artery; however the functional role of the endothelial NO release seem to be different in both arteries: Ach-induced relaxation is totally dependent of NO because the Ach-induced relaxation is inhibited when the synthesis of NO is blocked (Blanco-Rivero *et al.*, 2007), as occur in aorta from control rats, while in aorta from orchidectomized rats important relaxation exist even after NO synthesis was blocked (Ferrer *et al.*, 1999).

These differences could be due to the existence of regulatory mechanisms turned on by the loss of gonadal function that include, at least, prostanoids production.

#### 4. Androgens and prostanoids

Endothelial cells also release vasoconstrictor and vasodilator prostanoids, originated from the arachidonic acid metabolism through the cyclooxygenase (COX) pathway, to regulate vascular tone (Blanco-Rivero *et al.*, 2005; Feletou & Vanhoutte, 2006; Henrion *et al.*, 1997).

Two major isoforms of COX exist: COX-1 is expressed constitutively and is usually abundant in all animal and human endothelial cells, whereas endothelial COX-2 is induced mainly during inflammatory responses in nearly all animals. Depending on the vessel studied and the agonist used to activate cells, different prostanoids can be released and contribute to vasomotor response depending on the prostanoid receptor that result activated. Among all the prostanoids, one of the most studied is thromboxane A<sub>2</sub> (TXA<sub>2</sub>), which has been implicated as a mediator in diseases such as myocardial infarction, hypertension, stroke and bronchial asthma (FitzGerald *et al.*, 1987; Narumiya *et al.*, 1999; Noll & Luscher, 1998). However, little information is available on the effect of androgens on vascular effects of endogenous TXA<sub>2</sub>. Thus, orchidectomy has been reported to either decrease (Gonzales *et al.*, 2005) or not modify (Blanco-Rivero *et al.*, 2006) TXA<sub>2</sub> synthase expression. Likewise, the contractile effect induced by the TXA<sub>2</sub> mimetic, U-46619, was not modified by orchidectomy in mesenteric (Blanco-Rivero *et al.*, 2006) and cerebral (Gonzales *et al.*, 2005) rat arteries.

On the other hand, the role of COX-derivates, other than TXA<sub>2</sub>, such as prostaglandin (PG) I<sub>2</sub>, PGF<sub>2α</sub> and PGE<sub>2</sub>, which can act as vasoconstrictors under certain pathological conditions, is the objective of numerous studies (Félétou *et al.*, 2010; Gluais *et al.*, 2005; Rapoport & Williams, 1996). However, to the best of our knowledge studies analyzing the effect of endogenous male sex hormones in the involvement of these prostanoids in vascular function were lacking, and therefore this was one of our objectives. As above commented, we have previously demonstrated that EFS induced similar contractile response (Fig.1) in mesenteric arteries from control and orchidectomized rats, responses that appear to be mediated by NA release from adrenergic nerve terminals and the subsequent activation of α-adrenoceptors (Martín *et al.*, 2005); in addition, we found that the contractile response to exogenous NA was decreased by orchidectomy, suggesting the EFS could increase NA release in arteries from orchidectomized rats; however, we later demonstrated the EFS-induced NA release was not modified by orchidectomy (Blanco-Rivero *et al.*, 2006) which indicated that other vasoconstrictor factors could be released when the artery was electrically stimulated. We observed that the EFS induced a greater TXA<sub>2</sub> formation in arteries from orchidectomized than control rats, which was in line with other reports showing increased TXA<sub>2</sub> release after activation of muscarinic (Blanco-Rivero *et al.*, 2007) or α<sub>2</sub>-adrenoceptors (Blanco-Rivero *et al.*, 2006), and it confirmed the endothelial and smooth muscle cells as sources of TXA<sub>2</sub> production. The increased TXA<sub>2</sub> release could be the contractile factor that was released when the artery is electrically stimulated, and explains the non modification of the EFS-induced response in arteries from control and orchidectomized rats, in spite of the fact that the NA response was diminished in arteries from the latter animals.

It has been previously reported that prostanoids other than TXA<sub>2</sub>, i.e. prostaglandin (PG) I<sub>2</sub> increased neuronal NO release (Ferrer *et al.*, 2004) and taking into account that in our experimental conditions the main neurotransmitters involved in that response elicited by EFS were NO and NA, we investigated the regulation of the NA and NO release and function by TXA<sub>2</sub>. We observed that in arteries from control rats, the TXA<sub>2</sub> synthesis inhibition, with furegrelate, increased the neuronal NO release, which was in line with reports describing an inhibitory effect of TXA<sub>2</sub> on inducible (Yamada *et al.*, 2003) and endothelial (Miyamoto *et al.*, 2007) NO release. The vasodilator response induced by the NO donor, sodium nitroprusside was also increased by furegrelate. Concerning the effects of TXA<sub>2</sub> on NA release, inhibition (Nishihara *et al.*, 2000) or non-modification (Rump &

Schollmeyer, 1989) have been reported. In our experimental model, the inhibition of TXA<sub>2</sub> synthesis did not modify either NA release or its vasomotor effect, which indicated that endogenous TXA<sub>2</sub> did not alter the function of sympathetic innervations on arteries from control rats. By contrast, in arteries from orchidectomized rats, endogenous TXA<sub>2</sub> did not regulate either the release/function of neuronal NO or NA. The results obtained in mesenteric arteries from control animals explain the decreased EFS-induced response in the presence of furegrelate; however, did not explain the unaltered EFS-induced response in arteries from orchidectomized rats, since to remove a constrictor substance a decreased in EFS-induced vasoconstriction would be expected. Since cross-talk between TXA<sub>2</sub> and PGI<sub>2</sub> has been reported (Cheng *et al.*, 2002; Martorell *et al.*, 2008) and joint increases in PGI<sub>2</sub> and TXA<sub>2</sub> synthesis have been shown in pathological conditions (Caughey *et al.*, 2001; FitzGerald, 1991), the effect of TXA<sub>2</sub> on PGI<sub>2</sub> production was analyzed, as well as, its dependence of the hormonal status. We observed that the inhibition of endogenous synthesis of TXA<sub>2</sub> did not modify the release of PGI<sub>2</sub> in arteries from control rats, while it did greatly increase PGI<sub>2</sub> release in arteries from orchidectomized rats, which could work as a contractile factor after activation of TXA<sub>2</sub> receptors. Taking together these results, the loss of gonadal function in male rats increased the non-endothelial TXA<sub>2</sub> release in mesenteric arteries, and regulates the EFS-induced response through different mechanisms.

As previously commented, orchidectomy increased the TXA<sub>2</sub> release in endothelial-intact mesenteric arteries (Blanco-Rivero *et al.*, 2006; 2007). Similar effect was also observed in aorta segments from comparable animals (Martorell *et al.*, 2008), however, in this case the relaxation to ACh was increased. Based on this result, the level of expression of COX-2 as well as the production of prostanoids derived from COX-2 other than TXA<sub>2</sub> was investigated. We found that COX-2 expression, in contrast to observations in mesenteric artery (Blanco-Rivero *et al.*, 2006), was increased in aortas from orchidectomized rats indicating that endogenous male sex hormones act differently depending on the specific vessel. Our results also show that, in aorta from orchidectomized rats, COX-2 derivatives could also be increased and play a role in the regulation of vascular function. To test this hypothesis, we analyzed the effect of the COX-2 inhibitor NS-398 on the ACh-induced response. In contrast to our assumptions, we found that NS-398 did not modify the ACh-induced relaxation in either group of rats, apparently indicating the lack of participation of COX-2 derived products in the ACh response, probably due to the equilibrium between the inhibition of prostanoids formation and phosphodiesterase inhibition (Klein *et al.*, 2007), which allowed us to speculate that the contribution of different prostanoids to the vasodilator response mediated by ACh could be regulated by endogenous male sex hormones.

Once we had established that orchidectomy increased TXA<sub>2</sub> release, we analyzed the possible role of this prostanoid in the response to ACh by analyzing the effect of the TXA<sub>2</sub> synthase inhibitor, furegrelate, and the TP receptor antagonist, SQ29,548, on the vasodilator response to ACh. We observed that neither substance had any effect on the ACh-induced response in arteries from control animals, indicating that TXA<sub>2</sub> did not participate in that response, in agreement with reports in other rat strains (Gluais *et al.*, 2005; Rapoport & Williams, 1996). However, in arteries from orchidectomized rats, furegrelate enhanced the vasodilator response to ACh, showing a functional involvement of TXA<sub>2</sub>. The fact that the contractile response to the TXA<sub>2</sub> mimetic U-46619 was similar in arteries from control and orchidectomized rats demonstrated that sensitivity to TXA<sub>2</sub> is not modified by orchidectomy, which agrees with reports in cerebral (Gonzales *et al.*, 2005) and mesenteric

(Blanco-Rivero *et al.*, 2006) arteries; additionally, it also shows that differences in the TXA<sub>2</sub> involvement in the ACh-response are due to increased synthesis rather than increased sensitivity to TXA<sub>2</sub>. However, the incubation with SQ29,548 did not affect the ACh-induced relaxation, which seems to contradict the results obtained with furegrelate. However, since interactions among different prostanoids have been reported (Bachschnid *et al.*, 2005; Cheng *et al.*, 2002), it is possible to hypothesize that when TXA<sub>2</sub> synthesis is inhibited, the production of other prostanoids, which counterbalance the TXA<sub>2</sub> effect, could be increased. Therefore, we investigated the effect of inhibiting PGI<sub>2</sub> synthesis (with TCP) on the ACh-induced response, observing that it decreased the vasodilator response to ACh to a greater extent in arteries from orchidectomized than in those of control rats, which would indicate a greater involvement of this vasodilator prostanoid in the former arteries. Therefore, the release and vasomotor effect of PGI<sub>2</sub> was investigated. The ACh-induced PGI<sub>2</sub> release was increased in arteries from orchidectomized rats, probably due to the superoxide anion overproduction observed in aortas from orchidectomized rats (Blanco-Rivero *et al.*, 2006), supporting the concept of redox regulation of vascular prostanoid synthesis proposed by Bachschnid *et al.* (2005). Moreover, the increased production of PGI<sub>2</sub> is in line with that reported in human syndromes involving platelet activation in which PGI<sub>2</sub> biosynthesis is elevated along with TXA<sub>2</sub> (Caughey *et al.*, 2001; FitzGerald, 1991). It is known that PGI<sub>2</sub> can induce both vasodilation, through activation of prostacyclin receptors (IP) and thereby increasing cyclic-AMP, and vasoconstriction through activation of TP receptors (Blanco-Rivero *et al.*, 2005). In the present study, we found that exogenous PGI<sub>2</sub> induced relaxation in rat aorta, and that it was decreased in arteries from orchidectomized rats, which could be due to differences in the expression of IP receptors rather than differences in cell signalling operating after receptor activation; we have observed that the relaxation induced by the activator of adenylate cyclase, forskolin, was similar in arteries from control and orchidectomized rats (unpublished data).

Since considerable evidence exists for cross-talk between the TXA<sub>2</sub> and PGI<sub>2</sub> systems (Cheng *et al.*, 2002), we analyzed the functional effect of inhibiting the synthesis of both prostanoids. We observed that co-incubation of arteries with TCP plus furegrelate, or TCP plus SQ29,548, reversed the decreased response to ACh caused by TCP in arteries from control rats, showing the existence of a balance between TXA<sub>2</sub> and PGI<sub>2</sub> in these arteries. However, in arteries from orchidectomized rats, the co-incubation with TCP plus furegrelate did not modify the decreased ACh response caused by TCP, indicating the participation of prostanoids other than PGI<sub>2</sub> and TXA<sub>2</sub> that could induce contraction. Moreover, these prostanoids would activate TP receptors since co-incubation with TCP and SQ29,548 completely reversed the decrease in the ACh response induced by TCP.

Among COX-2 derivatives, other than TXA<sub>2</sub> and PGI<sub>2</sub>, that can activate TP receptors, PGE<sub>2</sub> is the most plausible candidate (Gluais *et al.*, 2005; Blanco-Rivero *et al.*, 2007) since the ACh-induced PGF<sub>2 $\alpha$</sub>  production and its vasoconstrictor effect were both very limited. Therefore, we investigated the ACh-induced PGE<sub>2</sub> release, as well as its vasoconstrictor effect. We found that both ACh-induced PGE<sub>2</sub> production and PGE<sub>2</sub>-induced vasoconstrictor response were greater in arteries from orchidectomized than in those of control rats. Consequently, the ACh-induced PGE<sub>2</sub> release, under inhibited synthesis of TXA<sub>2</sub> and PGI<sub>2</sub>, was analyzed. We found the ACh-induced PGE<sub>2</sub> production further increased, probably as a consequence of increased PGH<sub>2</sub> production and subsequent transformation into PGE<sub>2</sub> (Frein *et al.*, 2005); and, what is more important, the PGE<sub>2</sub> increase was more pronounced in arteries from orchidectomized than in those of control rats.

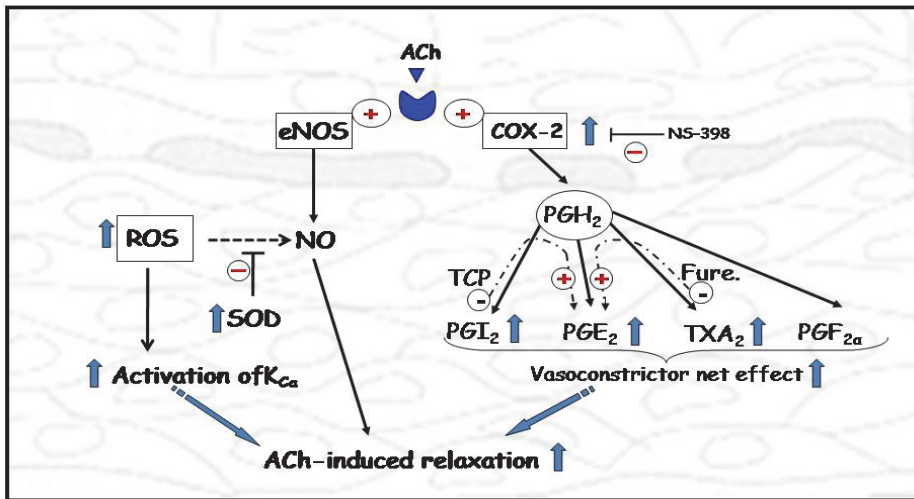


Fig. 6. Scheme showing the effect of orchidectomy on different mechanisms involved in the ACh-induced relaxation (see text for explanation).

This finding confirms our hypothesis that when the synthesis of PGI<sub>2</sub> and TXA<sub>2</sub> was inhibited, the release of PGE<sub>2</sub> was increased in arteries from orchidectomized rats, but raises the question as to why the PGE<sub>2</sub> produced in the presence of TCP plus furegrelate did not affect the ACh-induced relaxation in arteries from control animals. The possible explanation could be that the PGE<sub>2</sub> release was not sufficient to induce a vasomotor effect and/or the PGE<sub>2</sub>-induced contraction in arteries from control rats was diminished as a consequence of different expression of EP receptor subtypes. By itself, this finding is of physiological relevance, since PGE<sub>2</sub> release and the vasoconstrictor effect are both increased in orchidectomized animals. In summary, in rat aorta orchidectomy enhances COX-2 expression, and induces an imbalance in the production and function of vasodilator and vasoconstrictor prostanoids, in such a way that the vasoconstrictor prostanoids predominate in the latter group. Additionally, we have previously reported a decreased NO bioavailability in aorta from orchidectomized rats (Blanco-Rivero *et al.*, 2006) that would also counteract the vasodilator response to ACh. However, despite these findings, the vasodilator response to ACh is increased in aorta from orchidectomized rats, probably as a consequence of compensatory mechanisms, such as the activation of K<sub>Ca</sub> channels by superoxide anion, the formation of which is increased in orchidectomized rats (Ferrer *et al.*, 1999) (Fig. 6).

## 5. Androgens and NO release, oxidative stress and protein kinase C interactions

The results described above point to that orchidectomy increases the oxidative stress through superoxide anion formation, but in contrast to that expected, without modifying the NO release. What is the mechanism underlying this effect?

In several types of vascular diseases, protein kinase C (PKC) activation is involved in the induction of oxidative stress through the increased expression and activity of NADPH oxidase and the eNOS uncoupling (Hadi & Swaidi, 2007; Vanhoutte, 2001). But increased



of PKC activity by different reactive oxygen species has been also reported (Balafanova *et al.*, 2002; Bapat *et al.*, 2001; Oeckler & Wolin, 2000). PKC is a ubiquitous enzyme that was originally described as calcium-activated, phospholipid-dependent protein kinase. Molecular cloning and biochemical analysis have revealed a family of PKC subspecies with closely related structures (Newton, 1995; Nishizuka, 1992). The activity of these proteins is critical for signal transduction of a wide range of biological responses (Chen, 2003; Nishizuka, 1984). The pathways through PKC exerts its vascular effects include actions on ion channels, cytoskeleton and cell adhesion proteins, transcription factors, other kinases and other proteins (Spitaler & Cantrell, 2004; Ward *et al.*, 2004). The participation of PKC in vascular smooth muscle contraction is well established (Khalil & van Breemen, 1988; Salamanca & Khalil, 2005); in addition, different investigations have also reveal that PKC can phosphorylate nNOS in the central nervous system leading to an increase (Nakane *et al.*, 1991; Okada, 1995) or a decrease (Bredt *et al.*, 1992; Dawson *et al.*, 1993) in neuronal NO production.

On the other hand, influence of gender on PKC activity and expression had been studied (Kanashiro & Khalil, 2001). In that work, the authors observed a gender-specific reduction in vascular smooth muscle reactivity in female rats with intact gonadal function compared with males, that was associated with a reduction in the expression and activity of different  $\alpha$ -,  $\delta$ -, and  $\zeta$ -PKC isoforms; they also proposed that gender-specific differences in vascular reactivity and PKC activity were possible related to endogenous estrogen. However, little information is available on the specific effect of endogenous male sex hormones on PKC activity in vascular tissues.

As described in the section corresponding to neuronal NO release, we observed that the neuronal NO release was not modified by orchidectomy although the nNOS expression was diminished, which indicated that nNOS activity could be increased; regarding regulation of nNOS by male sex hormones both increase (Simoncini *et al.*, 2003; Weiner *et al.*, 1994) or decrease (Reynoso *et al.*, 2002; Singh *et al.*, 2000) have been reported. Since in our experimental model, orchidectomy induced an increased oxidative stress, we analyzed the possible differences in PKC activity as well as its involvement in neuronal NO release.

We observed that orchidectomy increased PKC activity in rat mesenteric arteries (Blanco-Rivero *et al.*, 2005), in contrast to the results obtained in aorta from Wistar-Kyoto rats (Kanashiro & Khalil, 2001); this discrepancy could be due to differences in the artery analyzed, since it has been described different enzyme properties and function in the same blood vessels from different species and in different vessels from the same species (Liou & Morgan, 1994; Kanashiro & Khalil, 1988; Khalil *et al.*, 1992). However, the fact that PKC was increased by orchidectomy is not totally surprising, since orchidectomy increased the formation of superoxide anion and peroxynitrite that has been described to act in cell signalling pathways, for instance, increased PKC activity (Balafanova *et al.*, 2002; Bapat *et al.*, 2001; Oeckler & Wolin, 2000).

The regulatory effect of PKC on nNOS activity in arteries from the orchidectomized and the control animals was analyzed by using DAF-2 as fluorescence probe to measure the modification in basal and EFS-induced NO release. The results showed that the PKC activator PDBu (Abdel-Latif, 1986; Nishizuka, 1984) induced a greater increase in EFS-induced NO release in arteries from control than from orchidectomized animals, while the PKC inhibitor, calphostin C (Kobayashi *et al.*, 1989), induced a stronger decrease in arteries from the control than the orchidectomized animals (Fig. 7).

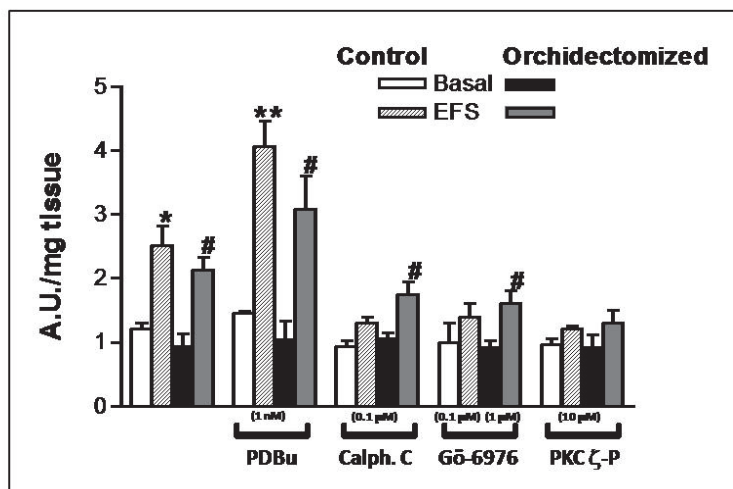


Fig. 7. Effect of PDBu, calphostin, Gö-6976 or PKC $\zeta$ -PI on the basal-and EFS-induced NO release in mesenteric arteries from control and orchidectomized rats. Results are expressed as arbitrary units (AU). Number of animals=4-7. \* $p$ <0.05, \*\* $p$ <0.01 vs the respective basal NO release from control rats; # $p$ <0.05 vs the respective basal NO release from orchidectomized rats (from Blanco-Rivero *et al.*, 2005).

These results indicate that PKC is involved in nNOS activation in arteries from both control and orchidectomized rats. Because the degree of PKC activation is already greater in arteries from orchidectomized rats, PDBu and calphostin C showed less ability to respectively increase or diminish NO release in arteries from orchidectomized than from control rats. Conventional and novel PKC isoforms are dependent on diacylglycerol (DAG) (Ward *et al.*, 2004) and, since we use the pharmacological mimetic of DAG, PDBu, we stimulate both isoforms. Although selective inhibitors for these isoforms do not exist (Davies *et al.*, 2000) it has been reported that certain compounds, such as the indolocarbazole Gö6976, are partially selective for conventional over the novel and atypical isoforms (Martiny-Baron *et al.*, 1993; Ward *et al.*, 2004). Consequently, we tested the effect of this inhibitor on nNOS activity, and observed that a concentration of 0.1  $\mu$ M Gö6976 inhibited the EFS-induced neuronal NO release in arteries from control arteries while it did not affect the neuronal NO release in arteries from orchidectomized rats. A higher Gö6976 concentration (1  $\mu$ M) was used but it only decreased the EFS-induced NO release in arteries from orchidectomized rats. All these results support the assumption that nNOS seems to be much more activated by PKC, and probably the conventional PKC isoforms, in arteries from the orchidectomized than from the control rats.

Since the atypical PKC  $\zeta$  isoform has been described in vascular smooth muscle as modulating vascular responses to different agents (Cogolludo *et al.*, 2003; De Witt *et al.*, 2001), the effect of this isoform on nNOS activity was analyzed. We found that PKC $\zeta$ -PI decreased EFS-induced NO release to a similar extent in arteries from control and orchidectomized animals. This result indicates that PKC  $\zeta$  isoform involvement in the regulation of neuronal NO release occurs and that this involvement is not modulated by endogenous male sex hormones.

Regarding endothelial NO release, it has been previously described that orchidectomy did not modify the Ach-stimulated endothelial NO release, despite the increased production of superoxide anion. Based on the results obtained about PKC regulation of nNOS activity, the action of PKC activation or inhibition on eNOS activity was also investigated in arteries from orchidectomized and control animals. The results obtained showed that neither the PKC activator, PDBu (Nishizuka, 1984), nor the PKC inhibitor, calphostin C (Kobayashi *et al.* 1989), modified ACh-induced NO release in arteries from control animals. In contrast, in arteries from orchidectomized rats, PKC activation increased the basal and the ACh-induced NO release while PKC inhibition more strongly decreased both basal and ACh-induced NO release (Fig. 8).

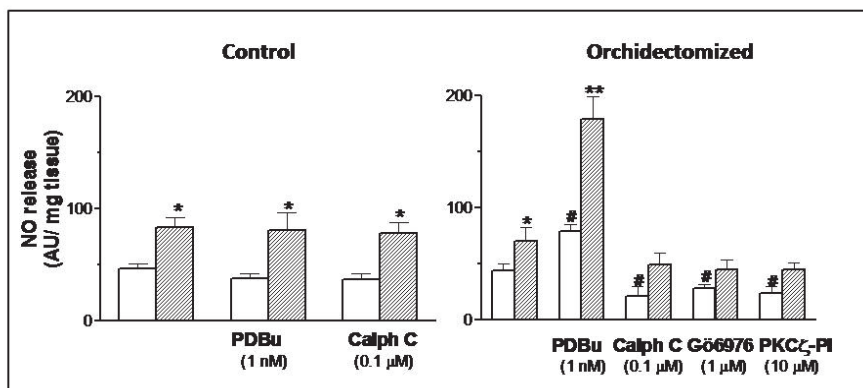


Fig. 8. Effect of PDBu, calphostin, Gö-6976 or PKC $\zeta$ -PI on the basal - (white columns) and Ach - (dashed columns) induced NO release in mesenteric arteries from control and orchidectomized rats. Results are expressed as arbitrary units (AU). Number of animals=8. \* $p < 0.05$  vs the respective basal NO release; # $p < 0.05$  vs basal NO release in non treated arteries (from Blanco-Rivero *et al.*, 2007).

These results indicate that PKC participates in eNOS activity only in arteries from orchidectomized rats, and also seem to indicate that PKC apparently did not regulate eNOS activity in arteries from control animals, which contrast with the results reported with nNOS (Blanco-Rivero *et al.*, 2005) in which we observed an nNOS activity modulation by PKC. In this respect, it is possible to speculate that very subtle differences could exist in the modulation of NOS isoforms. In this regard, it is important to keep in mind that regulatory mechanisms other than PKC could be working on eNOS, including different redox conditions (Polytarchou & Papadimitriou, 2005), phosphatases activity (Fleming *et al.*, 2001) and/or other kinases (Ferrer *et al.*, 2004) that, in their turn, regulate the intracellular environment and function. Additionally, since the pharmacological mimic of diacylglycerol, PDBu, stimulate conventional and novel PKC isoforms (Ward *et al.*, 2004) and since calphostin C is a non specific PKC inhibitor, we tested the effect of the PKC inhibitor Gö6976, which is partially selective for conventional over novel and atypical PKC isoforms (Martiny-Baron *et al.*, 1993, Ward *et al.*, 2004). We observed that this inhibitor also decreased basal and ACh-induced NO in arteries from orchidectomized rats, indicating the involvement of the conventional PKC isoforms in endothelial NO regulation. Since the

atypical PKC $\zeta$  isoform has been reported to modulate vascular responses (Damron *et al.*, 1998, De Witt *et al.*, 2001, Cogolludo *et al.*, 2003) and neuronal NO release (Blanco-Rivero *et al.*, 2005), we also tested the possible involvement of this isoform in endothelial NO release. The fact that PKC $\zeta$ -PI decreased the basal and ACh-induced NO release showed the participation of this isoform. Moreover, since the three PKC inhibitors that we used, calphostin C, Gö6976 and PKC $\zeta$ -PI, diminished both basal and ACh-induced NO release, it seems that eNOS, like nNOS (Blanco-Rivero *et al.*, 2005), would have already been activated by PKC in arteries from orchidectomized rats.

These results show that PKC activity is enhanced in mesenteric arteries from orchidectomized rats, and this increase would be responsible for the higher nNOS and eNOS activity.

## 6. Conclusions

Orchidectomy alters different cell signalling pathways that are involved in vascular tone regulation. Orchidectomy increases: (i) the formation of superoxide anion and peroxynitrite; (ii) the activity of PKC; and (iii) the expression of COX-2, the production of prostanoids derived from COX-2, as well as their vasoconstrictor effect. These aspects seem to be physiologically relevant, since the balance between vasodilator/vasoconstrictor prostanoids is lost in favour of vasoconstrictor substances in arteries from orchidectomized rats. This situation could indicate a disadvantage in cardiovascular function in the absence of male sex hormones, thereby suggesting that testosterone has a beneficial influence on the vasculature. However, in the animals used in our study (6 months old) several compensatory mechanisms are working: reactive oxygen species are able to induce relaxation; PKC positively regulates nNOS and eNOS activity ensuring the maintenance of NO release; the activity and expression of SOD are increased in an attempt to compensate for the increased superoxide anion production. This intriguing information makes it essential to perform studies in vascular function taking into account different cell signalling pathways that are working simultaneously. Future studies in the research field of androgens on cell signaling pathways are needed, since they will be of important interest to implement therapeutic strategies that could improve vascular function.

## 7. Acknowledgements

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# Orchidectomy Upregulates While Testosterone Treatment Downregulates the Expression of Ornithine Aminotransferase Gene in the Mouse Kidney

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## 1. Introduction

Ornithine aminotransferase (L-ornithine: 2-oxoacid aminotransferase, OAT, EC 2.6.1.13) plays crucial physiological roles in amino acid metabolism because this enzyme is at the crossroad of several pathways including those of L-arginine, L-ornithine, L-glutamate, L-glutamine, and L-proline. Specifically, OAT catalyzes the transamination of L-ornithine in the presence of  $\alpha$ -ketoglutarate to produce one molecule of L-glutamate and the unstable compound glutamate- $\gamma$ -semialdehyde that is spontaneously converted into  $\Delta^1$ -pyrroline-5-carboxylate. This latter molecule is further metabolized by the enzyme pyrroline-5-carboxylate dehydrogenase into a second molecule of L-glutamate (Wakabayashi, 2004). The enzyme is expressed in many mammalian tissues including the liver, the kidney, and the intestine which exhibit the highest OAT activities (Peraino & Pitot, 1963; Herzfeld & Knox, 1968; Sanada et al., 1970; Kasahara et al., 1986; Alonso & Rubio, 1989; Levillain et al., 2007; Ventura et al., 2009). These enzymes may not only display diverse tissue-specific physiological roles, but demonstrate marked sexual differences in expression and activity.

In rat kidneys, estrogen dramatically increased the expression of OAT and is responsible for the higher levels of OAT in female than in male rat kidney (Herzfeld & Knox, 1968; Lyons & Pitot, 1977; Mueckler & Pitot, 1983; Mueckler et al., 1984; Levillain et al., 2004). The presence of thyroid hormone is required for estrogen induction. These hormones exert a synergistic effect on the expression of OAT gene (Mueckler & Pitot, 1983). The expression of OAT gene during the rat postnatal development strongly supports the sexual dimorphism of OAT in kidney, but not in liver (Herzfeld & Knox, 1968; Herzfeld & Greengard, 1969). Taken together, the expression of OAT gene in the female rat kidney is naturally upregulated in the presence of estrogen.

The expression of OAT gene in the mouse kidney has been reported by different authors who independently measured OAT mRNA and protein levels or OAT activity (Alonso & Rubio, 1989; Natesan & Reddy, 2001; Yu et al., 2003; Levillain et al., 2005; Manteuffel-Cymborowska et al., 2005; Levillain et al., 2007; Ventura et al., 2009). Strong evidences

support a marked sexual dimorphism in the expression of OAT gene in the mouse kidney with three-fold higher levels in females than in males. A detailed study of the renal expression of OAT gene during the postnatal development of male and female mice revealed that puberty is the starter responsible for this sexual dimorphism (Levillain et al., 2007). In addition, the level of OAT protein has been inversely correlated with the plasma level of testosterone (Levillain et al., 2005).

The present study was designed to explore the mechanisms involved in the sexual dimorphism of the expression of OAT gene in the mouse kidney. We shall determine by *in vivo* studies at which level testosterone regulates the expression of OAT gene. To answer this question, male mice were subjected to orchidectomy and testosterone replacement. The renal expression of OAT gene was analyzed at the transcriptional, translational, and post-translational levels. The possible involvement of the eukaryotic initiation factor eIF4-E in the control of OAT gene expression was analyzed. We also explored the delay required for testosterone to induce a decrease in the expression of OAT gene in orchidectomized mice. For this, a time-course study was performed over a period of 32 hrs following a single injection of testosterone to castrated male mice. Finally, to explain the physiological role of testosterone on the expression of OAT specifically in the mouse kidney, we searched to identify androgen response elements (ARE) in the promoter of the murine OAT gene.

## 2. Material and methods

### 2.1 Animals and treatment

Eight- to nine-week-old adult male (35-40 g body weight) OF-1 Swiss (IOPS Caw) mice, from either Charles River Laboratories (L'Arbresle-sur-Orge, France) or Janvier (Le Genest-saint-Isle, France) had free access to tap water and standard food (2018 Teklad Global 18% Protein Rodent Diet, Harlan, Gannat, France). Animals were housed in a controlled environment maintained at  $21 \pm 1^\circ\text{C}$  with a 12-h light, 12-h dark cycle, lights on at 0600 h.

Twenty-four thirty-day-old male mice were subdivided into four groups of six mice: nonoperated (group I, control), sham-operated (group II) and two groups of orchidectomized mice. Eleven days later (*i.e.* 41 days after birth), mice of groups I, II, and III were sacrificed, whereas mice of group IV were sacrificed seven days later (*i.e.* 18 days after orchidectomy).

Twenty-four thirty-day-old male mice were subdivided into four groups of six mice: sham-operated (group V, control), 11-day orchidectomized (group VI), 11-day orchidectomized treated with sesame oil (group VII), and 11-day orchidectomized treated with testosterone + sesame oil (group VIII). Mice subjected to oil or testosterone treatment were injected subcutaneously with 150  $\mu\text{L}$  vehicle or testosterone propionate (3.1 mg/mL in sesame oil, *i.e.* approximately 15  $\mu\text{g/g}$  BW or 0.55 mg per mouse). All mice were sacrificed forty-eight hrs after the treatment.

Twenty thirty-day-old male mice were subjected to orchidectomy (11-day) and subdivided into five groups of four mice: 11-day orchidectomized (group IX, control) and four groups of mice treated with testosterone as described above (0.55 mg per mouse). Mice were sacrificed 8 hrs (group X), 24 hrs (group XI), 28 hrs (group XII), or 32 hrs (group XIII) after the treatment (Fig. 1). For these three protocols, half of the mice were purchased from Charles River Laboratories and the other from Janvier. Mice were equally distributed in the different experimental groups. Orchidectomy was carried out by the suppliers. Mice were anesthetized (ip) using 0.1 mL/30 g BW pentobarbital sodium (Nembutal 6%, Clin Midy, Paris, France) diluted 1:2 in 0.9% NaCl solution.



Fig. 1. Schema of the time-course study of testosterone effect on the expression of OAT gene.

Animal care complied with French regulations for the protection of animals used for experimental and other scientific purposes and with European Community regulations (Council of Europe N° 123, Strasbourg, 1985). The author (O. Levillain) is authorized by the "Direction Départementale des Services Vétérinaires" (authorization no. 69-33 and 69266391) and the local Animal Care Committee to use animals for these experiments (authorization no. 299 revised and no. BH 2009-15).

## 2.2 Sampling of kidneys and blood

The renal pedicle of each kidney was clamped and the kidney was rapidly removed, decapsulated; the blood contained in each kidney was removed with blotting paper (free-blood). The kidney was placed in a sterilized Eppendorf tube, frozen, and conserved at  $-80^{\circ}\text{C}$ .

Blood was collected in the vena cava of all mice with a 25-gauge needle (Neolus, VWR, Limonest, France) mounted on a 1-mL syringe (Terumo, VWR) prealably heparinized (Heparin, Roche, Meylan, France). Blood was immediately transferred in a cold BD Vacutainer tube, centrifuged at  $4,000 \times g$  for 20 min at  $4^{\circ}\text{C}$ . Plasma was frozen and stored in liquid nitrogen until testosterone and corticosterone measurement.

## 2.3 RNA extraction and semiquantitative RT-PCR

The steady state levels of OAT and cyclophilin A transcripts were estimated by semi-quantitative polymerase chain reaction (PCR) and quantitative PCR (qPCR) as described further in the text.

### 2.3.1 RNA extraction

Total RNA was extracted from whole kidney by using Trizol® according to the supplier's procedure. Briefly, kidneys were mixed in the proportion of 150 mg tissue per 1 mL Trizol® at  $4^{\circ}\text{C}$  with a Ultra-Turrax T10 (VWR, Fontenay-sous-Bois, France). RNAs were extracted with chloroform, purified by isopropanol precipitation, and washed with 70% ethanol. RNA pellets were resuspended in sterilized water (Eurobio, Courtaboeuf, France) and stored

frozen (-80°C). RNA concentration was determined from the absorbance at 260 nm and RNA purity the absorbance ratio at 260 and 280 nm (ratio  $\approx$  2) using a BioPhotometer (Eppendorf France S.A.R.L., Le Pecq, France). RNA integrity was checked by electrophoresis on 1% agarose gel (Eurobio).

### 2.3.2 Reverse transcription

Total RNA (1  $\mu$ g) was denatured for 5 min at 65°C and mRNAs were reverse-transcribed (60 min at 37°C) in 25  $\mu$ L final volume containing 5  $\mu$ L of M-MLV RT 5X buffer, 100 U moloney murine leukemia virus reverse transcriptase (Invitrogen, France), 15 nmoles of deoxynucleotide triphosphate and 1  $\mu$ g oligo dT (PCR) or 100 ng random primer (qPCR, Invitrogen). After RT, inactivation step was 15 min at 70°C. RT products were frozen (-30°C).

### 2.3.3 Polymerase chain reaction for PCR

The cDNA from 2.5  $\mu$ L of RT product were amplified by PCR in 47.5  $\mu$ L of PCR mixture containing 5  $\mu$ L of 10X Taq PCR buffer, 100 nmoles  $MgCl_2$ , 15 nmoles of deoxynucleoside triphosphate (Promega), 2.5 U Taq DNA polymerase, 15 pmoles corresponding to forward and reverse primers. Gene-specific oligonucleotide primers (20-22 nucleotides) were selected from the cDNA sequences of mouse OAT and rat cyclophilin A. Forward and reverse primers were designed using Primer3 software (v. 0.4.0) (<http://frodo.wi.mit.edu/primer3/>) and are shown in Table 1. Sequence identity between rat and mouse cyclophilin A was 91% for forward primer and 95% for reverse primer. The PCR program consisted of a denaturation step (2 min at 94°C), X cycles (see Table 1) and a final elongation step (10 min at 72°C). Each cycle included denaturation (1 min at 94°C), primer annealing (1 min at 60°C), and elongation (1 min at 72°C). Preliminary experiments were performed to determine the linear range of PCR amplification for each target mRNA. RT and PCR reactions were performed using a thermocycler (Mastercycler® Personal, Eppendorf, Le Pecq, France).

Gene	Accession number	Primer	Sequences (5' -> 3')	PCR product size (pb)	Cycle number
OAT	NM_016978.2	Forward	TCCAGGATACCTGACGGGAGTT	330	27
		Reverse	ATCTTGTCTGCGTTTTTCAGCAA		
eIF4-E	NM_007917.3	Forward	AGCAGAGTGGACTGCACTGA	394	27
		Reverse	GCAAGGACAATGCTGTGAAA		
Cyclophilin A	NM_017101	Forward	GTGGCAAAGTCCATCTACGGAG	265	24
		Reverse	CCACAGTCGGAGATGGTGATC		

Abbreviation: ornithine aminotransferase (OAT) and eukaryotic initiation factor eIF4-E (eIF4-E).

Table 1. Gene-specific sequences of PCR primers, predicted PCR products, and parameters for PCR analyses

### 2.3.4 Quantification of RT-PCR products

Fourteen  $\mu$ L of each PCR products containing 14% of a gel loading buffer (Blue juice 10X) were separated by electrophoresis on a 4% (w/v) agarose (Eurobio) gel prestained with



0.01% ethidium bromide. For quantitation of band intensities, pictures were taken with a Camera DC120 (Kodak) and intensities of the bands were determined with Kodak Digital Science 1D 2.0 (Kodak Scientific Imaging System). The housekeeping rat cyclophilin A gene was used as an internal control to normalize the target gene expression as previously reported (Bitoun et al., 2001).

### 2.3.5 Polymerase chain reaction for qPCR

Real-time PCR was performed in a MyiQ thermal cycler (Bio-Rad, Marnes La Coquette, France) using IQ SYBR Green Supermix (Bio-Rad). Primers specific to the mouse sequence of OAT and HPRT were designed using Primer3 software. The following qPCR conditions were used: 3 min at 95°C, followed by 40 cycles of denaturation for 10 s at 95°C and annealing/extension for 45 s at 60°C, according to the manufacturer's instructions. All samples were run in duplicate along with dilutions of known amounts of target sequence to quantify the initial cDNA copy number (Concentration = Efficiency<sup>ΔCt</sup>). The results are expressed as the ratio of the target gene over HPRT mRNA concentration which was verified to exhibit non-significant variation between the different groups of cDNAs.

Gene	Accession number	Primer	Sequences (5' -> 3')	qPCR product size (pb)
OAT	NM_016978.2	Forward	GGGCTCTTGTGAAACTCTGC	195
		Reverse	AGATGGGTCCGTTTCTCCTT	
HPRT	NM_013556	Forward	GTAATGATCAGTCAACGGGGGAC	177
		Reverse	CCAGCAAGCTTGCAACCTTAACCA	

Abbreviation: ornithine aminotransferase (OAT) and hypoxanthine guanine phosphoribosyl transferase (HPRT).

Table 2. Gene-specific sequences of PCR primers and predicted PCR products for qPCR analyses.

### 2.4 Protein extraction and western blot

Frozen kidneys were mixed at 4°C with a Turrax homogenizer at 100 mg frozen tissue per 2 mL of lysing buffer containing protease inhibitor (Laemmli, 1970). After centrifugation, the concentration of soluble proteins was determined using the Bradford protein assay (Bradford, 1976). Fifty or 100 µg samples of soluble proteins were subjected to 10% PAGE containing 0.1% SDS using 6 Watts per gel. Protein transfer and equal loading of proteins were visualized on the membranes with Ponceau S solution. Blocked immunoblots were incubated with primary polyclonal rabbit antibodies raised against rat/mouse-OAT (dilution 1:1,000), rat-aldose reductase (AR; EC 1.1.1.21, dilution 1:3,000) (Lambert-Langlais et al, 2009), and monoclonal mouse β-actin (dilution 1:2,000) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH; EC 1.2.1.12, dilution 1:170) antibodies, and then with a peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibodies (dilution 1:10,000) as previously described (Levillain et al., 2004, 2005). Antibody binding was revealed using chemiluminescence (ECL) Western Blotting Kit. Details for ECL detection and quantitation of the bands have been mentioned earlier (Levillain et al., 2004, 2005).

## 2.5 Measurement of OAT activity

OAT activity was measured in whole kidneys. Samples were mixed at 4°C with a Turrax homogenizer in a buffer (0.33 M sucrose, 5 mM HEPES, 1 mM EGTA, 1 mM DTT, and 0.5% Triton X 100). Homogenates were centrifuged 21,000 × *g* for 10 min at 4°C. The protein concentration was determined on the supernatant using the Bradford protein assay. OAT activity was determined using the enzyme assay previously described (Herzfeld & Knox, 1968; Peraino & Pitot, 1963). Briefly, the supernatant was incubated with a buffer composed of 75 mM potassium phosphate pH 8.0, 20 mM L-ornithine, 0.45 mM pyridoxal phosphate, 5 mM  $\alpha$ -aminobenzaldehyde, and 3.75 mM  $\alpha$ -keto-glutarate for 15 or 30 min at 37°C. Blanks did not contain  $\alpha$ -keto-glutarate. The reaction was stopped by adding trichloroacetic acid 40%. Samples were centrifuged 21,000 × *g* for 3 min at 4°C and absorbance was measured on the clear supernatant at 440 nm on a Hitachi U-1100 spectrophotometer (Meylan, France). Duplicate or triplicate samples and blanks were performed for all experiments.

## 2.6 Determination of testosterone and corticosterone levels

Testosterone was measured by radioimmunoassay after extraction by organic solvent and partition chromatography of the plasma samples as previously described (Dechaud et al, 1989). Corticosterone concentration was measured, after diethylether extraction, by radioimmunoassay using 1,2,6,7 [3H]-corticosterone and rabbit anti-corticosterone polyclonal antibody (Filipski et al, 2002).

## 2.7 Calculations and statistical analyses

The calculations were as follows: for each group of mice and for each protein, the mean intensity optical densitometry (IOD) of the bands was calculated. The mean IOD of the untreated (group I) and sham-operated (group V) mice were used as a reference (control). For each mouse, the IOD value of a given protein was divided by the mean IOD value of the control group. Consequently, this ratio value is 1 in each control group. Then, these ratios were related to those of  $\beta$ -actin and/or G3PDH.

OAT activity was expressed in absorbance per 15 min per mg soluble protein because we were unable to find a supplier to buy P5C as standart to convert the absorbance of P5C into molar unit. Depending on the material used or the physiological interest of expressing the data in other units, OAT activity is expressed in absorbance per 15 min per mg soluble proteins.

Results are expressed as means  $\pm$  SE. Non-parametric statistical tests were used to analyze the data. Where appropriate, statistical differences were assessed using the Kruskal-Wallis test at level significance of 95% and this test was followed by the U-Mann-Whitney test at level significance of 95% (StatView SE+Gr). For correlation analyses, the correlation coefficient  $r^2$  was calculated with Microsoft Excel, and *P* was determined from tables at the 95% level of significance.

## 2.8 Chemicals

Salts and most chemicals, Ponceau S solution, secondary anti-IgG antibodies, Kodak X-MAT film were purchased from Sigma (St Quentin Fallavier, France). Protease inhibitor cocktail were purchased from Boehringer Mannheim (Strasbourg, France). Agarose Seakem GTG was purchased from TEBU (Le Perray-en-Yvelines, France). ECL Western Blotting Kits, and ImagerMaster Total Lab v2.01 program were purchased from Amersham (Buckinghamshire, UK).

### 3. Results

#### 3.1 Technical verifications

##### 3.1.1 Semiquantitative RT-PCR

To quantify the levels of OAT and cyclophilin A mRNA by RT-PCR, we determined the appropriate number of amplification cycles to remain in the exponential phase of the amplification process and avoid saturation (Table 1). For each mRNA studied a single RT-PCR product was obtained. The amplified RT-PCR products were of the expected size (Table 1). The level of the mRNA of a housekeeping gene (cyclophilin A) did not vary significantly in our experimental conditions (data not shown).

##### 3.1.2 OAT activity assay

The renal cortex of male and female mice was dissected and prepared to quantify OAT activity. To determine the conditions for assay of OAT activity, the incubation time was tested every 5 min up to 30 min and the protein concentration varied from 0 to 400  $\mu\text{g}$  per sample. OAT activity increased linearly with the concentration of soluble proteins up to 400  $\mu\text{g}$  in male mouse cortex and 300  $\mu\text{g}$  in that of the female when the incubation time was fixed at 30 min (Fig.2 left, male:  $r^2=0.99809$  and female:  $r^2=0.99583$ ,  $P < 0.01$ ). OAT activity increased linearly in proportion with the incubation time up to 30 min when the concentration of soluble proteins was fixed at 140  $\mu\text{g}$  in both male and female mice (Fig. 2 right, male:  $r^2=0.99352$  and female:  $r^2=0.9972$ ,  $P < 0.01$ ). Constantly, OAT activity was more than three-fold higher in female than in male mouse kidney. The sex-differential OAT activity found in the present results is in good agreement with published data (Levillain et al., 2007; Ventura et al. 2009).

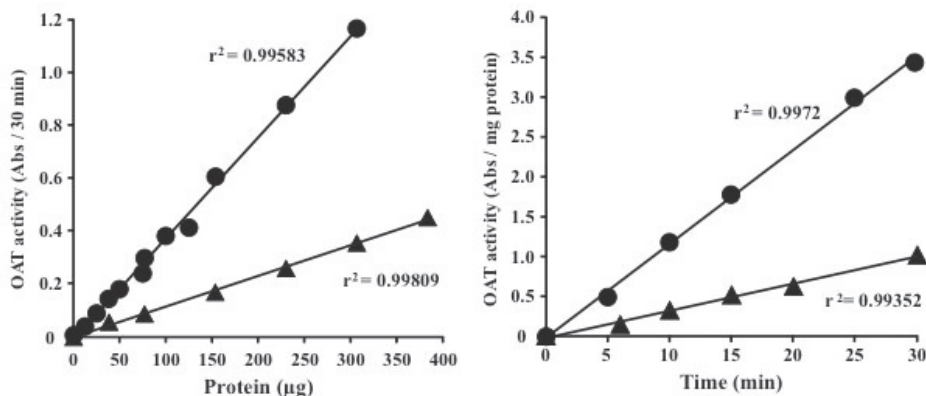


Fig. 2. Technical verifications to determine the conditions for OAT assay.

Closed circles: female mouse kidney and closed triangles: male mouse kidney. In all cases, the coefficient of correlation was statistically significant ( $P < 0.01$  or less).

#### 3.2 Influence of orchidectomy on the expression of OAT gene

A complete analysis of the expression of OAT gene in the mouse kidney was undertaken by analyzing the levels of mRNA and protein, and measuring OAT activity. The level of OAT

mRNA, protein, and enzyme activity did not differ between control and sham-operated male mice. Surgery did not affect the expression of OAT gene in the mouse kidney (Fig. 3). In contrast, orchidectomy provoked a sharp increase in OAT mRNA, protein, and enzyme activity. In details, in castrated mice killed 11 day after the surgery, the level of OAT mRNA, OAT protein, and OAT activity were increased by 3.95-fold (Kruskal-Wallis,  $P < 0.035$  followed by Mann Whitney,  $P < 0.0495$ ), 2.13-fold (Kruskal-Wallis,  $P < 0.0004$  followed by Mann Whitney,  $P < 0.0039$ ), and 2.27-fold (Kruskal-Wallis,  $P < 0.018$  followed by Mann Whitney,  $P < 0.05$ ), respectively as compared to the sham-operated mice (Fig. 3). When orchidectomized mice were killed 18 days after the operation, OAT gene remained still highly expressed, nevertheless, its expression had a tendency to be higher than at day 11. The level of OAT mRNA was 4.44-fold higher than that of the sham-operated mice (Mann Whitney,  $P < 0.0495$ ), but did not differ from that of the mice killed on day 11. Similarly, the mean value of OAT protein was higher on day 18 than on day 11, but it did not reach the statistical level of significance ( $P < 0.0547$ ). In contrast, OAT activity was significantly higher on day 18 than day 11 as compared to the sham-operated mice (Mann Whitney,  $P < 0.0495$ ). The level of OAT mRNA was quantitated either by RT-PCR (Fig. 3) or by q-PCR (Fig. 4). The two methods gave similar results and patterns (Figs. 3 and 4). Different house-keeping genes were used to prove that the effect of castration were independent of the gene of reference. Orchidectomy provoked a 4.9- and 4.7-fold increase in the level of OAT mRNA in 11d- and 18d-castrated mice as compared to the sham-operated male mice. (Fig. 4; Kruskal-Wallis,  $P < 0.0001$  followed by Mann Whitney,  $P < 0.0027$  or less in both cases).

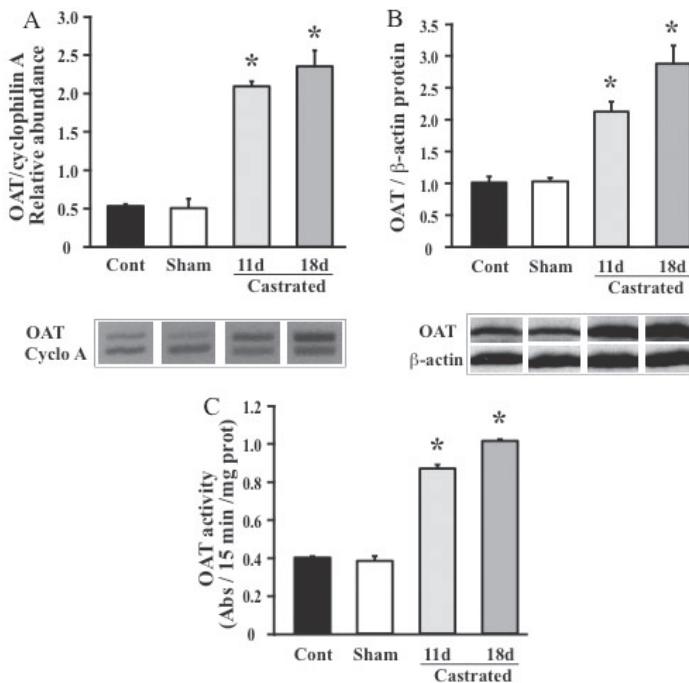


Fig. 3. Effect of orchidectomy on the expression of ornithine aminotransferase gene in the mouse kidney.

The values are expressed as means  $\pm$  SE. A: The level of OAT mRNA were analyzed by semi-quantitative RT-PCR. The amplified cDNAs were separated by agarose-gel electrophoresis. Band intensities were quantified and reported relative to the cyclophilin A band (n=3 mice per group). B: Immunoblot assessing the abundance of OAT and  $\beta$ -actin proteins in mouse kidneys. Immunoblots were loaded with samples of 100  $\mu$ g soluble proteins (n=6 mice per group).  $\beta$ -actin was used as control of protein loading and transfer. C: OAT activity was measured in mouse kidneys (n= 4, 3, 3, 3 mice per group, respectively). To simplify the Figure, a representative RT-PCR or immunoblot that corresponds to one mouse from each group was shown. Abbreviations: control (Cont), sham-operated (Sham), and 11- and 18-day orchidectomized mice (11d and 18d, respectively), cyclophilin A (Cyclo A), ornithine aminotransferase (OAT), and absorbance (Abs). Differences between groups were statistically analyzed by Kruskal-Wallis test and followed by Mann-Whitney test. \*,  $P < 0.05$  or less.

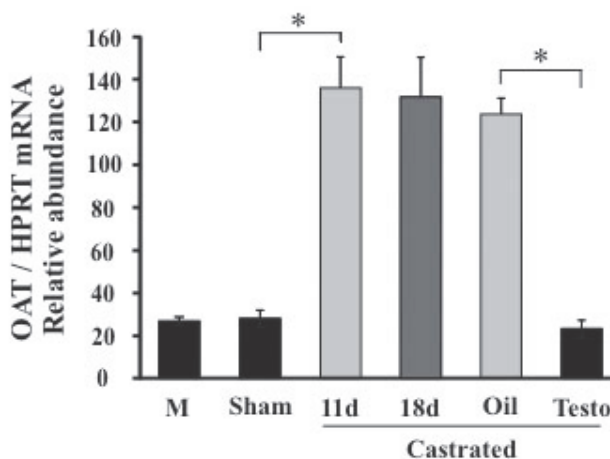


Fig. 4. Relative levels of ornithine aminotransferase mRNA in mouse kidneys.

The values are expressed as means  $\pm$  SE. The relative mRNA levels of OAT were determined by qPCR in the kidneys of male (M), sham-operated (Sham), 11- and 18-day orchidectomized mice (11d and 18d, respectively), castrated mice treated with oil as solvent (Oil), and castrated mice supplemented with testosterone dissolved in oil (Testo). Abbreviations: ornithine aminotransferase (OAT), and hypoxanthine guanine phosphoribosyl transferase (HPRT). Differences between groups were statistically analyzed by Kruskal-Wallis test ( $P < 0.0001$ ) and followed by Mann-Whitney test. \*,  $P < 0.0039$  or less. Number of mice used: 11, 6, 9, 7, 6, and 6, respectively.

### 3.3 Influence of testosterone on the expression of OAT gene

Given that orchidectomy provoked a sharp increase in the expression of OAT gene in the mouse kidney, we hypothesized that androgens control and down-regulate the expression of this gene. To verify our hypothesis, castrated male mice were subjected to testosterone-treatment. An additional group of castrated mice was subjected to sesame oil-treatment inasmuch as oil was used as a solvent to dissolve testosterone. The expression of OAT gene was analyzed at the transcriptional and traductional levels and OAT activity was measured. In

this new study, our results confirmed that castration of male mice provoked an overexpression of OAT gene (Fig. 5). The levels of OAT mRNA, protein, and enzyme activity were increased and strongly resemble those presented in Figure 3. However, the level of OAT mRNA was increased only by two-fold in kidneys of castrated mice of the second study, (Fig. 5) as compared to four-fold for the first study (Fig. 3). In kidneys of castrated mice treated with a single injection of sesame oil, the level of OAT mRNA, protein, and OAT activity did not differ from those of the castrated mice (Fig. 5). In contrast, a single injection of a physiological dose of testosterone decreased by 2-fold the level of OAT mRNA (Kruskal-Wallis,  $P < 0.036$  followed by Mann Whitney,  $P < 0.0495$ ), OAT protein by 1.58-fold (Kruskal-Wallis,  $P < 0.032$  followed by Mann Whitney,  $P < 0.05$ ), and OAT activity by 1.62-fold (Kruskal-Wallis,  $P < 0.0045$  followed by Mann Whitney,  $P < 0.0209$ ) (Fig. 5). The level of OAT mRNA was also quantitated either by RT-PCR (Fig. 5) or by q-PCR (Fig. 4). Different house-keeping genes were used to prove that the effect of castration were independent of the gene of reference. Again, the same pattern was found with the two methods (Figs. 4 and 5). Oil-treatment had no effect whereas testosterone-treatment provoked a 5.3-fold decrease in the level of OAT mRNA as compared to oil-treated mice. (Fig. 5; Kruskal-Wallis,  $P < 0.0001$  followed by Mann Whitney,  $P < 0.0039$ ). The plasma levels of corticosterone had a tendency to increase after orchidectomy and decrease in testosterone-treated mice. Unfortunately, the difference did not reach statistical significance (Kruskal-Wallis,  $P < 0.2435$ ). The plasma level of corticosterone were inversely correlated with those of testosterone (Fig. 5 and Levillain et al. 2005).

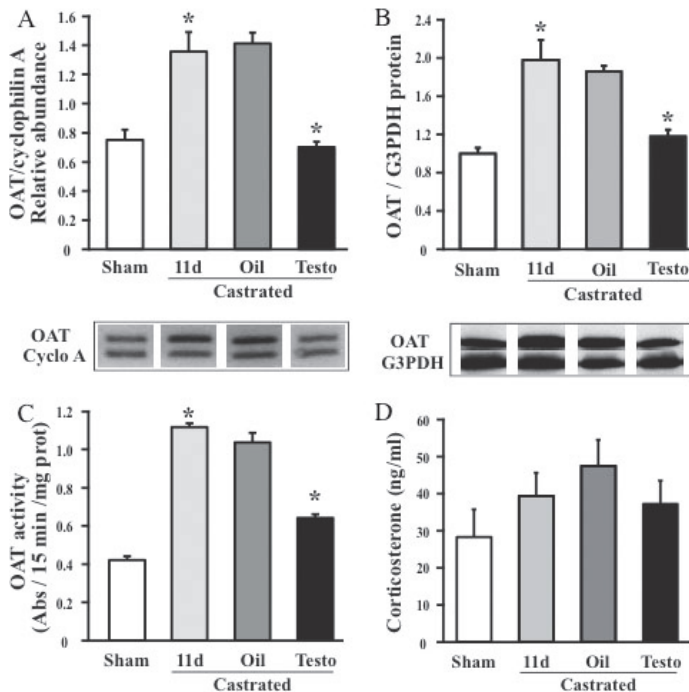


Fig. 5. Effect of testosterone treatment on the expression of ornithine aminotransferase gene in the mouse kidney.

The values are expressed as means  $\pm$  SE. A: The level of OAT mRNA were analyzed by semi-quantitative RT-PCR. The amplified cDNAs were separated by agarose-gel electrophoresis. Band intensities were quantified and reported relative to the cyclophilin A band (n=3 mice per group). B: Immunoblot assessing the abundance of OAT and G3PDH proteins in mouse kidneys. Immunoblots were loaded with samples of 100  $\mu$ g soluble proteins (n=3 mice per group). G3PDH was used as as control of protein loading and transfer. C: OAT activity was measured in mouse kidneys (n=4 mice per group). D: Plasma levels of corticosterone (n = 9, 10, 10, and 9 mice, respectively). To simplify the Figure, a representative RT-PCR or immunoblot that corresponds to one mouse from each group was shown. Abbreviations: sham-operated (Sham), and 11-day orchidectomized mice (11d), cyclophilin A (Cyclo A), ornithine aminotransferase (OAT), G3PDH (glyceraldehyde-3-phosphate dehydrogenase), Testosterone (Testo), and absorbance (Abs). Differences between groups were statistically analyzed by Kruskal-Wallis test and followed by Mann-Whitney test. \*,  $P < 0.05$  or less.

### 3.4 Influence of testosterone on the expression of eIF4-E gene

The eukaryotic initiation factor eIF4-E has been reported to be rate-limiting for OAT translation (Fagan et al., 1991). We analyzed whether the level of eIF4-E mRNA was altered by orchidectomy and testosterone replacement. Our data show that, in the mouse kidney, the level of eIF4-E mRNA had a tendency to be decreased by orchidectomy and enhanced by testosterone replacement. Nevertheless, the difference did not reach statistical significance (Fig. 6, Kruskal-Wallis,  $P < 0.1574$ ).

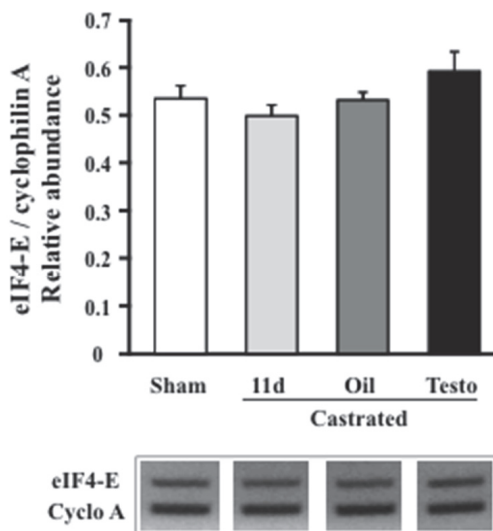


Fig. 6. Effect of testosterone treatment on the expression of eIF4-E gene in the mouse kidney.

The values are expressed as means  $\pm$  SE. The levels of eIF4-E mRNA were analyzed by semi-quantitative RT-PCR. The amplified cDNAs were separated by agarose-gel electrophoresis. Band intensities were quantified and reported relative to the cyclophilin A band (n=3 mice

per group). To simplify the Figure, a representative RT-PCR that corresponds to one mouse from each group was shown. Abbreviations: sham-operated (Sham), and 11-day orchidectomized mice (11d), cyclophilin A (Cyclo A), eukaryotic initiation factor eIF4-E (eIF4-E), and oil + testosterone (Testo). Differences between groups were statistically analyzed by Kruskal-Wallis test,  $P = 0.1574$ .

### 3.5 Time course changes in the expression of OAT gene

This experiment was performed to determine the delay requested for testosterone to down-regulate the expression of OAT gene in the mouse kidney. The time-course effect of testosterone on the expression of OAT gene was analyzed at the transcriptional and post-translational levels 8, 24, 28, and 32 hrs following hormone administration. Eight hours following testosterone treatment, the level of OAT mRNA was reduced by 8%, but did not reach statistical significance (Fig. 7A). In contrast, 24, 28, and 32 hrs after testosterone replacement, the renal levels of OAT mRNA were decreased by 45%, 40%, and 36%, respectively (Kruskal-Wallis  $P < 0.0028$  followed by Mann Whitney,  $P < 0.0209$  for each case). The pattern of OAT activity along this time-course study strongly resembled that of OAT mRNA except that the decrease in OAT activity was about 2-fold lower than that of OAT mRNA (Fig. 7B). Indeed, 24, 28, and 32 hrs after testosterone administration, OAT activities were decreased by 20%, 21%, and 23%, respectively (Kruskal-Wallis  $P < 0.0071$  followed by Mann Whitney,  $P < 0.0209$  for each case). The efficiency of the hormonal treatment was checked by measuring testosterone. Our results show that testosterone was very high 8 hrs after a single injection of the hormone and regularly decreased over the experimental period. Thirty-two hours after the treatment, testosterone was about 2-fold higher than the physiological concentration of testosterone reported for control male mice (Levillain et al., 2007). Corticosterone were quantified in the same mice to determine whether testosterone modified the plasma level of corticosterone. A high concentration of corticosterone was detected 8 hrs after testosterone treatment (Fig. 7C). One day later (24 and 28 hrs), the plasma level of corticosterone were decreased by 3-fold whereas at 32 hrs, it increased again by 2-fold (Fig. 7D). Unfortunately, given that it was impossible to have access to the animal room during the night, no mice could be sampled during the dark period.

## 4. Discussion

OAT plays a pivotal role in the intermediary metabolism because this enzyme is at the crossroad of several pathways. OAT controls the production of glutamate and ornithine, competes with the polyamine pathway, and may be a source of carbon for the renal gluconeogenesis. These pathways are essential for many physiological roles including growth and energy supply. Several reports clearly documented the hormonal regulation of OAT gene in the rat liver and kidney. In this species, the renal expression of OAT gene is up-regulated by estrogens and triiodothyronine (Herzfeld & Knox, 1968; Lyons & Pitot, 1977; Mueckler & Pitot, 1983; Mueckler et al., 1984). During the post-natal development of the rat kidney, the sexual dimorphism of the expression of OAT gene increased in parallel with the endogenous synthesis of estrogens (Herzfeld & Knox, 1968). In contrast, testosterone affected OAT activity neither in liver nor in kidneys of male and female rats (Herzfeld & Knox, 1968). The authors concluded that estrogen naturally control and upregulate the expression of OAT



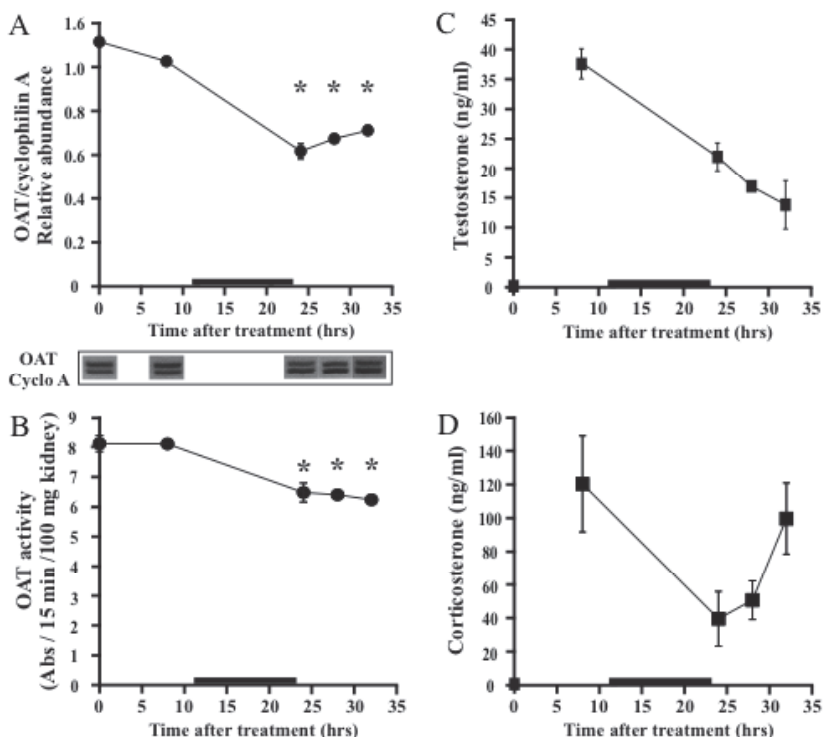


Fig. 7. Time course effect of testosterone on the expression of ornithine aminotransferase gene in the mouse kidney. A single dose of testosterone was injected to castrated-male mice. Mice were killed 8, 24, 28, and 32 hrs after the hormonal-treatment. Untreated castrated mice were used as controls. The values are expressed as means  $\pm$  SE, (n=4 mice per group). A: The level of OAT mRNA were analyzed by semi-quantitative RT-PCR. The amplified cDNAs were separated by agarose-gel electrophoresis. Band intensities were quantified and reported relative to the cyclophilin A band. To simplify the Figure, a representative RT-PCR that corresponds to one mouse from each group was shown. B: OAT activity was measured in mouse kidneys. C: Plasma levels of corticosterone. D: Plasma levels of testosterone. The black bar represents the dark period (night). Differences between groups were statistically analyzed by Kruskal-Wallis test and followed by Mann-Whitney test. \*,  $P < 0.0209$  in all cases.

gene in the female rat kidney. As reported for the rats, the mouse kidney also exhibits a sexual dimorphism in the expression of OAT gene. Although the hormonal regulation of this gene has been poorly studied in both liver and kidney of male and female mice, it has been clearly shown that the endogenous production of testosterone was responsible for a decrease in the renal expression of OAT gene (Levillain et al., 2007). Interestingly, in contrast to the rats, ovariectomy altered neither the level of OAT protein nor OAT activity in the murine kidney (Levillain et al., 2007). Estrogens do not control the renal expression of OAT in the mouse kidney. Consequently, testosterone naturally controls and down-regulates the expression of

OAT gene in the male mouse kidney. Furthermore, orchidectomy completely abolished the sexual dimorphism of the renal expression of OAT gene (Levillain et al., 2007). The present findings confirm these statements. Moreover, our results show that testosterone negatively regulated the expression of OAT gene at the transcriptional level. Indeed, the levels of OAT mRNA, protein, and enzyme activity were markedly increased following castration. The castrated male mice were killed either 11 days or 18 days after the surgery to completely deplete the endogenous pool of testosterone. Plasma testosterone levels showed that all mice were testosterone-free as soon as 11 days after castration (Levillain et al., 2005).

To further explore the mechanisms involved in the regulation of OAT gene by testosterone in the mouse kidney, experiments were conducted to test the consequence of testosterone replacement on the expression of OAT gene at the transcriptional, translational, and post-translational levels. The dose of testosterone injected was calculated to reach a physiological plasma level of testosterone. The efficiency of testosterone to lower the level of OAT mRNA in the mouse kidney was confirmed. These results demonstrate that testosterone regulates OAT gene expression at the transcriptional level. The regulation of OAT gene at the transcriptional level has been also reported in the rat tissues (Mueckler & Pitot, 1983; Mueckler et al., 1983, 1984). The levels of OAT protein and OAT activity were also diminished by a single injection of testosterone, however, this decrease was less pronounced than that of OAT mRNA. This difference may be explained by the long half-life of OAT protein. The half-life of OAT protein is estimated at about 48 hrs (Ip et al., 1974). The time-course study of the effect of testosterone on the expression of OAT gene in castrated male mice also supports this timeline with the level of OAT mRNA decreasing more rapidly than OAT activity. This experiment also revealed a lag-time of 8 hrs or more from testosterone administration before detecting significant changes in the level of OAT mRNA. Similar results were observed with injection of testosterone to female mice (paper in preparation). This delay from administration to mRNA expression suggests that several genomic and/or non-genomic events may take place in the signaling cascade of testosterone (Heinlein & Chang, 2002). One hypothesis is that a target molecule such as the eukaryotic initiation factor eIF4-E is rate-limiting for OAT translation (Fagan et al., 1991). However, in our experiments, the level of eIF4-E mRNA was altered neither by orchidectomy nor by testosterone replacement.

In order to explain a direct genomic action of testosterone, an attempt was made to identify ARE on the promoter of the mouse OAT gene. We carried out an *in silico* search for homology to the consensus ARE motifs by selecting either the left half-site 5'-AGAACA-3' or the right half-site 5'-TGTTCT-3' motifs by search computational using MatInspector (<http://www.genomatix.de/>) (Beato et al., 1989; Fabre et al., 1994; Roche et al., 1992; Wang et al., 2007; Merkulov & Merkulova, 2009). Several ARE (5'-AGAACAnnnTGTTCT-3') and glucocorticoid responsive element (GRE, 5'-GGTACAnnnTGTTCT-3') which share the same sequence 5'-TGTTCT-3' (Nelson et al., 1999, Verrijdt et al., 2003) were identified on the promoter of the mouse OAT gene. Interestingly, four GRE have been found in the human OAT gene (Zintz & Inana, 1990). Further experiments will be required to determine the functionality of these elements in response to hormone administration.

Recently, it has been reported that, in Swiss CD1 mice, sex hormones influence the weight of the adrenal gland and the plasma level of corticosterone (Bastida et al, 2007). Indeed, the weight of the female adrenal gland was 2.8-fold higher than that of the male (Bastida et al, 2007). Orchidectomy enhanced the weight of the male adrenal gland by 2.1-fold whereas

testosterone treatment decreased by 2.6-fold the weight of the female adrenal gland which did not differ from that of the control male adrenal gland (Bastida et al, 2007). The plasma level of corticosterone was 2.8-fold higher in the females as compared to the males (Bastida et al, 2007). These results led us to hypothesize that testosterone may control the plasma level of corticosterone which may secondarily regulate the expression of OAT gene in the mouse kidney. For this reason the plasma level of corticosterone were measured in the different groups of mice. Our results did show a trend for the plasma levels of corticosterone to be higher in orchidectomized mice than in untreated male mice and in castrated male mice treated with testosterone, however, with a low sample number and high standard error the difference did not reach statistical significance. Contrarily, when analyzing the time course effect of testosterone 8 hrs after the hormonal treatment the plasma level of corticosterone and testosterone were both very high suggesting that testosterone does not control the rate of synthesis and release of corticosterone. The presence of GRE and ARE in the promoter of the mouse OAT gene offers new insight on the hormonal regulation of this gene. Further experiments utilizing male mice subjected to both orchidectomy and adrenalectomy may afford a model to resolve this point.

Hormonal regulation of OAT gene by steroids can impact human disease model, such as prostate cancer (PCa). Androgen receptor (AR) targets have been recently identified in a model of androgen-dependent (LNCaP) and a model of castrate-resistant (C4-2B) human PCa cell lines (Jariwala et al., 2007) with OAT expressed at a higher level in the castrate resistant (C4-2B) cells. Expression of OAT was repressed by siRNA in C4-2B cells demonstrating that OAT was an AR regulated gene, although it did not respond to dihydrotestosterone (DHT). Furthermore, the expression of OAT was analyzed in primary PCa tumors and primary PCa tumors after 3 months of androgen ablation therapy. OAT appears to be negatively regulated by the AR *in vivo*.

In conclusion, in the mouse kidney, testosterone down-regulates the expression OAT gene at the transcriptional level. This finding may have interesting consequences for human diseases as reported for prostate cancer.

## 5. Acknowledgements

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## **Part 4**

### **Insulinomas**





# Diagnosis and Treatment of Insulinomas in the Adults

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## 1. Introduction

Insulinomas are rare endocrine tumours developed from pancreatic beta cells. Their incidence is about 1 in 250,000 patient-years (Cryer 2008) (0.396 per 100,000 person-years for two decades, 1967-1986) (Service et al. 1991). The median age at surgical diagnosis was found to be 47 years (8 to 82), 59% being female patients. In a series of 33 patients, the age at the time of diagnosis was 57 +/- 16 years (mean +/- SD) (range: 18-85 years) and 66% were female patients (Vezzosi et al. 2007). 90% of insulinomas are single, benign and sporadic tumours that are located in the pancreas. The diagnostic and therapeutic strategy of benign sporadic insulinomas has been now established by a recent expert consensus (Cryer et al. 2009). However, some issues remain unaddressed regarding the diagnosis and the treatment of insulinomas. More rarely, in about 10% of insulinoma patients, the insulinoma is part of MEN-1. Such patients often present with multiple insulinomas and other secreting or non-secreting endocrine tumours. Finally, a particular condition is malignant insulinoma, also found in about 10% of insulinoma patients. There are no recommendations regarding the particular conditions represented by insulinomas in MEN-1 and malignant insulinomas.

The aim of this chapter is to give an updated and detailed view of the medical management of adult patients with insulinoma regarding the diagnosis (diagnosis of hypoglycemia related to endogenous hyperinsulinism, differential diagnosis, topographic assessment) and the treatment (surgery, medical therapies), including the therapeutic strategy and the possibilities of long-term medical treatment in inoperable patients. The first part of the chapter will be focused on the most frequent case, i.e. single benign sporadic insulinoma. The remaining parts of the chapter will deal with specific issues and concerns regarding malignant insulinomas, insulinomas in genetic disorders, and the rare cases of nesidioblastosis in the adults.

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## 2. Single benign sporadic insulinomas

### 2.1 Diagnosis of hypoglycaemia related to endogenous hyperinsulinism

#### 2.1.1 Clinical symptoms of insulinomas

The clinical symptoms of insulinomas are heterogeneous and may differ among the patients. There is no specific clinical symptom. Hypoglycaemia results in autonomic and neuroglycopenic symptoms. Autonomic symptoms comprise adrenergic symptoms, such as palpitations and tremor, and cholinergic symptoms, with sweating, hunger, sometimes paresthesias. Neuroglycopenic symptoms comprise severe weakness, and many psychiatric and neurological manifestations, the most commonly reported being behavioural changes, confusion, agitation or slow reaction patterns, blurred vision, and finally seizures, transient loss of consciousness or hypoglycaemic coma. Hypoglycaemic coma is typically sudden, with agitation, sometimes pyramidal signs, and hypothermia; it may be profound and prolonged and therefore may be a cause of neurologic and cognitive sequelae and may occasionally lead to brain death. The combination of autonomic and moderate neuroglycopenic symptoms strongly suggests the diagnosis of hypoglycaemia. However, in insulinoma patients, autonomic symptoms may often be lacking. Neuroglycopenic symptoms are more specific of hypoglycaemia related to insulinoma, but may be mild, or more often misleading. Therefore measurement of plasma glucose concentrations should be recommended in all patients with such symptoms, either in a patient without any known neurological or psychiatric disorder, or in a patient in whom such disorder had been previously controlled. Hypoglycaemic spells typically occur suddenly after a period of fast, several hours after the last meal, or after physical exercise. Some of them have been reported to occur within the hours following a meal or without any relationship with meal time, so that symptoms occurring in non fasting patients cannot rule out the diagnosis of insulinoma. In a series of 214 insulinoma patients with a reliable clinical history, symptoms of hypoglycaemia were reported exclusively in the fasting state in 73%, postprandial state in 6%, and both fasting and postprandial state in 21% (Placzkowski et al. 2009). Weight gain is found only in 25% of patients.

Rapid (or rarely delayed) resolution of symptoms after glucose administration is part of Whipple's triad, which makes the diagnosis of hypoglycaemia when it has been assessed reliably; the triad comprises clinical symptoms of hypoglycaemia, the finding of a low plasma glucose concentration at the time of clinical symptoms, and subsiding of clinical symptoms after glucose administration.

In rare pregnant patients with insulinoma, symptoms may resemble those of emesis in early pregnancy and may subside in late pregnancy as a consequence of insulin resistance, then are unmasked after delivery (Diaz et al. 2008), as reported in a patient who presented with coma and clonic seizures 14 hours after delivery (Christiansen & Vestergaard 2008).

#### 2.1.2 Biological diagnosis of insulinomas

##### Plasma glucose concentrations

Since the clinical symptoms lack diagnostic specificity, measurement of plasma concentration of glucose is mandatory (ideally at the time of occurrence of clinical symptoms) and must be performed in venous blood samples with a reliable laboratory method. In order to avoid pseudohypoglycaemia, i.e. a consequence of glucose metabolism by blood cells in the tube containing the blood sample, the blood sample should be collected in a tube with a glycolysis inhibitor (NaF). Glucose concentrations are measured using reference method with hexokinase.

The threshold to define hypoglycemia remains controversial. According to recent expert recommendations (Cryer et al. 2009) a 0.55 g/L threshold must be selected. However, the glycaemic threshold that can cause clinical symptoms is very different among individuals, and a plasma concentration of glucose below 0.7 g/L at the time of clinical symptoms is thought to be enough, if the other criteria of Whipple's triad are fulfilled, to warrant further investigation. A plasma glucose concentration above 0.7 g/L during a symptomatic episode indicates that those symptoms are not the result of hypoglycaemia.

Since even values below 0.55 g/L can be found in some healthy individuals, it is also recommended by the expert consensus that such levels be taken into account for further evaluation only in patients who had presented Whipple's triad. On the other hand, very rare insulinoma patients were found to be asymptomatic and did not fulfil Whipple's triad. Among our patients (Vezzosi et al. 2007), the diagnosis of insulinoma was made on the basis of repeated plasma glucose concentrations of 0.40-0.55 g/l after overnight fasts in one patient, though she never described any clinical symptom. In many insulinoma patients, the glycaemic threshold for clinical symptoms is shifted to very low glucose levels, so that they may be asymptomatic at the time of plasma glucose concentrations below 0.55 and even 0.45 g/L; most of these patients experienced some clinical symptoms suggestive of hypoglycaemia, but even if they present with fasting glucose levels below 0.55 g/L, Whipple's triad has not been assessed in most of them, and awaiting Whipple's triad to be fulfilled would make the diagnosis be delayed. The finding of a spontaneous plasma glucose concentration below 0.55 g/L is rare in normal subjects; during a 72-hour fast test, plasma glucose concentrations do not reach values below 0.45 g/L in most controls, and do not drop below 0.4 g/L in controls (Vezzosi et al. 2007). Therefore we think that patients with spontaneous plasma glucose concentrations below 0.55 g/L warrant further investigation, even when they do not fulfil all the criteria of Whipple's triad, and that such evaluation is mandatory in patients with plasma concentrations below 0.45 g/L, provided that plasma glucose concentration has been measured reliably in a venous sample.

Confirmation of hypoglycaemia in a venous sample is the first requirement for the biological diagnosis of insulinoma.

If a venous sample cannot be collected when hypoglycaemia occurs spontaneously, a 72 hour-fast test is to be performed.

A detailed protocol for the fast test has first been described by the Mayo Clinic group (Service 1995)(Service 1999) and more recently by an expert consensus (Cryer et al. 2009). The patient is allowed to drink calorie-free beverages. Samples are to be collected every 6 hours until the plasma glucose concentration is less than 0.6 g/L if the patient remains asymptomatic, then the frequency of sampling is increased (every 1 or 2 hours). Serum insulin, C-peptide, proinsulin and beta-hydroxy-butyrate are to be measured in all the samples taken at the time when plasma glucose concentration drops below 0.6 g/L. At the end of the fast test, a sample is collected to measure oral hypoglycaemic agents; a glucagon test (1.0 mg intravenously) has also been recommended. Insulin antibodies should also be measured.

The criteria used to decide to stop the fast test before 72 hours have been modified recently. According to previous recommendations (Service 1995), the fast test is to be stopped if the patient has symptomatic hypoglycaemia with a plasma glucose concentration of 0.45 g/l or less, or if the plasma glucose concentration drops below 0.4 g/L. According to the recent recommendations (Cryer et al. 2009) the fast test can be stopped: 1) when Whipple's triad is observed; 2) when plasma glucose concentrations drop below 0.55 g/L in a patient who had previously experienced Whipple's triad; 3) if plasma beta-hydroxy-butyrate levels rise above 2.7 mmol/L.

Awaiting that plasma glucose concentrations drop below 0.45 g/L in an asymptomatic patient to stop the fast test, instead of stopping the fast when plasma glucose is 0.46-0.54 g/L, leads to an improvement in diagnostic specificity regarding the diagnosis of a hypoglycaemic disorder. In our own data, out of 67 controls, 31 reached glucose plasma concentrations of 0.46-0.54 g/L during a 72-hour fast test, and only 4 reached values within the 0.40-0.45 g/L range (0.41-0.43 g/L) after 24 hours of fast. No control subject reached plasma glucose concentrations below 0.4 g/L. Among 55 patients who were diagnosed to have insulinoma in our institution between 1997 and 2010, and who underwent a fast test, only one patient reached a plasma glucose concentration below 0.55 g/L (0.53 g/L) without further decrease in glucose concentration, and all the other patients who had a positive fast test reached glucose concentrations below 0.45 g/L; the fast test was negative in one patient. In addition, using a 0.45 g/L glucose level threshold increases the diagnostic specificity of concomitant serum insulin, C-peptide and proinsulin levels for the diagnosis of insulinoma (see below). Therefore we think that if the patient remains asymptomatic, the fast test is to be prolonged at least until reaching plasma glucose concentrations below 0.45 g/L, the diagnostic specificity being about 100% if such levels are reached.

In most insulinoma patients such plasma glucose concentrations are reached within the first 48 hours of the test (Hirshberg et al. 2000; Vezzosi et al. 2007), though the 72 hour-fast test was reported to be necessary to provide a clear diagnostic conclusion in a few patients (Service 1999; Service & Natt 2000). Moreover, additional criteria for the diagnosis of insulinoma using proinsulin and beta-hydroxy-butyrate levels and plasma glucose response to intravenous glucagon were established during a 72-hour (not 48-hour) fast test for patients who did not reach plasma glucose concentrations below 0.45 or 0.55 g/L. Thus in most patients the test to be performed is the classical 72 hour-test. However, 48 hours of fast test are enough to provide diagnostic evidence in almost all insulinoma patients who have a positive fast test.

In the patients who experience symptoms of hypoglycemia only within the hours following a meal, a meal test is to be performed with serial measurement of plasma concentrations of glucose, insulin, C-peptide and proinsulin during the 5 hours following the ingestion of a standardized meal. A mixed meal similar to that which the patient reports to have caused the clinical symptoms is to be used, or a commercial formula mixed meal. Samples should be collected every 30 minutes and those collected for insulin, C-peptide and proinsulin measurement should be sent for analysis only if the sample has been taken at the time when plasma glucose concentration was found to be below 0.6 g/L. Measurement of oral hypoglycaemic agents and anti-insulin antibodies must also be performed if Whipple's triad is demonstrated (Cryer et al. 2009). Post-prandial hypoglycaemia without fasting hypoglycaemia is now known to occur in some insulinoma patients (6% according to Placzkowski et al. 2009) and in patients with noninsulinoma pancreatogenous hypoglycaemia (Service et al. 1999), a syndrome which is part of nesidioblastosis (see below). Others have reported false negative tests in insulinoma patients who are correctly diagnosed after a meal test or oral glucose tolerance test (Sjoberg & Kidd 1992) (Izumiya et al. 2006; Kar et al. 2006). A mixed meal test was shown to be preferable to the oral glucose tolerance test (Hogan et al. 1983), so that only the meal test was recommended in the 2009 expert consensus.

The next requirement for the diagnosis of insulinoma is to provide evidence for inappropriate insulin secretion at the time of hypoglycaemia.

### **Criteria for inappropriate insulin secretion (hypoglycaemia related to endogenous hyperinsulinism)**

According to the expert consensus, the diagnosis of hypoglycaemia related to endogenous hyperinsulinism can be made when serum insulin concentration is equal to or greater than 3 mIU/L, serum C-peptide concentration is equal to or greater than 0.6 ng/mL (0.2 nmol/L), and serum proinsulin concentration is equal to or greater than 5 pmol/L at the time of hypoglycaemia, with venous plasma glucose concentrations less than 0.55 g/L. During a fast test, beta-hydroxy-butyrate levels of 2.7 mmol/L or less and an increase in plasma glucose of at least 0.25 g/L after intravenous glucagon indicate mediation of hypoglycaemia by insulin.

#### **Insulin**

Serum insulin concentrations can be artifactually modified by haemolysis, heterophilic antibodies, and endogenous anti-insulin antibodies. Red cells contain an insulin-degrading enzyme which may lead to underestimation of the actual insulin concentrations on haemolysed samples. Most insulin assays are calibrated against the standard IRP 66/304 preparation, and the new 83/500 standard is not yet used by all insulin assays. As a consequence of the differences between the standards and the methods employed, the conversion factor from mIU/L to pmol/L varies from 6.0 to 7.5. In the 2009 expert consensus, the threshold of 3 mIU/L is equal to 18 pmol/L.

Insulin concentrations had been measured for long with non-specific assays that yielded cross-reactions with proinsulin. The insulin assays that are now in use have negligible cross-reaction with proinsulin. Though the 3 mIU/L threshold (which replaces a 6 mIU/L threshold previously established with less specific insulin assays) was established for insulin-specific assays, it is known now that the finding of low insulin levels with such assays cannot rule out the diagnosis of insulinoma (Vezzosi et al. 2003). 11-35% patients with insulinoma were found to have insulin levels below the recommended diagnostic threshold (3 mIU/L) at the time of hypoglycemia when insulin was measured with insulin-specific assays, the percentage of values below 3 mIU/L depending on the assay employed (Vezzosi et al. 2007). On the other hand, while the specificity of the 3 mIU/L threshold was found to be 100% with a plasma glucose concentration threshold of 0.45 g/L for the diagnosis of hypoglycaemia related to endogenous hyperinsulinism, it was lowered to 87% for glucose levels ranging from 0.46 to 0.54 g/L (personal data). The sensitivity and specificity for serum insulin concentrations with a threshold level of at least 3 mIU/L at the time of plasma glucose concentrations below 0.6 g/L were found to be 93% and 95%, respectively (Placzkowski et al. 2009).

#### **C-peptide**

C-peptide and proinsulin levels at the time of hypoglycemia have better diagnostic accuracy than insulin levels. C-peptide assays are calibrated against the 1<sup>st</sup> standard IRP 84/510 (WHO). According to the expert consensus (Cryer et al. 2009), serum C-peptide levels of at least 0.6 ng/mL concomitant with plasma glucose concentrations below 0.55 g/L are one of the criteria that make the diagnosis of hypoglycaemia related to endogenous hyperinsulinism. In 33 patients who reached plasma glucose concentrations below 0.45 g/L during a fast test, all insulinoma patients had C-peptide levels above 0.6 ng/mL, and only one patient who had previously been treated by left-sided pancreatectomy for nesidioblastosis and who had recurrent hypoglycaemia did not reach the 0.6 ng/mL threshold at the time of hypoglycaemia below 0.45 g/L (Vezzosi et al. 2007). The diagnostic accuracy is improved when blood glucose levels drop below 0.45 g/L: for such blood glucose levels, the sensitivity

and specificity of C-peptide (with a threshold of 0.6 ng/mL) are close to 100%, whereas we observed a false positive rate of 9% for C-peptide for concomitant plasma glucose concentrations of 0.46-0.54 g/L (personal data). It had been previously shown that for the 0.50-0.60 g/L (O'Brien et al. 1993) or 0.45-0.60 g/L (Vezzosi et al. 2007) range for plasma glucose concentrations, there was a considerable overlap between patients and controls regarding C-peptide levels. In the study by Placzowski et al. the sensitivity of the 0.6 ng/mL threshold for the diagnosis of insulinoma is 100% if concomitant plasma glucose is below 0.6 g/L, while its specificity is 60% for concomitant glucose levels below 0.6 g/L and 78% for concomitant glucose levels of 0.5 g/L or less (Placzowski et al. 2009). This argues for prolonging the fast test until a glucose level of 0.45 g/L or less is reached.

### **Proinsulin**

A high proportion of proinsulin is known to be secreted by insulinomas. Intact proinsulin enzymatic processing leads to split 32,33 proinsulin or split 65,66 proinsulin, then to des-31,32-proinsulin (the molecule with the highest serum concentration) and des-64,65-proinsulin and finally to insulin and C-peptide. Proinsulin assays may measure either intact proinsulin (most of them measuring both intact proinsulin and des-64,65-proinsulin) or "total proinsulin" (i.e. intact proinsulin and the other molecules, mostly des-31,32-proinsulin). Therefore the results depend strongly on the method employed. In addition, proinsulin should be measured in serum samples, since results observed in plasma samples may be different (and higher).

According to the 2009 consensus (Cryer et al. 2009), proinsulin levels of at least 5 pmol/L concomitant with plasma glucose concentrations below 0.55 g/L are one of the criteria that make the diagnosis of hypoglycaemia related to endogenous hyperinsulinism. Whether this 5 pmol/L threshold refers to assays measuring "total proinsulin" or "intact proinsulin", or to a specific assay, has not been specified. We used the Human Proinsulin RIA kit (Linco Research), which measures "total proinsulin" (in fact intact proinsulin and des-31,32-proinsulin, the cross-reactions with des-64,65-proinsulin, insulin and C-peptide being < 0.1%), to measure proinsulin in 33 patients with hypoglycaemia related to endogenous hyperinsulinism and 67 controls who underwent a 72-hour fast test. 100% sensitivity and specificity were found using a 5 pmol/L threshold for serum proinsulin and a 0.45 g/L threshold for plasma glucose concentration (Vezzosi et al. 2007). The specificity would have been much lowered if the 5 pmol/L threshold had been applied to the 0.46-0.54 g/L range for plasma glucose concentrations: 9 out of 31 control subjects (29%) who reached this range of plasma glucose concentrations during the prolonged fast were found to have proinsulin levels above 5 pmol/L (personal data). For this threshold of 5 pmol/L, Placzowski et al reported a 100%-sensitivity for the diagnosis with plasma glucose concentrations below 0.6 g/L, whereas the specificity was only 68% and 78% with glucose levels below 0.6 g/L and below 0.5 g/L, respectively (Placzowski et al. 2009). This also argues for prolonging the fast test until a glucose level of 0.45 g/L or less is reached.

In addition, serum proinsulin levels above 22 pmol/L can make the diagnosis of hypoglycaemia related to endogenous hyperinsulinism when concomitant plasma glucose concentration is below 0.6 g/L with 74% sensitivity and 100% specificity. Moreover, in non-obese subjects (body mass index less than 30 kg/m<sup>2</sup>) the diagnostic specificity of a 22 pmol/L for serum proinsulin levels measured after an overnight fast was found to be 100%, while its sensitivity was 70%, with concomitant low or normal plasma glucose

concentrations (Vezzosi et al. 2007). This 22 pmol/L (0.2 ng/mL) threshold for proinsulin had been previously reported by Hirshberg et al. (Hirshberg et al. 2000). In obese or insulin-resistant subjects, proinsulin levels after an overnight fast exceeding 22 pmol/L do not make the diagnosis of insulinoma: in a group of 110 obese subjects who did not present with glycaemic disorders, 23 (21%) had proinsulin levels above 22 pmol/L after an overnight fast (Vezzosi et al. 2007). In conclusion, serum proinsulin levels after an overnight fast below 22 pmol/L do not rule out the diagnosis of insulinoma, while if they are above 22 pmol/L in a non-obese, non-insulin-resistant subject they should lead to a high suspicion of insulinoma, but to date, this can be used as a diagnostic criterion only if plasma glucose levels do not exceed 0.6 g/L.

There are some limitations regarding the insulin, C-peptide and proinsulin threshold levels for the diagnosis of hypoglycaemia related to endogenous hyperinsulinism.

The recommended diagnostic thresholds for serum insulin, C-peptide and proinsulin levels are known to be valid only in patients with normal liver and renal function, and there is no study regarding their validity in patients who have concurrent endocrine disorders liable to modify insulin resistance or sensitivity and glucose counter-regulation. In addition, there is no specific study regarding their validity for the diagnosis of recurrence of insulinoma after partial pancreatectomy.

In 33 patients with hypoglycaemia related to endogenous hyperinsulinism, the only patient who did not present with a C-peptide level of at least 0.6 ng/mL at the time of hypoglycaemia below 0.45 g/l during a fast test was a patient who had recurrence of nesidioblastosis one year after undergoing left-sided pancreatectomy; we speculated that a lower glucagon production could explain why severe hypoglycaemia was associated with lower insulin and C-peptide levels than in the other patients (Vezzosi et al. 2007). In addition, three of our patients with malignant insulinomas, in whom total remission had been initially achieved by partial pancreatectomy, did not fulfil the criteria for C-peptide levels when they were found to have recurrence of tumour-induced hypoglycaemia (personal data).

Insulin clearance takes place in the liver (50% of portal insulin is cleared by the liver) and in the kidney (50% of peripheral insulin removal) both by diffusion and receptor-mediated transport, the major route of clearance being receptor-mediated degradation by the renal tubular epithelial cells; the kidney also removes 70% of C-peptide by renal filtration. Therefore, measuring insulin and C-peptide levels in patients with severe renal insufficiency is not helpful; in such patients it has been recommended to use beta-hydroxy-butyrate levels and glucose response to glucagon at the end of a fast test to provide evidence for insulin-mediated hypoglycaemia (Basu et al. 2002).

### **Beta-hydroxy-butyrate**

Another valuable tool for the diagnosis of insulinomas is measurement of plasma beta-hydroxy-butyrate level during the fast test. Insulin is known to suppress ketogenesis. According to the 2009 consensus (Cryer et al. 2009), during a fast test, beta-hydroxy-butyrate levels of 2.7 mmol/L or less indicate mediation of hypoglycaemia by insulin.

A progressive rise in beta-hydroxy-butyrate levels during the fast test was found to rule out insulinomas (Service & O'Brien 2005). A plasma level of beta-hydroxy-butyrate exceeding 2.7 mmol/L was found to rule out the diagnosis of insulinoma when blood glucose levels were 0.50-0.60 g/L during a 72-hour fast test with both sensitivity and specificity close to

100% (O'Brien et al. 1993). There is no study specifying the validity of this criterion for another range of plasma glucose concentrations. Placzowski et al. reported 100% sensitivity and specificity for the diagnosis of insulinoma with beta-hydroxy-butyrate levels of 2.7 mmol/L or less during a fast test with concomitant plasma glucose concentrations below 0.6 g/L or below 0.5 g/L (Placzowski et al. 2009), but the results are not detailed. There are two case reports of insulinoma patients with false negative results of beta-hydroxy-butyrate levels: an 80-year-old female patient with plasma glucose concentrations of 0.48 g/L (2.7 mmol/L), serum C-peptide of 0.93 ng/mL (0.31 nmol/L) and beta-hydroxy-butyrate levels of 3.0 mmol/L after 72 hours of fast (Wiesli et al. 2004b) and a 49-year-old man with plasma glucose concentrations of 0.32 g/L, serum C-peptide levels of 1.2 ng/mL, and beta-hydroxy-butyrate levels of 3.2 mmol/L (Soh & Kek 2010). Among our patients (Vezzosi et al. 2007), one patient with a malignant insulinoma was found to have, during a fast test, plasma glucose concentrations of 0.36 g/L, serum C-peptide levels of 1.7 ng/mL, plasma proinsulin levels of 37 pmol/L (which led to the unequivocal diagnosis of inappropriate insulin secretion) and plasma beta-hydroxy-butyrate levels of 3.8 mmol/L. We hypothesized that severe hypoglycaemia below 0.45 g/L could result in a greater secretion of counter-regulatory hormones thereby leading to an increase in beta-hydroxy-butyrate levels; lipolysis may escape the inhibitory effect of insulin during hyperinsulinic hypoglycaemia (Lucidi et al. 2010). However we observed a patient with a malignant insulinoma whose lowest plasma glucose concentration during a 72 hour-fast test was 0.53 g/L after 58 hours of fast, with concomitant serum levels of insulin, C-peptide and proinsulin of 13 mIU/L, 2.4 ng/mL, and 36 pmol/L (which gave diagnostic evidence), respectively, and plasma beta-hydroxy-butyrate levels above 2.7 mmol/L. At the end of the 72 hour-fast test, beta-hydroxy-butyrate levels reached the value of 7.0 mmol/L in this patient, with concomitant concentrations of 0.68 g/L for plasma glucose, 4.0 mIU/L for serum insulin, 1.2 ng/mL for serum C-peptide and 25 pmol/L for serum proinsulin (personal data).

In conclusion, though being very valuable, the threshold of 2.7 mmol/L or less for beta-hydroxy-butyrate levels does not yield 100% sensitivity and specificity. Based on the observed results there are several caveats: 1) beta-hydroxy-butyrate levels may lose part of their diagnostic accuracy when plasma glucose concentrations are below 0.45 g/L and are of minor interest for this range of glucose levels; 2) proinsulin levels must be taken into account before ruling out the diagnosis of insulinoma in patients whose plasma glucose concentrations are within the 0.45-0.60 g/L range even when plasma beta-hydroxy-butyrate levels reach values above 2.7 mmol/L; 3) patients with recurrent insulinoma or nesidioblastosis after partial pancreatectomy may not fulfil the usual diagnostic criteria for insulin-mediated hypoglycaemia during the fast test. Whether a disruption in the progressive elevation of beta-hydroxy-butyrate levels concomitant with lower glucose levels during the fast test (with an insulin secretory burst) should lead to suspicion of insulinoma remains to be determined.

### Glucagon test

According to the 2009 expert consensus (Cryer et al. 2009) an increase in plasma glucose of at least 25 mg/dL (0.25 g/L, 1.4 mmol/L) after intravenous glucagon indicate mediation of hypoglycaemia by insulin. This refers to the evaluation protocol established by the Mayo Clinic group (O'Brien et al. 1993) : the glucagon test is performed at the end of a prolonged fast test, i.e. either at the time of occurrence of symptomatic hypoglycaemia with concomitant



plasma glucose concentrations of 0.45 g/L (2.5 mmol/L) or less, or after 72 hours of fast: plasma glucose responses to the intravenous injection of 1 mg glucagon are measured at 10 min-intervals for 30 min. Under these conditions, plasma glucose response to glucagon was found to be higher in insulinoma patients than in controls with a range of maximal increase in plasma glucose concentrations of 1.4-5.4 mmol/L in insulinoma patients and of 0.1-1.3 mmol/L in controls, as a consequence of a lesser depletion of hepatic glycogen in insulinoma patients during the fast, which is related to the higher insulin secretion in the patients. This allowed a clear distinction between patients and controls who has reached plasma glucose concentrations below 3.3 mmol/L (0.6 g/L) during the fast test (O'Brien et al. 1993).

On the other hand, the glucagon test has long been known to be a provocative test in insulinoma patients: glucagon may trigger severe hypoglycaemia related to insulin secretion in insulinoma patients. This provocative test was found to be helpful for the diagnosis of insulinoma in patients with a negative fast test (Wiesli et al. 2004b; Soh & Kek 2010).

### **Other diagnostic criteria for hypoglycaemia related to endogenous hyperinsulinism**

\* Regarding the various tests employed in the evaluation of a patient with suspected hypoglycaemic disorder, only the fast test, with a glucagon test at its termination, in patients with symptoms of fasting hypoglycaemia, and the mixed meal test in patients with postprandial hypoglycaemia are recommended by the 2009 expert consensus (Cryer et al. 2009).

# Physical exercise is known to result in hypoglycaemia in many insulinoma patients. A physical exercise was found to be necessary in some insulinoma patients to make the correct diagnosis (Jarhult et al. 1981). The percentage of patients with negative fast test and meal test who could be diagnosed to have insulinoma by a physical exercise test remains to be established. In selected patients a "physical exercise test" under medical supervision, with at least serial measurements of plasma glucose and serum insulin, C-peptide and proinsulin could be considered.

# Cohn's diet (1200 kcal with 50 g of carbohydrates followed by a 36 hour-fast) is no more in use and has been replaced by the 72 hour-fast test.

# The oral glucose tolerance test was found to be of interest in patients with a negative fast test (Wiesli et al. 2004b) (Kar et al. 2006), but it was shown to be less accurate than the mixed meal test (Hogan et al. 1983) and is not recommended by the 2009 expert consensus (Cryer et al. 2009). 10% of healthy persons were reported to have a plasma glucose nadir less than 0.5 g/L during this test while being asymptomatic and without presenting with Whipple's triad (Lev-Ran & Anderson 1981). Whether such subjects may have some subtle abnormality regarding glucose regulation may still be debated, but this test was not considered as a tool to provide diagnostic evidence for a hypoglycaemic disorder.

# The C-peptide suppression test by insulin-induced hypoglycaemia may give results that do not clearly distinguish all patients from controls, and its interpretation may be difficult, since the results depend on several parameters including gender and body mass index (Service et al. 1992). A more sophisticated C-peptide suppression test, combined with the euglycaemic clamp, was successfully employed for the diagnosis of insulinoma, but it cannot be recommended for clinical practice (Gin et al. 1987).

\* Regarding the diagnostic criteria for inappropriate insulin secretion, previously employed ratios (insulin/glucose ratio, Turner's index) are no more in use and do not bring greater diagnostic accuracy than the presently-used criteria (Vezzosi et al. 2007) (Service 1995). Though insulinoma secrete a high proportion of proinsulin, the proinsulin/insulin ratio

does not bring more valuable information than proinsulin levels for the diagnosis of insulinoma (Vezzosi et al. 2007).

## **2.2 Differential diagnosis**

### **2.2.1 Evaluation of the patient**

In an adult patient clinically otherwise healthy who presents with severe hypoglycemia, the diagnosis of insulinoma must be considered as very likely, provided that the patient does not use insulin or other hypoglycaemic agents.

Apart from “reactive functional hypoglycaemia” (see below), which is a controversial and heterogeneous medical entity without actual hypoglycaemia below 0.55 g/L, other actual causes of hypoglycaemia are often observed in patients presenting with other clinical symptoms related to their disease: alcohol ingestion, critically ill patients or septicemia, severe prolonged undernutrition, liver or renal deficiency, adrenal insufficiency, hypopituitarism, IGF-II or pro-IGF-II secreting tumours, or very rare patients with other tumour secretion (IGF-I, GLP1 and somatostatin).

Thus the first step is to record carefully the history of the patient including exposure to any medication, and the possible access to hypoglycaemic agents, and to perform a clinical physical examination.

Then the causes of hypoglycaemia are identified mainly on the basis of insulin, C-peptide and proinsulin levels at the time of hypoglycaemia. Additional biological evaluation comprises measurement of oral hypoglycaemic agents (at the time of hypoglycaemia) and anti-insulin antibodies (at any time) in plasma samples in patients who are found to have insulin-mediated hypoglycaemia. Other plasma measurements will be made if there is clinical presumption of a specific disease, for instance diseases known to lead to hypoglycaemia mediated by IGF-II or incompletely processed IGF-II (“big IGF-II”), exceptionally IGF-I. Finally, according to the 2009 expert consensus (Cryer et al. 2009), “a test of adrenocortical function is reasonable”.

### **2.2.2 Reactive “functional hypoglycaemia”**

Reactive “functional hypoglycaemia” is a controversial entity, and its existence is still debated. The first definition of “reactive functional hypoglycaemia” had been based on the belief that hypoglycaemic disorders could be divided into disorders responsible for fasting hypoglycaemia (mainly insulinoma) and disorders responsible for reactive hypoglycaemia (mainly “functional hypoglycaemia”). It is now known that many organic disorders may result both in fasting and/or reactive hypoglycaemia. However, many patients present with dizziness or minor autonomic symptoms within the hours following a meal, and most of them do not meet the criteria for actual hypoglycaemia, i.e. they do not have actually low plasma glucose levels below 0.55 g/L, and do not necessarily fulfil Whipple’s triad. The so-called “reactive functional hypoglycaemia” may be the only likely diagnosis for some of these patients, but glucose levels do not drop below 0.55 g/L, and it cannot be responsible for symptoms of neuroglycopenia. It could reflect various mechanisms: 1) rapid changes in plasma glucose concentrations after ingestion of nutrients with a high glycaemic index; 2) a plasma glucose threshold for autonomic symptoms higher than that of other subjects 3) various reactions after ingestion of nutrients, including mechanisms differing from changes in plasma glucose levels. The patients are asked to reduce their intake of simple carbohydrates while increasing that of complex carbohydrates, and to divide their daily

nutrient intake into three meals and two or three snacks. Repeated symptoms suggesting hypoglycaemia despite correct nutrition warrant further investigation.

Actual reactive insulin-mediated hypoglycaemia is observed in patients with dumping syndrome (see below, causes of hypoglycaemia differing from insulinomas), and this must be distinguished from “reactive functional hypoglycaemia”.

### **2.2.3 Causes of hypoglycaemia differing from insulinomas**

#### **Medications known to be used by the patient**

The first step of any evaluation is to check whether some of the medications ordered to the patient can cause hypoglycaemia. Insulin, insulin analogues or insulin secretagogues (sulfonylureas, glinides) are the most common causes of hypoglycaemia. Some patients may be mistaken and accidentally take inappropriate doses of medications, or undergo concurrent disorders that increase the bioavailability of the drug. Though metformin cannot induce hypoglycaemia, as confirmed by the expert consensus (Cryer et al. 2009), it has been reported to cause severe hypoglycaemia in the 1995 UK prospective diabetes study (Cryer et al. 2009) and in an elderly patient (Zitzmann et al. 2002). Thiazolidine-diones, alpha-glucosidase inhibitors, GLP1-receptor agonists and dipeptidyl peptidase IV inhibitors do not induce hypoglycaemia themselves but increase the risk of hypoglycaemia when combined with an insulin secretagogue or insulin. Other medications are known to be liable to induce hypoglycaemia, mostly in aged patients and patients with associated diseases, or combination of treatments, or renal failure. Miscellaneous mechanisms may be involved: increased insulin release, reduced insulin clearance, interference with glucose metabolism, liver or renal toxicity (Ben Salem et al. 2011).

A list of the most commonly employed medications liable to induce hypoglycaemia has been given in the 2009 consensus (Cryer et al. 2009): some of them are associated with a risk of hypoglycaemia with moderate quality of evidence: cibenzoline, gatifloxacin, pentamidine, quinine, indomethacin. Other medications are involved with a lesser quality of evidence. The most commonly reported offending drugs appeared to be quinolones, pentamidine, quinine, beta blockers, angiotensin-converting enzyme agents, and IGF (Murad et al. 2009). Salicylates in large doses (4-6 g/day) may produce rarely hypoglycaemia in adults, and more often in children, by reducing basal glucose production and possibly increasing sensitivity to insulin (Cryer 2008). Disopyramide (Marks & Teale 1999), non-steroid anti-inflammatory drugs, analgesics, antidepressants (Ben Salem et al. 2011) and antifungal agents (Lionakis et al. 2008) have been reported to induce hypoglycaemia. However hypoglycaemia appears to be rare with such medications, unless employed in combination with other medications or in patients with underlying diseases. Recently tyrosine kinase inhibitors were found to decrease plasma glucose concentrations (Agostino et al. 2010). Patients with huge non functional endocrine tumours and multiple liver metastases may be at risk of developing hypoglycaemia when treated by long-acting somatostatin analogues, as a result of reduced glucagon and GH secretion, impairment of hepatic glucose output and delay in intestinal absorption of carbohydrates (Unek et al. 2009).

#### **Patient with known or obvious acute or chronic disorder**

In such patients the diagnosis is often clinically obvious. Concurrent intake of hypoglycaemic agents must always be screened for, and in patients without any treatment

with insulin secretagogues, measurement of insulin, C-peptide, proinsulin and (whenever possible) beta-hydroxy-butyrate levels at the time of hypoglycaemia below 0.55 g/L will generally rule out insulin-mediated hypoglycaemia, unless the underlying disorder (especially in renal failure and acute liver failure) is liable to alter insulin, C-peptide and proinsulin metabolic processing and clearance.

### **Alcohol**

Alcohol ingestion is known to cause hypoglycaemia. Serum insulin and C-peptide are appropriately low during episodes of alcohol-induced hypoglycaemia (Marks & Teale 1999). Ethanol inhibits gluconeogenesis (Cryer 2008) and hormonal counterregulation to hypoglycaemia (Marks & Teale 1999; Cryer 2008), and can exert influences on pancreatic microcirculation, resulting in a massive redistribution of blood flow from the exocrine into the endocrine part of the pancreas, augmenting late-phase insulin secretion (Huang & Sjöholm 2008). In patients presenting in coma from alcohol-induced hypoglycaemia, recovery after glucose injection is immediate, with no relapse if there is no concurrent cause of hypoglycaemia; recovery is delayed when brain swelling has occurred, which requires specific treatment. Glucagon is not recommended (Marks & Teale 1999). Alcohol-induced hypoglycaemia typically occurs after a binge of alcohol consumption within 6 to 36 hours generally in a patient who presents glycogen depletion, and ethanol is measurable in blood but poorly correlates with glucose levels; it may also be a late feature of alcoholic keto-acidosis and ethanol may be undetectable in blood samples (Marks & Teale 1999).

### **Acute hepatic failure**

Acute hepatic failure, with massive and rapid liver destruction, rather than common forms of cirrhosis and hepatitis, can lead to severe hypoglycaemia. Massive destruction of the liver tissue and defective glucose storage in extrahepatic organs are thought to be the main mechanisms, but non-insulin hypoglycaemic factors secreted by the damaged liver might be involved, since reversal of fulminant hepatitis-associated hypoglycaemia was observed at the anhepatic stage of liver transplantation (Ilan et al. 1996).

### **Renal failure**

Hypoglycaemia is not rare in renal failure and its pathogenesis involves generally several mechanisms (Arem 1989; Basu et al. 2002). Mechanisms directly related to renal failure are involved: reduced renal gluconeogenesis, impaired renal insulin degradation and clearance. Additional mechanisms may be involved, such as medications, sepsis, poor nutrition with deficiency of precursors of gluconeogenesis, impaired glycogenolysis, defective counterregulation with frequent autonomic dysfunction, concurrent disorders such as concomitant liver disease, congestive heart failure, or an associated endocrine deficiency. In patients undergoing haemodialysis a particular mechanism is postdialysis glucose-induced hyperinsulinemia, caused by a high glucose content in the dialysate (Arem 1989; Basu et al. 2002). On the other hand dialysis with glucose-free solution results in frequent hypoglycaemia, whereas using glucose-added dialysis solution at 90 mg/dL was found to reduce the number and severity of hypoglycaemic episodes (Burmeister et al. 2007). Since clearance of insulin, C-peptide and proinsulin is impaired in renal failure, beta-hydroxy-butyrate during a fast test and glucose response to glucagon were reported to be the best biological parameters for the differential diagnosis of insulinoma in patients with renal failure (Basu et al. 2002).

**Heart failure**

Hypoglycaemia has been occasionally reported in patients with congestive heart failure. Its pathogenesis is unknown, probably multifactorial, and may involve inhibited gluconeogenesis (Cryer 2008).

**Inanition**

Inanition related hypoglycaemia, with low insulin, C-peptide and proinsulin levels has been found in some patients with very severe malnutrition, such as patients with very severe chronic anorexia nervosa (Rich et al. 1990; Yanai et al. 2008). The occurrence of inanition-related severe hypoglycaemia in a patient with anorexia nervosa is thought to imply a grave prognosis (Rich et al. 1990).

**Adrenal or pituitary failure**

Adrenal and/or pituitary failure does not result in plasma glucose concentrations below 0.55 g/L unless associated with other disorders or hypoglycaemic medications. Plasma glucose concentrations of 0.6 g/L may be found in such patients, and are often associated with hyponatremia and/or typical clinical symptoms of the disease. As stated in the 2009 expert consensus (Cryer et al. 2009), adrenocortical failure without associated conditions is unlikely to induce hypoglycaemia below 0.55 g/L, and a low cortisol level at the time of hypoglycaemia may be the consequence of a lower glycaemic threshold to stimulate cortisol in patients with recurrent hypoglycaemia without adrenal failure.

**Other causes****Low insulin, C-peptide and proinsulin levels**

\* Hypoglycaemia related to non-insulin secreting tumours : IGF-II and precursors (“big IGF-IIs”)

If insulin, C-peptide and proinsulin levels are low at the time of hypoglycemia, the diagnosis of non pancreatic IGF-II (or pro-IGF-II) –secreting tumour must be considered. However, one must bear in mind that rare insulin-specific assays may not be able to identify insulin analogues (see below, “factitious hypoglycaemia”). Patients with IGF-II secreting tumours have low IGF-I levels and increased IGF-II/IGF-I ratios, which are pathognomonic of the diagnosis. IGF-II precursors (“big IGF-IIs”) do not complex normally with IGF-BPs, thus can reach more readily target tissues, which is thought to be the mechanism of hypoglycaemia in most patients. Enhanced mRNA expression of IGF-II and defective expression of prohormone convertase 4, a potential protease responsible for IGF-II precursor processing, were found in a patient with pleural solitary fibrous tumour (Tani et al. 2008). Glucose consumption is also thought to be a mechanism of hypoglycaemia in such tumours, and could be the major mechanism in some patients without evidence for IGF-II secretion, in whom PET-scan with 18-Fluoro-deoxy-glucose showed preferential tumour glucose uptake (Habra et al. 2010). Most IGF-II secreting tumours are large tumours that can be found on clinical examination or chest X-ray. IGF-II related hypoglycaemia is well known in patients with pleural solitary fibrous tumour (Doege-Potter syndrome). Solitary fibrous tumour is a rare mesenchymal neoplasm composed of CD34-positive fibroblastic cells; it can be either pleural or extrapleural, with abdominal locations, of whom one was found to grow in the bladder (Bruzzzone et al. 2010). IGF-II secreting mesenchymal neoplasms are numerous, and also comprise synovial sarcomas, myxoid liposarcomas, GISTs, malignant peripheral nerve sheath tumours, chondrosarcomas, undifferentiated pleomorphic sarcomas, Ewing’s

sarcomas and tenosynovial giant cell tumours (Steigen et al. 2009). We observed hypoglycaemia with suppressed insulin, C-peptide and proinsulin levels and high pro-IGF-II levels in a patient with a malignant meningioma and lung and pleural metastases (unpublished observation). Rare cases of adrenocortical carcinomas have also been reported to be responsible for hypoglycaemia (Cryer 2008). One exceptional patient with an islet-cell tumour of the pancreas was reported to have IGF-II-related hypoglycaemia (Chung et al. 2008).

\* Other tumours (non-insulin, non-IGF-II secreting tumours)

Exceptionally, tumour-induced hypoglycaemia was found to be related to hormone secretions that differed from insulin or IGF-II: IGF-I, in a metastasizing undifferentiated large-cell carcinoma of the lung (Nauck et al. 2007), somatostatin in an ovarian teratoma (Gregersen et al. 2002). The latter patient had alternate episodes of hyper- and hypoglycaemia, with only reactive post-prandial hypoglycaemia and a negative fast test; insulin levels were low at the time of hyperglycaemia, and were not reported at the time of hypoglycaemia. It was hypothesized that somatostatin led to suppression of glucagon secretion to a greater extent than that of insulin secretion in the post-prandial period, thereby leading to reactive hypoglycaemia. Another ovarian tumour was reported to induce hypoglycaemia by secreting GLP-1 and somatostatin (see below).

**High insulin levels with low C-peptide and proinsulin levels**

\* Factitious hypoglycaemia related to insulin and insulin analogues

In clinical practice, the finding of high insulin levels with concomitant low C-peptide and proinsulin levels is almost specific of the diagnosis of surreptitious insulin injections (Grunberger et al. 1988). It must be known that some insulin-specific assays may not be able to identify insulin analogues (Sapin 2003; Neal & Han 2008); insulin assays that can identify the presence of insulin analogues are to be preferred.

\* Anti-insulin receptor antibodies

Such antibodies were found mostly in patients with immunity disorders, either before or after the use of medications like methimazole (Redmon & Nuttall 1999). Many of these patients have type B insulin resistance and diabetes mellitus, but some of them develop hypoglycaemia. Anti-insulin receptor antibodies bind to the insulin receptor, may mimic insulin action and cause fasting hypoglycaemia; also, they may inhibit insulin binding thereby inhibiting insulin clearance and increasing insulin levels, while the resulting hypoglycaemia suppresses beta-cell secretion and C-peptide levels (Taylor et al. 1989). Spontaneous remissions and improvement after glucocorticoid treatment have been reported (Taylor et al. 1989).

\* Insulin receptor mutation

Exceptionally patients may present an insulin receptor mutation, which is transmitted as an autosomal dominant trait, and delays insulin clearance, resulting in hypoglycaemia with inappropriate insulin levels after an overnight fast, and low C-peptide levels, with increased insulin/C-peptide ratio (Hojlund et al. 2004), or reactive insulin-mediated hypoglycaemia; in such patients the fast test is positive for hypoglycaemia ( $< 0.4$  g/L), but insulin, C-peptide and proinsulin are suppressed during a prolonged fast. This disease can be considered to be one of the established genetic causes of nesidioblastosis (see below).

### High insulin, C-peptide and proinsulin levels

If insulin, C-peptide and proinsulin levels are elevated at the time of hypoglycemia, insulinoma is the most likely diagnosis. One must remind that some insulinoma patients do not have elevated insulin levels when insulin is measured with insulin-specific assays (Vezzosi et al. 2003). When an insulinoma is not found by imaging techniques, two very different diagnoses must be suspected: factitious hypoglycemia related to sulfonylurea or glinide, or nesidioblastosis, which is rare in adult patients. Exceptionally, anti-insulin antibodies and extra-pancreatic non-islet cell insulin-secreting tumours may also be found; sometimes a genetic metabolic disorder which has been overlooked at a younger age may be diagnosed in an adult patient. Finally, a particular condition is reactive insulin-mediated hypoglycaemia in patients with dumping syndrome; some of these patients were reported to have post-gastric bypass nesidioblastosis.

#### \* Factitious hypoglycaemia related to sulfonylureas or glinides

A plasma sample must be collected to measure oral hypoglycaemic agents (ideally all available sulfonylureas and glinides) with specific methods at the time of symptomatic hypoglycaemia. This is the only way to distinguish factitious (or accidental) hypoglycaemia related to insulin secretagogues from the endogenous abnormal insulin secretion observed in patients with insulinomas and nesidioblastosis. The diagnosis is generally suspected in patients whose relatives are diabetic or health professionals, but it may be very difficult (Hirshberg et al. 2001).

#### \* Anti-insulin antibodies

Anti-insulin antibodies responsible for hyperinsulinaemic hypoglycaemia have been rarely found (Taylor et al. 1989; Redmon & Nuttall 1999) generally in patients with autoimmune disorders who developed such antibodies spontaneously or a few weeks after specific treatments for autoimmune diseases, especially medications with a sulphhydryl group, or in very rare cases of myeloma (Halsall et al. 2007). Anti-insulin antibodies must be measured in patients with hypoglycaemia related to endogenous hyperinsulinism, not necessarily during hypoglycaemia (Cryer et al. 2009). Insulin levels are often very high, partly as a consequence of an interference in the insulin assay by the anti-insulin antibody. Most patients have reactive hypoglycaemia, which could be a consequence of meal-induced exaggerated insulin secretion, overcoming the buffering effect of the antibody (Taylor et al. 1989); however some patients also have fasting hypoglycaemia (Basu et al. 2005). The natural course is not predictable, some patients may be controlled by frequent meals and many experienced remission some months after the withdrawal of the drug responsible for the onset of the disorder, but others required glucocorticoids or even plasmapheresis.

#### \* Non islet cell insulin-secreting tumours and GLP-1 secreting tumour

Non islet cell insulin-secreting tumours are quite exceptional. They must be distinguished from "ectopic insulinomas" which are extrapancreatic islet-cell tumours located in the proximity of the pancreas. Insulin-mediated hypoglycaemia has been reported in the following tumours : a small cell carcinoma of the cervix (Seckl et al. 1999), a neuroectodermal brain tumour (Nakamura et al. 2001), and a neuroendocrine carcinoma of the gallbladder (Ahn et al. 2007).

Todd et al reported a GLP-1 and somatostatin-secreting ovarian tumour (Todd et al. 2003). The patient had alternate episodes of hyper- and hypoglycaemia, with a negative fast test.

Insulin-mediated hypoglycaemia was thought to be a consequence of GLP-1 and somatostatin secretion; since the effects of GLP-1 on insulin secretion are dependent on glucose levels, the inhibitory effects of somatostatin on glucagon secretion may have played a role in inducing reactive hypoglycaemia.

#### \* Genetic metabolic disorders

Some patients with hypoglycaemia related to metabolic genetic disorders may occasionally be diagnosed to have such disorders when they are adults. Some of these disorders can be considered as possible causes of nesidioblastosis (Christesen et al. 2008; Palladino & Stanley 2011) (Flanagan et al. 2011b) (Flanagan et al. 2011a) (see last section of this chapter): 1) physical exercise-induced hypoglycaemia caused by failed silencing of monocarboxylate transporter 1 in pancreatic beta cells; 2) patients with mostly post-prandial (but also fasting) symptoms of hypoglycaemia in mutations of insulin receptor (see above), activating mutation of glucokinase with some patients diagnosed only as adults (Christesen et al. 2008), and hyperinsulinism-hyperammonemia syndrome due to activating mutations of glutamate dehydrogenase; 3) patients with mostly fasting (and also post-prandial) hypoglycaemia due to mutations in the genes of the subunits of K-ATP channel (ABCC8, KCNJ11) and mutations of 3 hydroxy-acyl-CoA dehydrogenase (HADH) gene. Among 79 patients admitted in our department between 1990 and 2010 for organic causes of hypoglycaemia (69 of them with insulinomas) only one had hyperinsulinism-hyperammonemia syndrome (diagnosed at the age of 30), with an activating mutation of glutamate dehydrogenase, and another one had two heterozygous mutations of the ABCC8 gene (diagnosed at the age of 47). Both patients had been treated since early childhood for epilepsy.

Other patients may present with other metabolic disorders such as disorders of the glycogen metabolic pathway. Some patients with almost asymptomatic forms of glycogen storage disease type Ia may develop hepatocellular adenomas and carcinomas as adults (Cassiman et al. 2010).

A detailed review of these genetic disorders in childhood has been made by Palladino (Palladino et al. 2008).

#### \* Reactive hypoglycaemia in patients with dumping syndrome

Postprandial hypoglycaemia caused by rapid emptying of the gastric remnant has long been known to be a possible complication of gastric resection; it is associated with various symptoms, including symptoms unrelated to hypoglycaemia, which are termed dumping syndrome. Many, but not all patients with post-gastric bypass were found to have reactive severe insulin-mediated hypoglycaemia a few months or years after bypass surgery. Continuous glucose monitoring can be a useful tool to identify hypoglycaemia in patients after bariatric surgery (Hanaire et al. 2010). The abnormal insulin responses to intraarterial calcium injection and the histopathological findings in 6 patients, compared to pancreata from patients with pancreatic cancer, led to the conclusion that such patients might have gastric bypass-induced nesidioblastosis (Service & O'Brien 2005) (see below). This conclusion was challenged, since Meier JJ et al. did not confirm the histopathological diagnosis of nesidioblastosis in the 6 patients, when compared to pancreata collected at autopsy; they only observed a greater beta-cell nuclear diameter than in controls, and this nuclear diameter was positively correlated with BMI, if the BMI used for the patients in the correlation was the BMI observed prior to bypass surgery (Meier et al. 2006). Goldfine AB et al reported increased GLP-1 levels after a liquid mixed-meal in patients who had post-gastric bypass surgery hypoglycaemia, in comparison with those who did not have



hypoglycaemia (Goldfine et al. 2007). GLP-1 is thought to be unable to induce hypoglycaemia in patients with normal beta cells, but high GLP-1 levels might override glucose-dependency and cause hypoglycaemia (Toft-Nielsen et al. 1998). The degree of post-prandial GLP-1-induced glucagon suppression (which is also dependent on glucose, GLP-2 and GIP levels) might play a role in the resulting post-prandial glucose levels. Insulin-mediated hypoglycaemia after gastric bypass surgery is not associated with overexpression of GLP-1 receptor in islets (Reubi et al. 2010). McLaughlin et al reported immediate reversal of reactive hypoglycaemia by feeding a patient with Roux-en-Y gastric bypass through a gastrostomy tube, which argued against a structural pancreatic abnormality in this patient and provided major evidence for a functional dysregulation of post-prandial insulin secretion in patients with gastric bypass-induced hypoglycaemia (McLaughlin et al. 2010). Nevertheless such patients often require insulin-suppressing treatments (diazoxide, calcium channel blockers, somatostatin agonists) (Tack et al. 2009) (Vella & Service 2007). Acarbose, which delays absorption of simple carbohydrates and may reduce GLP-1 secretion, was successfully employed (in combination with dietary advices) (Tack et al. 2009; Hanaire et al. 2010) but it was found relatively ineffective by others (Vella & Service 2007). A patient who had dumping syndrome after partial gastric resection was finally admitted in our department, 20 years after gastric surgery, for severe reactive insulin-mediated hypoglycaemia, with a negative fast test, and normal pancreas on CT-scan; hypoglycaemia was controlled by diazoxide, not by octreotide. Partial (distal) pancreatectomy has been performed to control hypoglycaemia in some of these patients, as in patients with nesidioblastosis. Since insulinomas may present with GIP and/or GLP-1 receptors, occurrence of insulin-mediated hypoglycaemia after bypass surgery or gastrectomy can be caused by an insulinoma. Radiological evaluation is of course mandatory in such patients.

### 2.3 Topographic assessment

The topographic assessment of an insulinoma must be performed only after the biological diagnosis of hypoglycemia related to inappropriate insulin secretion has been confirmed. Insulinomas are generally thought to be difficult to localize due to their small size. Intra-operative palpation and ultrasound examination of the pancreas had been thought to be the best methods to detect insulinomas. Using such methods intra-operatively remains mandatory, but to date careful radiological examination must be performed to localize insulinomas before surgery in order to avoid failure of surgery to cure the disease and re-operations. The imaging techniques now in use have greatly improved, so that most insulinomas are detected preoperatively.

High definition multidetector helical CT-scans have become widely available since the early 2000s, so that CT-scan is now again the first examination that must be performed to localize insulinomas. Multidetector CT-scan allows optimization of the scan protocols (with very rapid scan times reducing movement artefacts), and arterial and portal venous phase images on thin-sections. A meticulous technique is mandatory and detailed recommendations have been made regarding the examination procedure (Rockall & Reznick 2007) (Sundin et al. 2009). Most insulinomas are small, isodense with the pancreas on pre-contrast images, then hypervascular on arterial phase images, but sometimes are more easily detected on portal venous phase images. More rarely, the tumours are hyperdense to the pancreas, or have nodular calcification, or appear hypovascular, cystic or hypodense after injection of contrast medium. Exceptionally, ectopic insulinomas are located in the proximity of the pancreas or

of the liver. While the sensitivity of non helical CT had been 29%, with multidetector helical CT-scan a meticulous technique can result in 94 % sensitivity (Rockall & Reznick 2007). CT-scan can show metastases (see below, malignant insulinomas)

MRI is often complementary to CT, in order to confirm a suspected lesion on CT, or to search for a tumour that CT-scan has not been able to localize; combining both methods (CT and MRI) improves the accuracy for detection of insulinomas. Detailed technical information has been provided (Rockall & Reznick 2007). Temporal resolution and spatial resolution are greater with CT than with MRI, but MRI allows high resolution contrast-enhanced imaging. Pancreatic endocrine tumours generally appear hypo-intense on T1-weighted sequences and hyperintense on T2-weighted sequences. On pre-contrast imaging, fat suppressed T1 and fat saturation T2 may improve visualization of the tumours, which appear of low intensity on T1 and have a bright T2 signal. There is a marked homogeneous enhancement after injection of gadolinium, which may in some cases render the low-intensity tumour isointense and less detectable (Rockall & Reznick 2007). The sensitivity of MRI for detection of pancreatic endocrine tumours was first reported to be less than that of CT, but is now similar to that of CT: a sensitivity of up to 94% has now been reported (Thoeni et al. 2000). MRI has also proven very helpful in the diagnosis of hepatic metastases of malignant insulinomas (see below, malignant insulinomas).

Trans-abdominal ultrasound examination is traditionally thought to be of low sensitivity to detect pancreatic endocrine tumours like insulinomas: the sensitivity was as low as 20 % in the series reported in the 1990s, with a maximum of 66-80% (Grant 1999). Insulinomas generally appear as small hypoechoic solid masses. The limitations of ultrasound examination are related to body habitus and tumour size; also, the presence of bowel obscures the left upper quadrant, and insulinomas located in the body and the tail of the pancreas may be difficult to visualize. Water intake by the patient allows to use the stomach as an acoustic window and may improve the detection. A sensitivity of up to 79.3% has been reported with recent material for transabdominal ultrasound (Oshikawa et al. 2002) (Rockall & Reznick 2007).

More recently, contrast-enhanced ultrasonography has been reported to detect insulinomas in a high number of patients. Very promising results were found by An L et al (An et al. 2010), who reported the results obtained with a second-generation sonographic contrast agent (SonoVue) in 31 patients with hypoglycaemia related to endogenous hyperinsulinism, among whom 27 had a solitary insulinoma, 3 had multiple insulinomas, and 1 had no definite tumour during laparoscopic evaluation. The results of contrast-enhanced ultrasonography were in agreement with surgical findings except in three patients: one solitary tumour had been localized in the head of the pancreas and was found in the duodenum ligament; one tumour had been visualized whereas no tumour was found during surgery; in the patient with 6 insulinomas, only 2 tumours had been visualized. Thus the diagnostic sensitivity and localization specificity were 33 (89.2%) and 32 (86.5%) of 37 surgically verified insulinomas, respectively. The enhancement pattern of insulinoma was fast wash-in and slow wash-out. The insulinomas displayed homogeneous hypervascularity in the earlier arterial phase and had still a hyperenhancing pattern in the late phase. This ultrasound non invasive examination could probably be developed in the next years.

Endoscopic ultrasound has been reported to be the reference method to localize insulinomas in the 1990s: it allows to use a high frequency US probe in close proximity to the pancreas, and in expert hands its sensitivity has been shown to be 79-100% (Rosch et al. 1992) (McLean & Fairclough 2005). It may show very small tumours (about 3 mm in size) in the head of the

pancreas, such tumours being impalpable at surgery. A lower sensitivity for pancreatic tail lesions (37-50%) has been reported (Ardengh et al. 2000). Pancreatic nodularities may be mistaken for insulinomas (Kann et al. 2003). Endoscopic ultrasound is an invasive method, which requires sedation, and its results are operator-dependent. Therefore it is now a second-line examination after conventional imaging. Even if recent CT-scan techniques lead to localization of most insulinomas, endoscopic ultrasound still brings the advantage of making a better evaluation of the lesions (especially if they are multiple, as often in MEN-1) and of their proximity with pancreatic ducts and vessels, and it also enables the operator to make biopsies.

Intra-operative ultrasound is mandatory during surgery of insulinomas in order to localize impalpable tumours. High-frequency intra-operative ultrasound has been shown in expert hands to improve detection of small tumours in the pancreatic head and multiple pancreatic lesions to up to 97% (Grant 1988) (Grant 1999). Intra-operative ultrasound examination of the pancreas is also mandatory (using a specific material) during laparoscopic surgery of insulinomas. Despite the high sensitivity of intra-operative ultrasound, a thorough pre-operative examination is necessary to localize insulinomas, in order to maximize the chance for a successful initial resection, minimize the risk of re-operation with its possible morbidity, help in the choice of the surgical technique and make laparoscopic surgery easier when it appears to be the possible choice to remove the insulinoma.

Nuclear imaging of insulinomas is also possible. One of the first functional imaging method was based on the fact that most endocrine tumours display somatostatin receptors, which led to the use of Octreoscan\* scintigraphy (111In-DTPA-octreotide). On the other hand, sst2A receptors were found in only about 66% of insulinomas (Reubi & Waser 2003). Recommendations have been made regarding the protocol (Balon et al. 2001; Bombardieri et al. 2003). Because of competition of the somatostatin analogues at the receptor site, somatostatin analogue treatment should be withdrawn before performing Octreoscan scintigraphy, though there are contradictory data (Dörr et al. 1993). The sensitivity for tumour detection is poor in patients with insulinomas (20-50%) (Krenning et al. 1993) in comparison with other endocrine tumours. However it was 80% in a series of 14 insulinoma patients with the use of SPECT (single-photon emission computed tomography) and an injected activity of 250 MBq of Octreoscan instead of the commonly recommended activity of 150-200 MBq (Schillaci et al. 2000). We only found a 24% sensitivity and neither pre-treatment with octreotide nor the lack of expression of sst2A receptors could account for all the negative scans (Vezzosi et al. 2005). In our experience, Octreoscan scintigraphy proved to be useful in a patient with an ectopic insulinoma, which was located about 2 cm below the body of the pancreas. Using the same technique for Octreoscan scintigraphy, 89.1% of non-insulinoma well-differentiated digestive endocrine tumours were detected in our centre. Small tumours cannot be detected by Octreoscan scintigraphy (Dromain et al. 2005). Octreoscan displays a lower affinity for sst2 receptors than octreotide (Reubi et al. 2000). Specific problems of imaging techniques may play a role in this low detection of insulinomas, even in sst2-positive insulinomas (Vezzosi et al. 2005). Octreoscan scintigraphy may help in localizing metastases, but MRI is better than Octreoscan scintigraphy for detection of liver metastases of endocrine tumours (Dromain et al. 2005). To date, the systematic use of Octreoscan scintigraphy in insulinoma patients is questionable.

Positron emission tomography (PET) with 18-F-labelled deoxy-glucose (FDG) has not proven advantageous for imaging endocrine tumours with the exception of poorly

differentiated, highly proliferative tumours (Pasquali et al. 1998; Sundin et al. 2009). The unstable glucose levels in insulinoma patients may also increase the difficulties.

Since pancreatic endocrine tumours are part of the APUDomas because of their ability for amine precursor uptake and decarboxylation through the action of aromatic amino acid decarboxylase, <sup>18</sup>F-labelled-DOPA was also employed as a tracer. The first results in 10 patients with hyperinsulinemic hypoglycaemia were very encouraging. The pancreatic lesion was localized in 9/10 patients with subsequent confirmation by histological analysis (Kauhanen et al. 2007). The following studies did not confirm these results. A 27% sensitivity was reported for detection of pancreatic endocrine tumours (Montravers et al. 2009). In six patients with hyperinsulinemic hypoglycaemia (4 solitary insulinomas, 1 diffuse beta-cell hyperplasia, 1 malignant insulinoma), F-DOPA-PET was positive in only one case and it underestimated the extent of the disease in the malignant insulinoma with liver metastases (Tessonnier et al. 2010). More data are necessary to conclude, but to date F-DOPA PET does not appear to be a major help in managing pancreatic endocrine tumours.

The first results achieved with a radiolabelled GLP-1 analogue seem promising. Insulinomas were reported to be characterized by a very high incidence of GLP-1 receptors, these receptors being expressed in a particularly high density (Reubi & Waser 2003). A radiolabelled GLP-1 analogue (<sup>111</sup>In-DOTA-exendin-4) was employed in 6 patients (5 with pancreatic insulinomas, 1 with an extrapancreatic insulinoma). Scintigraphy with GLP-1 analogue correctly localized the insulinoma in all 6 patients, while CT-scan was positive in 1, MRI-scan in 1 and endoscopic ultrasound in 4. Four patients had an intense uptake 4 hours after injection, and the other 2 patients showed demarcation between tumour and kidney only at late scans 3-7 days after injection (Christ et al. 2009). Interestingly, while exendin-4 does not induce hypoglycaemia in normal subjects, the insulinoma patients experienced a decrease in plasma glucose levels, with a nadir 40 min after injection, and an exogenous glucose infusion was necessary in 3 patients (for a maximum of 120 min), so that scintigraphy with radiolabelled exendin-4 appears to be also a provocative test in some insulinoma patients, and plasma glucose levels must be monitored after injection.

Finally trans-hepatic pancreatic venous sampling, to screen for an insulin concentration gradient, or calcium injection after selective catheterization of the arteries supplying the pancreas combined with measurement of insulin levels in the right hepatic vein can be used in difficult cases. The latter method was first described by Doppman et al (Doppman et al. 1993). A recent report by an expert team has evaluated the diagnostic accuracy of this technique (Guettier et al. 2009). A 2-fold or greater step-up in the right hepatic vein insulin concentration from baseline at times 20, 40 or 60 seconds after injection was considered to be positive. When a positive response was found at more than one injection site, the dominant site was used to predict tumour localization. In the absence of anatomical variants, a positive response to calcium injection in the gastroduodenal artery or superior mesenteric artery predicts a head-neck tumour, a positive response to the injection into the proximal splenic or the midsplenic artery predicts a body-tail tumour, and a response to calcium injection into the proper hepatic artery represents liver metastases. In 45 patients with surgically proven insulinomas, 38 (84%) localized to the correct region; in 5/45 (11%) the result was falsely negative, and 2/45 (4%) had false-positive localizations. Thus in expert hands selective intra-arterial calcium stimulation is a valuable tool to localize insulinomas when conventional imaging is negative. However, as pointed out by the expert group

(Guettier et al. 2009), there are several caveats, because this technique is based on the following 3 assumptions: 1) a 2-fold insulin increase is pathological; 2) normal beta cells do not show a positive response to calcium injection; 3) insulinomas always respond to calcium injection. All 3 assumptions can be challenged on the basis of the available data. According to Guettier's report, there was no explanation for 3 false-negative cases of insulinomas. A 2-fold increase in insulin levels was found in two or more vessels in 24 of 45 cases, which could suggest that some normal pancreatic areas respond to calcium injection, as already reported by Wiesli (Wiesli et al. 2004a), who recommended that only a 5-fold increase should be considered as a positive response if insulin levels are measured with an insulin-specific assay. This invasive test should probably be a last-line investigation in patients with hypoglycaemia related to endogenous hyperinsulinism that cannot be controlled easily by a medical treatment. Within the last 10 years, we used this method according to the modified protocol described by O'Shea (O'Shea et al. 1996) in only two patients, who were finally found to have nesidioblastosis (with two heterozygous mutations in the ABCC8 gene in one patient) with false positive "pseudo-insulinoma" results.

A recent study (Druce et al. 2010) compared the diagnostic accuracy of most methods employed to localize insulinomas in the years 1990-2009 in 36 patients, among whom 30 were treated by surgery. Among these 30 patients, 25 had CT and 28 had MRI with successful localization in 16 (64%) and 21 (75%) by CT and MRI, respectively. Together this led to 80% of successful localizations. Radiolabelled octreotide scanning was positive in 10 out of 20 cases (50%). Endoscopic ultrasound identified 17 tumours in 26 patients (65.4%). 27 patients had selective intra-arterial calcium stimulation: 6 (22%) did not localize, 17 (63%) were correctly localized, and 4 (15%) gave discordant or confusing results. Thus combination of CT and MRI predicts tumour localization with high accuracy, Octreoscan and endoscopic ultrasound are less helpful but could be valuable in selected cases, and calcium stimulation may provide an additional functional perspective.

In most patients, by now, multidetector CT-scan (with trans-abdominal ultrasound and/or MRI as complementary techniques) employed by an experienced radiologist will localize insulinomas, and must be considered as a first-line examination, while contrast-enhanced ultrasound needs further evaluation.

## **2.4 Treatment**

### **2.4.1 Surgery**

Surgery remains the only curative treatment of insulinomas. Long-term remission can be achieved by surgery in 95% of patients according to a recent study (Zhao et al. 2011). Two different types of surgery can be performed : minimal resection i.e. either tumour enucleation whenever it is possible or central pancreatectomy, or a more extended resection, i.e. left-sided pancreatectomy or pancreatico-duodenectomy. The type of surgery depends on the size and the location of the tumour and of its proximity with specific anatomical structures (pancreatic duct, vessels, adjacent organs). The improvement in the pre-operative imaging techniques enables the surgeon to have an accurate pre-operative topographic assessment and to decide the surgical approach pre-operatively. However intra-operative bidigital palpation and ultrasound remain valuable (Fendrich et al. 2009).

Whenever it is possible, tumour enucleation is to be chosen (Crippa et al. 2007). It allows to cure the patient in most cases with minimized risks of post-operative pancreatic exocrine deficiency and diabetes mellitus. It must be performed only to remove small tumours on the surface of the pancreas, with a distance of more than 2-3 mm between the

tumour and the pancreatic duct (Finlayson & Clark 2004). Providing that the tumour present with the above mentioned characteristics, and that surgery is performed by experienced surgeons, the risk of pancreatic fistula is not higher than that observed in larger resections of the pancreas (Kooby et al. 2008). When tumour enucleation is not possible, central pancreatectomy for a tumour in the pancreatic neck or adjacent body is preferred by several groups (Muller et al. 2006; Crippa et al. 2007; Zhao et al. 2011), in order to preserve a functional pancreatic gland, and to reduce the risks of post-operative pancreatic exocrine deficiency and diabetes mellitus (Crippa et al. 2007; Hirono et al. 2009). A larger pancreatic resection, i.e. left-sided pancreatectomy, ideally spleen-preserving, or pancreaticoduodenectomy, is preferred when the insulinoma is in close proximity to the pancreatic duct in order to lower the risk of pancreatic fistula (Carrere et al. 2007; Nikfarjam et al. 2008). If a plane between the tumour capsule and the pancreatic parenchyma cannot be easily identified, resection is indicated instead of enucleation (Fendrich et al. 2009). A large resection is also preferable for big invasive insulinomas that are suspected to be malignant.

When enucleation is possible, or even when left-sided pancreatectomy is to be performed, laparoscopy is now employed (Crippa et al. 2007). It reduces the duration of the stay in the hospital and improves the post-operative quality of life (Zhao et al. 2011). It must be performed only by experienced surgeons. It can be employed only if the insulinoma has been accurately localized preoperatively. It does not allow intra-operative bi-digital palpation of the pancreas. Laparoscopic ultrasound can be now performed in many expert centres and can localize the insulinoma and evaluate its proximity with the pancreatic duct and the possibility of performing tumour enucleation.

When no insulinoma was found intra-operatively, blind distal pancreatectomy had been recommended several years ago, but to date, such procedure must not be performed (Hirshberg et al. 2002), due to its short-term and long-term morbidity and its frequent failure to achieve a cure of the disease. If no insulinoma is found, it is recommended to stop the operation, then to perform new investigations in order to localize the insulinoma, including invasive techniques.

Morbidity and mortality depend on the type of surgery. Mortality is almost 0% for enucleation, but may reach 1-2% for left-sided pancreatectomy and up to 4-5% for pancreaticoduodenectomy. The most frequent short-term complication of pancreatic surgery is pancreatic fistula. Pancreatic fistulae are more frequent after enucleation or left-sided pancreatectomy, but their consequences are more severe after pancreaticoduodenectomy, due to infectious or haemorrhagic complications. They occur globally in 3-60% of cases, depending on the definition (Pannegeon et al. 2006; Yoshioka et al. 2010; Zhao et al. 2011). Most of them are asymptomatic and no additional treatment is necessary. The clinical prevalence of pancreatic fistulae is about 14% (Zhao et al. 2011). Other complications are intra-abdominal abscess (6.6%), pulmonary infections (3.7%), wound infection (2.5%), delayed gastric emptying (2.2%), abdominal bleeding (1.3%) acute pancreatitis (0.6%) and pulmonary embolism (0.6%) (Zhao et al. 2011). Long-term complications also depend on the type of surgery. Pancreatic exocrine deficiency and diabetes mellitus almost never occur after enucleation or central pancreatectomy. Pancreaticoduodenectomy results in exocrine pancreatic deficiency in 60% of cases, and left-sided pancreatectomy may lead to 5-10% diabetes mellitus. Therefore, limited pancreatic resection such as tumour enucleation or central pancreatectomy are preferred whenever they are technically possible.

### 2.4.2 Medical treatment

A medical treatment must be given to insulinoma patients in order to control the hypoglycaemia while the patient is awaiting surgery. A long-term medical treatment is given only if surgery is technically impossible or contra-indicated.

In some patients a medical treatment can result in controlling the hypoglycaemia. Evaluation of the occurrence of hypoglycaemia could probably be improved by continuous glucose monitoring (Munir et al. 2008). However sudden occurrence of severe hypoglycaemic spells cannot be ruled out, so that even if medications seem to be effective, surgery must not be delayed or cancelled when a surgical cure is possible. Dietary advice is useful but generally not sufficient to avoid hypoglycaemia. The patient must be advised regarding the symptoms suggesting hypoglycaemia, and what must be done when such symptoms occur. Self monitoring of glucose levels is recommended in order to detect asymptomatic episodes of hypoglycaemia and to prevent occurrence of hypoglycaemic spells, by treating asymptomatic abnormal lowering of glucose levels. The patients should be advised regarding everyday personal safety, to avoid possible consequences of dizziness and loss of consciousness.

Diazoxide (a benzothiazide) allows direct suppression of insulin secretion by beta cells through its effect on K-ATP channel. It stimulates hepatic gluconeogenesis and lowers glucose utilization by muscle cells (Altszuler et al. 1977). It can be given bid or tid at a total daily dose of 150-400 mg in most cases. It can control symptomatic hypoglycaemia in 50-60% of the patients (Stefanini et al. 1974). Adverse effects have been reported in about half of the patients. They comprise mainly fluid retention with oedemas, hypokalemia, digestive intolerance with nausea, cutaneous rashes and hirsutism. Diuretics may be added to treatment with diazoxide in order to control oedemas, and thiazides are known to potentiate the anti-hypoglycemic effects of diazoxide, but may induce electrolytic disorders or cause worsening of hypokalemia.

Somatostatin analogues can achieve normalization of plasma glucose levels in 50-60% of the patients (Vezzosi et al. 2008). There are only few adverse effects, mostly digestive intolerance with diarrhoea and steatorrhea. We did not observe a tachyphylaxis phenomenon when using somatostatin analogues in insulinoma patients. The dose of octreotide that was found to control the hypoglycaemia had to be determined on an individual basis, since the doses varied between 50 and 2000 µg per day. Somatostatin analogues inhibit insulin secretion mainly through their effects on sst2A and sst5 receptors, which were found in 70% of insulinomas (Bertherat et al. 2003). A short 100 µg octreotide test, not Octreoscan uptake, was predictive of the long-term efficacy of octreotide treatment on hypoglycaemia. This could be explained by the differing affinities of Octreoscan and octreotide for sst2 receptor (Reubi et al. 2000; Vezzosi et al. 2008). Worsening of hypoglycaemia after administration of somatostatin analogues has been reported (Healy et al. 2007). Such phenomenon could be explained by glucagon suppression by somatostatin analogues. It has not been observed in all series (Vezzosi et al. 2008).

Glucocorticoids may be used to normalize plasma glucose levels in insulinoma patients (Novotny et al. 2005). They decrease insulin secretion and increase peripheral insulin resistance. They are associated with several well-known adverse effects and they cannot be recommended as a first-line medication or for long-term use. Other medications have been employed with variable results, e.g. calcium channel blockers like verapamil (Stehouwer et al. 1989), and also phenytoin and propranolol. To date, m-TOR inhibitors have only been used in metastatic malignant insulinomas (see below).

### 3. Particular conditions

#### 3.1 Malignant insulinomas

Malignant insulinomas represent 5-10% of insulinomas (Danforth et al. 1984; Service et al. 1991). The clinical and biological diagnostic criteria of insulinoma do not differ from those of benign insulinomas. On the other hand, there are specific issues in malignant insulinomas, regarding the diagnosis of malignancy, and the therapeutic management.

The histological diagnosis of malignancy is difficult. The only definite basis is the presence of a metastasis.

In clinical practice, a malignant insulinoma is generally a single large (> 4 cm) tumour. In most cases there are synchronous metastases, which allow to make the diagnosis of malignancy. The metastases are generally located in lymph nodes or in the liver. Thus careful radiological evaluation is valuable for the diagnosis of malignancy. Liver metastases of malignant insulinomas may present with a specific aspect on CT-scan (Atwell et al. 2008) and MRI (Sohn et al. 2001) with perilesional steatosis, due to a local effect of insulin. However metastases may be difficult to identify on CT-scan; a combination of pre-contrast, hepatic arterial-dominant phase and portal venous phase imaging improves the sensitivity for detection of liver metastases, since the lesion may be seen on only one of the three phases (Rockall & Reznek 2007), and large lesions may become necrotic and unenhanced by contrast agents. MRI, ultrasound and contrast-enhanced ultrasound may improve the detection of liver metastases. Typical liver metastases appear as low-signal intensity on T1 and high signal intensity on T2-weighted images, and are hypervascular on hepatic arterial dominant post-gadolinium images (Bader et al. 2001; Rockall & Reznek 2007). Nuclear imaging with Octreoscan can also be used, but false negative results are observed in small liver metastases, and MRI is better than Octreoscan scintigraphy for liver metastases detection (Dromain et al. 2005). However, Octreoscan uptake provides a tool to select patients for treatment with radio-labelled somatostatin analogues (Reubi et al. 2005). We did not confirm that Octreoscan uptake was predictive of the efficacy of somatostatin analogues on hypoglycaemia (Vezzosi et al. 2008; Maiza et al. 2011), unlike what had been previously shown in secreting endocrine tumours (Lamberts et al. 1990).

More rarely, the diagnosis of malignancy is suspected on the basis of the histo-pathological findings and it is confirmed later by occurrence of metastases. Apart from metastases and invasion of adjacent organs, suspicion of aggressive tumour behaviour is aroused by a tumour size > 2 cm, angioinvasion, and high proliferative activity (Kloppel 2007; Kloppel et al. 2009). A review of classification systems has been made by Klimstra et al (Klimstra et al. 2010).

The diagnosis of malignancy may be more difficult in some patients when the insulinoma has a benign presentation, even on the histo-pathological findings; in 48 operated insulinoma patients, with a median follow-up of 42 months, 2 patients initially diagnosed to have benign insulinomas were finally found to develop metastases (personal data). Therefore a prolonged follow-up is advisable. Since some patients with previous partial pancreatectomy may not fulfil the typical criteria for the diagnosis of insulinomas, recurrence of even mild clinical symptoms must not be neglected. Tumours of "uncertain behaviour" warrant a long-term clinical, biological and radiological follow-up. The follow-up of benign insulinomas after surgical remission is usually thought to be unnecessary (Arnold et al. 2009) but on the basis of our experience, we would recommend a clinical and biological follow-up and at least a radiological evaluation 1-2 years after surgery.

Regarding the treatment, malignant insulinomas can represent a double therapeutic challenge, i.e. that of the control of tumour progression, and the control of symptomatic



hypoglycaemia. Controlling the hypoglycaemia must not be neglected, since uncontrolled hypoglycaemia results in increased morbidity and mortality.

Regarding the tumour process, 10 year-survival is about 30% in patients with metastatic insulinomas (Service et al. 1976), with very heterogeneous courses. Some patients may present a very slow progression rate, even with a metastatic disease, and have long-term survival, whereas other patients present with rapid tumour progression and poor short-term survival rate. Predictive factors for rapid tumour progression remain to be established. They could be similar to those found for endocrine bronchial or digestive carcinomas, which comprise the presence of extra-hepatic metastases, the number of liver metastases, the proliferative index Ki67, the spontaneous progression of tumour volume in 3-6 months (Greenberg et al. 1987; Pape et al. 2004; Lepage et al. 2007). The anti-tumour strategy will not be detailed. Since malignant insulinomas are rare, there is no specific prospective study on this particular topic, so that the therapeutic strategy is similar to that of non secreting pancreatic endocrine carcinomas. Whenever it is possible, surgery must aim at totally removing the detectable lesions. However even in selected patients, post-operative complementary treatments are necessary, due to a frequent underestimation of liver metastases. In addition, even when total removal of the tumour has been performed with a R0-resection, recurrence is frequent (about 60% after a follow-up of 3 years) and the recurrence-free median survival is 5 years (Danforth et al. 1984). Treatments other than surgery combine local or regional treatments such as intra-arterial chemotherapy or chemoembolization of liver metastases (Roche et al. 2003) and radiofrequency (Berber et al. 2002), and systemic treatments such as radionuclide systemic administration (Ong et al. 2010), cytotoxic chemotherapy (traditional combination of streptozotocin, doxorubicin and 5-fluoro-uracil and more recently, capecitabine and temozolomide) (Moertel et al. 1992; Strosberg et al. 2008; Strosberg et al. 2011), or targeted therapies (Kulke et al. 2006; Dimou et al. 2010)(Raymond et al. 2011; Yao et al. 2011).

Symptomatic treatment of insulinomas aims at achieving short-term control of the hypoglycaemia while awaiting the effects of anti-tumour treatment or when anti-tumour treatments do not prove to be effective. Surgical removal of the tumour and its metastases is valuable in order to control the hypoglycaemia. Even a reduction of the tumour volume may result in reduction or transient subsiding of symptoms of hypoglycaemia (Sarmiento et al. 2002). A medical treatment is performed if there is no possible surgical cure, or as a complementary therapeutic approach. One should choose as first-line therapies medications that can achieve short-term control of hypoglycaemic spells, and do not jeopardize (by their possible adverse effects) the following use of other treatments. A few recent studies have addressed the issue of the control of severe hypoglycaemic spells in patients with inoperable malignant insulinomas (Bourcier et al. 2009; Ong et al. 2010; Maiza et al. 2011). All the medications used to treat hypoglycaemia in benign insulinomas can be employed, but in most cases, a combination of several hyperglycaemic medications is necessary (Vezzosi et al. 2008). Diazoxide or somatostatin analogues (2-3 subcutaneous injections daily, or long-acting forms, or continuous subcutaneous administration with a portable pump) may result in reduction or disappearance of hypoglycaemic spells in patients with metastatic insulinomas. Radiolabelled somatostatin analogues (especially the Lutetium labelled derivative of octreotide) may prove helpful in long-term control of hypoglycaemia and tumour progression in some patients (Ong et al. 2010). Calcium channel blockers like verapamil gave disappointing results (Stehouwer et al. 1989). Glucocorticoids were found to be effective on hypoglycaemia in malignant insulinomas, and some patients were improved even on doses as low as 2.5 mg of

prednisone per day (Novotny et al. 2005; Starke et al. 2005). m-TOR inhibitors seem now to be very promising regarding the control of the hypoglycaemia, in addition to their anti-tumour effects (Bourcier et al. 2009; Kulke et al. 2009). They can suppress insulin synthesis and secretion and increase insulin-resistance, thereby resulting in euglycaemia in patients with malignant insulinomas (Di Paolo et al. 2006). Chemoembolization of liver metastases can achieve the control of the hypoglycaemia (Moscetti et al. 2000). Repeated chemoembolization procedures are often necessary. Combining the use of continuous subcutaneous administration of a high dose of octreotide as a first-line treatment, followed if necessary by chemoembolization of liver metastases could achieve control of hypoglycaemic spells in a few inoperable patients (Maiza et al. 2011). Continuous glucose infusion may be necessary in some patients, while awaiting the efficacy of the other treatments. Vitamin B1 supplementation should be systematically implemented for patients with severe recurrent hypoglycaemia requiring large infusions of glucose, in order to prevent Wernicke's encephalopathy (Grunenwald et al. 2009).

### 3.2 Insulinomas as part of a genetic disorder

This section will be focused on 4 genetic disorders that are known to be responsible for pancreatic endocrine tumours in adult patients: multiple endocrine neoplasia 1 (MEN-1, OMIM # 131100), Von Hippel Lindau's disease (VHL, OMIM # 193300), tuberous sclerosis (TS) (a disease that is known to involve a dysregulation of the mTOR pathway) (TSC1 and TSC2 genes, OMIM # 191100 and # 191092), and neurofibromatosis type 1 (NF1, OMIM # 162200). Of these 4 disorders, MEN-1 is the most frequent cause of pancreatic endocrine tumours, particularly insulinomas, followed by order of decreasing frequency, by VHL, NF1 and TS (Jensen et al. 2008). It can be noticed that though pancreatic endocrine tumours are found in VHL patients, to our knowledge, there is no case report of a VHL patient with insulinoma. These 4 genetic disorders are caused by mutations in tumour-suppressor genes and are transmitted as autosomal dominant traits. Genetically-determined insulinoma does not differ from sporadic insulinoma regarding the clinical and biological diagnostic criteria. On the other hand, the therapeutic strategy may be different, particularly in MEN-1 insulinomas, since insulinomas are often multiple or associated with other secreting or non secreting endocrine tumours.

MEN-1 is related to a mutation in the menin gene, which maps to chromosome 11q13. Its prevalence is 1-10/100,000. The functional physiological role of menin has not yet been well established. Menin interacts with numerous proteins that play a role in transcriptional regulation, genomic stability, cell division and control of cell cycle (Balogh et al. 2006; Yang & Hua 2007). The major MEN-1 related endocrine disorders are pituitary adenomas, parathyroid adenomas or parathyroid hyperplasia, and secreting or non secreting pancreatic endocrine tumours. Regarding the secreting pancreatic endocrine tumours, the most frequent are gastrinomas (54%, 20-61% of patients) and insulinomas (18%, 7-31% of patients) (Jensen et al. 2008). MEN-1 related insulinomas occur generally in younger patients than sporadic insulinomas, with a greater prevalence in patients aged 25-35 years, but the age of occurrence is very different from patient to patient (5-80 years) (Cougard et al. 2000; Machens et al. 2007). Cystic pancreatic endocrine neoplasms could be more frequent in MEN-1 than in sporadic cases (Bordeianou et al. 2008).

The major characteristic of MEN-1 related insulinomas is that they are generally multiple tumours, so that diffuse microadenomatosis can be observed in the entire pancreatic gland, with multiple infracentimetric lesions of the pancreas (Anlauf et al. 2006). Often in such

cases there are also secreting or non secreting endocrine tumours that are more than 1 cm in size and are distributed in the whole pancreatic gland. Thus managing MEN-1-related insulinomas is often managing multiple pancreatic endocrine tumours, that may secrete insulin and other hormones (gastrinoma, glucagonoma...) or may be non secreting endocrine tumours (Cougard et al. 2000).

The natural course of endocrine disorders in MEN-1 patients was previously thought to start almost always by occurrence of primary hyperparathyroidism (Marx et al. 1998). However others have reported cases of MEN-1 revealed by a secreting pancreatic endocrine tumour; hyperparathyroidism had not been identified prior to the diagnosis of the secreting pancreatic tumour, because it had a mild or asymptomatic presentation (Thakker 2000). Thus insulinoma was the endocrine tumour that revealed MEN-1 in 14-30% of patients (O'Riordain et al. 1994; Cougard et al. 2000). Therefore, apparently sporadic insulinomas in young patients and multiple pancreatic lesions warrant to screen for MEN-1 even if there is no hyperparathyroidism.

The major issue is that of the treatment, since the multiple lesions increase the difficulty in achieving surgical cure of the disease. There is a mean of 3 pancreatic endocrine tumours (1 to 10) in the reported series of MEN-1 patients with pancreatic lesions who underwent surgery (Bartsch et al. 2005; Triponez et al. 2006). When managing an insulinoma patient with multiple pancreatic tumours, there could be several therapeutic strategies: 1. to perform total pancreatectomy with its associated morbidity and mortality rates; 2. to perform an extended resection of the pancreatic gland with enucleations of the tumours on the remaining pancreatic gland, which is also associated with significant morbidity and adds a risk of tumour recurrence. 3. to perform selective enucleations of the detected tumour(s), which increases the risk of re-operation for other tumours, and also may sometimes result in removing a non-secreting tumour, and not the insulin-secreting tumour if it has not been detected because of its smaller size; this could warrant a functional study of the lesions (with preoperative trans-hepatic venous sampling or arterial calcium injection, or intra-operative measurement of insulin) 4. to choose a medical therapeutic approach in responders who do not seem to have malignant lesions. In an operable insulinoma patient, curative surgery is generally attempted for the following two reasons. First, the prevalence of malignant insulinomas is similar in MEN-1 patients and in patients with sporadic insulinomas (Jensen et al. 2008). The prognosis of MEN-1 depends on the progression of malignant pancreatic endocrine tumours (Jensen et al. 2008). Second, in insulinoma patients, even in a patient who is thought to be controlled by a medical treatment, the sudden occurrence of severe symptomatic hypoglycaemia cannot be excluded, with its potentially serious and even fatal consequences. Therefore there is a general agreement regarding the necessity to perform surgery in MEN-1 patients with insulinomas. The extent of surgery remains debated. It mainly depends on the number and the location of the lesions, and the possibility to enucleate the lesion. One of the major advantages of preoperative endoscopic ultrasound could be in the evaluation of MEN-1 patients, since it can visualize even small lesions in the head of the pancreas (Proye et al. 2004). Left-sided pancreatectomy with enucleation of the tumours located in the pancreatic head is generally recommended in patients with MEN-1 related insulinomas, whenever it is possible (Cougard et al. 2000). Tumor enucleation gives acceptable results if the choice of the lowest morbidity rates is made. In those patients who present with a small number of pancreatic tumours, surgery may be less extensive and single or multiple tumour enucleation can be performed, when it is technically possible. The patient must be informed about the risk of recurrence and re-operation.

VHL disease is caused by a mutation in the VHL gene, which maps to chromosome 3p25. Its prevalence is 2 to 3/100,000. It is responsible for occurrence of renal cysts and cancers, hemangioblastomas in the central nervous system, pheochromocytomas and paragangliomas, and pancreatic tumours (Barontini & Dahia 2010). Pancreatic tumours and cysts are reported in 35-77% patients with VHL (Mukhopadhyay et al. 2002), but to date, to our knowledge, there is no case report of VHL-related insulinoma. The mean age of VHL patients who present with a pancreatic lesion varies between 29 and 38 years; unlike what is found MEN-1 patients with pancreatic endocrine tumours, 70% of VHL patients with pancreatic tumours have a single pancreatic tumour (Libutti et al. 2000; Blansfield et al. 2007). Pancreatic endocrine tumours represent only 9-17% of pancreatic lesions in VHL patients (Mukhopadhyay et al. 2002), most of them being non secreting tumours (Libutti et al. 2000). It has also been reported that pancreatic lesions in VHL patients were significantly associated with renal cancers. Since insulinomas are obviously rare in VHL, it seems reasonable to screen for VHL in an insulinoma patient only if the insulinoma is associated with other typical VHL lesions, particularly renal cancer.

Tuberous sclerosis is a phakomatosis characterized by cutaneous (facial angiofibromas also termed "adenoma sebaceum", hypomelanotic macules also termed "ash leaf spots", fibrous raised discoloured forehead plaques, and periungual fibromas), neurological (mental retardation, seizures, subependymal nodules and cortical/subcortical tubers), and ocular (hamartomas, also termed "phakomas") manifestations. The patients also present with renal cysts and angiomyolipomas and cardiac rhabdomyomas. TS is caused by mutations either in the TSC 1 gene (that encodes hamartin, and maps to chromosome 9q34) or in the TSC2 gene (that encodes tuberin, and maps to chromosome 16p13.3) (Lodish & Stratakis 2010). Its prevalence is about 1/10,000 (Osborne et al. 1991). The occurrence of insulinomas in TS patients has been reported very rarely (Davoren & Epstein 1992; Kim et al. 1995; Boubaddi et al. 1997; Eleddrisi et al. 2002; Dworakowska & Grossman 2009). It is generally found in patients with mutations in the TSC2 gene (Merritt et al. 2006). In all reported cases, the insulinoma was found in patients aged more than 20, many years after that the diagnosis of TS had been made. Thus insulinoma is never the first manifestation of TS. Consequently, there is no reason to screen for TS in insulinoma patients. On the other hand, in a patient who is known to have TS, the re-occurrence of neurological or psychiatric symptoms warrants measurement of plasma glucose concentration.

Neurofibromatosis type 1 (NF1) ("Von Recklinghausen's disease") is caused by a mutation in the NF1 gene which maps to chromosome 17q11.2 and encodes a protein named fibromin. The main clinical signs are cutaneous "café-au-lait" spots, neurofibromas, Lisch nodules on iris surface, optic gliomas and bone dysplasia. Its prevalence is 1 in 4000 to 5000. The most frequent endocrine tumour is pheochromocytoma. Pancreatic endocrine tumours are rare (0-10%). Digestive tumours in NF1 are gastrointestinal stromal tumours (GIST), and duodenal somatostatinomas that characteristically cause biliary dilatation (Miettinen et al. 2006). Insulinomas are exceptional in NF1 (Perren et al. 2006).

### 3.3 Nesidioblastosis

There is no general agreement regarding the definition of adult nesidioblastosis. Nesidioblastosis represents a state of diffuse beta cell hyperfunction (though not necessarily uniform in all cases), also termed "non-insulinoma persistent hyperinsulinemic hypoglycaemia (NIPHH)" (Christesen et al. 2008). Such patients have insulin-mediated hypoglycaemia, either in the fasting or in the postprandial state. It also comprises cases

reported as “non insulinoma pancreatogenic hypoglycaemia syndrome (NIPHS)”, i.e. patients who only suffer from reactive insulin-mediated hypoglycaemia, who have a negative fast test without definite pancreatic endocrine tumour, and without mutations in the ABCC8 and KCNJ11 genes (Service et al. 1999). Though rare, it is not quite exceptional among adult patients with hypoglycaemia related to endogenous hyperinsulinism: 4/128 (3%) patients (Raffel et al. 2007), 15/232 (6%) patients (Anlauf et al. 2005b), and even 5/32 (16%) patients (Witteles et al. 2001). We have made the diagnosis of idiopathic nesidioblastosis only in 4 patients for 20 years (1990-2010), while during the same 20 years 69 patients were diagnosed to have insulinomas in our institution; in addition, two other adult patients who had presented symptoms since the early childhood were found to have ABCC8 or glutamate dehydrogenase gene mutations.

Adult nesidioblastosis is likely to represent a heterogeneous disorder and whether it has specific histological correlates remains debated. The first histological reported features were islet cell enlargement, beta cells budding off ductular epithelium, and islets in apposition to ducts. Such aspects were found in an autopsy series in 36% of subjects without hypoglycaemia (Karnauchow PN 1981), whereas using morphometric criteria, patients with NIPHH could not be distinguished from controls (Goudswaard et al. 1984). In 15 adults with nesidioblastosis, blinded interobserver analysis showed beta-cell hypertrophy with enlarged and hyperchromatic nuclei; the interobserver analysis revealed 100% specificity and 87.7% sensitivity for these criteria (Anlauf et al. 2005b). The degree and extent of these features vary much more from patient to patient than in newborns (Kloppel et al. 2008). The interpretation of histological findings is not unequivocal, as shown by the divergent conclusions regarding 6 patients with reactive insulin-mediated hypoglycaemia after bypass surgery (Service et al. 2005; Meier et al. 2006). In addition, Meier et al. reported a positive correlation between beta cell nuclear diameter and body mass index, so that the actual meaning of an increased nuclear size in beta cells can be debated. In 4 operated patients who all had transient remission of hypoglycaemia for about one year after left-sided pancreatectomy, the histological conclusions were nesidioblastosis in 2 patients, micronodular islet cell hyperplasia in one case, and presence of some enlarged nuclei in rare pancreatic areas in one patient (who had two heterozygous mutations of the ABCC8 gene) (personal data).

Nesidioblastosis must not be confused with the recently identified pathological entity called insulinomatosis (Anlauf et al. 2009) which has been described in 14 patients, and is characterized by the synchronous and metachronous occurrence of insulinomas, multiple insulinoma precursor lesions, and rare development of metastases (all but one patient had benign disease), but common recurrent hypoglycaemia.

Nesidioblastosis may be related to well identified genetic disorders with mutations in genes that regulate beta cell insulin secretion (Flanagan et al. 2011a; Flanagan et al. 2011b; Palladino & Stanley 2011) (see above, differential diagnosis) and in such cases, it may occasionally be diagnosed in adult patients (Christesen et al. 2008). It has also been reported to be an adult-onset disease occurring in patients who had been previously treated by insulin, or a sulfonylurea, and in patients with MEN-1, Zollinger-Ellison and Werner-Morrison syndromes (Service et al. 1999). A higher rate of nesidioblastosis in MEN-1 adult patients has not been confirmed by all studies (Anlauf et al. 2005a). None of our patients with adult nesidioblastosis was found to have MEN-1. Conversion of insulin-dependent diabetes mellitus into nesidioblastosis has also been reported (Raffel et al. 2006). Recently, it has been found in patients after gastric bypass surgery (0.5-8 years after surgery in 5/6

patients) but whether such patients have actual structural pancreatic abnormalities is still debated (see differential diagnosis, dumping syndrome). Most reported patients with adult-onset nesidioblastosis do not have any particular medical history and do not present with mutations known to be associated with hypoglycaemia in young children.

The diagnosis of nesidioblastosis is made in patients with definite biological evidence of hypoglycaemia related to endogenous hyperinsulinism (as in insulinoma patients, see biological diagnosis) in the absence of insulin secretagogues, autoimmune hypoglycaemia, and detectable pancreatic tumour. There is neither clinical nor biological basis to make a distinction between insulinoma and nesidioblastosis. Patients with nesidioblastosis may have a positive fast test (as in all our patients), though patients with NIPHS have negative fast tests and only reactive insulin-mediated hypoglycaemia; on the other hand, very rare insulinoma patients may present with a negative fast test, and some of them have only reactive insulin-mediated hypoglycaemia. The diagnostic specificity of multi-site insulin stimulation by intra-arterial calcium injection is not fully established: such insulin stimulation after intra-arterial calcium injection was found in normal pancreatic areas for unknown reasons (Wiesli et al. 2004a; Guettier et al. 2009), and has been reported in patients with factitious hypoglycaemia related to insulin secretagogues (Hirshberg et al. 2001). In addition, intra-arterial calcium stimulation may provide a pseudo-insulinoma response pattern in patients with adult nesidioblastosis, maybe because nesidioblastosis is not evenly distributed in the pancreas. One must also be reminded that nesidioblastosis has been found in patients who had concurrent insulinoma (Bright et al. 2008) (Service et al. 1999). Slight focal pancreatic abnormalities have been reported with radionuclide imaging (Kauhanen et al. 2007).

The natural course of adult-onset nesidioblastosis is not well established. Most patients require a medical and/or surgical treatment. In an obese patient with idiopathic nesidioblastosis who had been treated by left-sided pancreatectomy and high doses of octreotide, hypoglycaemia finally subsided during the perimenopausal years, and changed to mild type-2 diabetes mellitus. No similar change was observed in other patients with nesidioblastosis, though the dose of diazoxide could be decreased in one patient during the perimenopausal years (unpublished personal data).

A medical treatment is the first-line therapeutic approach for nesidioblastosis, but it may not be sufficient to control the hypoglycaemia, and pancreatic surgery must often be performed. Diazoxide remains the reference medication for nesidioblastosis, and is often effective to control the hypoglycaemia, at least after partial pancreatectomy (Arao et al. 2006). Long-term treatment with somatostatin analogues has also been reported to be effective, without the side effects of diazoxide (Vezzosi et al. 2008). One of our patients was treated with continuous subcutaneous administration of a high dose of octreotide throughout her pregnancy, without any detectable maternal or foetal adverse effect (Boulanger et al. 2004). Calcium channel blockers have been employed (Witteles et al. 2001) (Vella & Service 2007). Pancreatic surgery, guided by the results of transhepatic venous sampling or those of insulin stimulation after selective intraarterial calcium injection, is often performed. The extent of surgery is controversial, in order to achieve a total control or a significant improvement of hypoglycaemia, without pancreatic insufficiency (Raffel et al. 2007). Selective intra-arterial calcium injection usually helps in choosing the extent of pancreatectomy (Toyomasu et al. 2009). Total pancreatectomy cannot be recommended, since complete or acceptable control of the hypoglycaemia can be achieved in most patients by partial pancreatectomy followed if necessary by a medical treatment. Spleen-preserving distal pancreatectomy is often performed unless the results suggest a proximal pancreatic

origin for excess insulin secretion (Starke et al. 2006). Four of our patients with nesidioblastosis were treated by left-sided pancreatectomy; all of them had transient remission of hypoglycaemia for about one year, then acceptable control was observed with diazoxide or octreotide, which proved to be more effective to control hypoglycaemia after partial pancreatectomy. 70%-distal pancreatectomy was performed in 5 patients with nesidioblastosis (Witteles et al. 2001) resulting in long-term remission in 3 patients, and some recurrences of hypoglycaemia in 2 patients, successfully treated by calcium channel blockers, with a follow-up of 1.5-21 years. Thus 60-75% resection of the pancreatic gland, guided by insulin venous gradient or insulin stimulation by selective intraarterial calcium injection, and followed if necessary by a medical therapy, appears to be a reasonable treatment for adult nesidioblastosis when enough efficacy cannot be achieved by medical therapy alone.

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## **Part 5**

### **Aging and Endocrinology**



# Natural Flavonoids in StAR Gene Expression and Testosterone Biosynthesis in Leydig Cell Aging

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## 1. Introduction

It is well known that blood testosterone level declines during the course of male aging (Feldman et al., 2002; Harman et al., 2001), a phenomena that is associated with the decreases in bone density, muscle mass and strength, sexual function and other physiological parameters (Kaufman & Vermeulen, 2005; Matsumoto, 2002; Vermeulen, 2000). Previous studies reported that serum testosterone concentrations were lower in the male patients with Alzheimer's disease in comparison to non-demented and age-matched men (Hogervorst et al., 2001; Moffat et al., 2004). Further studies observed that supplementation with testosterone in rats reduced  $\beta$ -amyloid peptide and hyperphosphorylation of  $\tau$ -protein, two biomarkers of the disease (Gouras et al., 2000; Papasozomenos & Shanavas, 2002; Ramsden et al., 2003). The studies suggest that low blood testosterone is a possible risk factor for the development of Alzheimer's disease (Rosario & Pike, 2008). The decline in blood testosterone is a progressive process in male aging. Several longitudinal studies on the blood testosterone of aging males indicated that the incidence of hypogonadism increased with age (Feldman et al., 2002; Harman et al., 2001). In addition, many pathological and stress-related factors may accelerate this process. Therefore, delaying the decline in blood testosterone is clinically significant for the health of aging males suffering from hypogonadism.

For delaying the decline in testosterone, understanding the mechanisms responsible for the decline is important. The studies in the last decades reported multiple factors and alterations in aging process that affect the levels of blood testosterone (Wang & Stocco, 2005). The studies further indicated that the primary reason for the decline is the decrease in testosterone biosynthesis during male aging (Chen et al., 1994). Testosterone is principally synthesized in testicular Leydig cells from the substrate cholesterol and released into the blood circulation (Miller, 1988). The rate-limiting step in testosterone biosynthesis is the transfer of cholesterol to the mitochondrial inner membrane to initiate the steroidogenic process in Leydig cells (Stocco & Clark, 1996). This step is regulated by the steroidogenic acute regulatory (StAR) protein, a critical factor in steroid hormone biosynthesis that controls the cholesterol transfer to the mitochondrial inner membrane (Clark et al., 1994; Lin et al., 1995; Wang et al., 1998). However, StAR protein also declines in Leydig cell aging and

the mitochondrial cholesterol transfer is defective in aged Leydig cells (Culty et al., 2002; Leers-Sucheta et al., 1999; Luo et al., 2001). Therefore, understanding the mechanism for the decline in StAR gene expression becomes an important consideration in the research on the age-related decline in testosterone biosynthesis.

Further studies observed that cyclooxygenase-2 (COX2) in Leydig cells increased in male aging, and the increase in COX2 depressed StAR gene expression and testosterone production. Inhibition of COX2 activity in aged Leydig cells reversed the declines in StAR protein and testosterone production. In addition, feeding aged rats with a COX2 inhibitor reversed the decreased StAR protein and blood testosterone concentrations (Wang et al., 2005). These observations suggest a novel mechanism involving COX2 in the age-related decline in testosterone biosynthesis. The results from the studies indicate that it is possible to delay the decline using COX2 inhibitors. However, aging is a long process and long-term application of pharmacological levels of COX2 inhibitors is limited by their potential side effects. In search for safe and practical approaches, a group of natural flavonoids in food and food supplements has been identified being able to block the COX2-dependent signaling. These flavonoids acted at the different steps of the COX2 signaling pathway and significantly enhanced StAR gene expression and testosterone biosynthesis in Leydig cells. This chapter will describe the mechanism involving COX2 in the declines in StAR gene expression and testosterone biosynthesis in Leydig cell aging. The effects of the flavonoids on this mechanism will be reviewed. The potential application of the natural flavonoids in delaying the declines in StAR gene expression and testosterone biosynthesis will be discussed.

## **2. Luteinizing hormone-induced signaling in StAR gene expression and testosterone biosynthesis in Leydig cell aging**

StAR gene expression and testosterone biosynthesis in testicular Leydig cells are mainly regulated by luteinizing hormone (LH) secreted from pituitary gland. LH stimulation of Leydig cells induces two messengers: cyclic AMP (cAMP) and arachidonic acid (AA). These two messengers transduce signals to the nucleus through two separated pathways to co-regulate StAR gene expression. Both pathways are required with neither one alone being sufficient for LH-induced StAR gene expression and testosterone production (Wang & Stocco, 1999). In addition, these two pathways transduce both positive and negative signals to regulate StAR gene expression. The negative signals increase in Leydig cell aging, resulting in decreases in StAR gene expression and testosterone biosynthesis.

### **2.1 LH-induced positive signaling through cAMP-protein kinase A pathway**

It is documented that LH-induced signaling through cAMP-protein kinase A (PKA) pathway phosphorylates the transcription factors regulating StAR gene expression (Reinhart et al., 1999b). PKA-phosphorylation is important for the activities of several groups of transcription factors, including cAMP-responsive element binding (CREB) protein, steroidogenic factor-1 (SF-1) and GATA-4, which bind on StAR promoter to induce StAR gene transcription. The cAMP-responsive element (CRE)-like sites were found in StAR promoter DNA sequences. The results from electrophoretic mobility shift assays (EMSA) demonstrated the binding of CREB to StAR promoter (Manna et al., 2002; Manna et al., 2003). PKA-phosphorylation is critical for the activity of CREB (Meyer & Habener, 1993; Montminy et al., 1986), because the phosphorylation of CREB is needed for the recruitment

of its cofactor CBP (CREB binding protein) that is involved in the transcriptional activity of CREB (Chrivia et al., 1993; Mayr & Montminy, 2001; Mayr et al., 2001). SF-1 binding sites were also located on StAR promoter (Sandhoff et al., 1998). Binding of SF-1 to these binding sites is crucial for StAR gene transcription (Caron et al., 1997a; Caron et al., 1997b; Sandhoff et al., 1998; Sugawara et al., 1997). PKA-phosphorylation of SF-1 protein was detected at serine and threonine residues (Zhang & Mellon, 1996), which was further confirmed by the study with R2C rat Leydig cells (Carlone & Richards, 1997). It appears that PKA-phosphorylation is needed for the maximal activity of SF-1 in the regulation of StAR gene transcription (Lopez et al., 2001; Sugawara et al., 1996). GATA-4 is another transcription factor binding on StAR promoter to regulate StAR gene expression. A GATA-4 binding site was found at -61 to -66 in StAR promoter sequences (Silverman et al., 1999; Silverman et al., 2006). Stimulation of MA-10 mouse Leydig cells with cAMP dramatically increased phosphorylated GATA-4 protein (Tremblay et al., 2002), resulting in the increase in StAR promoter activity. The essential role of PKA-phosphorylation in the activity of GATA-4 was further confirmed by over-expression of PKA catalytic subunit. While expression of PKA catalytic subunit significantly increased GATA-4-supported StAR promoter activity, the increased promoter activity was reversed by co-expression of the protein kinase inhibitor (Tremblay et al., 2002).

The signaling through cAMP-PKA pathway is able to increase the activities of a group of transcription factors that bind on StAR promoter and regulate StAR gene transcription, including C/EBP $\beta$  (Christenson et al., 1999), Sp-1 (Sugawara et al., 2000), activator protein-1 (AP-1) (Manna et al., 2004), and sterol regulatory element binding protein (SREBP) (Shea-Eaton et al., 2001), by inducing the interaction and cooperation among them. The cAMP or PKA-induced interactions among these transcription factors generated synergistic effects that increased the cAMP-induced StAR gene transcription and steroid hormone production in Leydig cells (Reinhart et al., 1999a; Silverman et al., 1999; Silverman et al., 2006; Sugawara et al., 2000; Tremblay et al., 2002).

In addition, it was observed that cAMP stimulation of Leydig cells reduced DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1) protein. DAX-1 is a strong transcriptional repressor that binds to a hairpin structure of StAR promoter between -20 to -61 and depresses StAR gene expression (Zazopoulos et al., 1997). The reduction of DAX-1 by cAMP stimulation results in dramatic increases in StAR gene expression and steroid hormone production in Leydig cells (Jo & Stocco, 2004). Although it is still not clear how the signaling through cAMP-PKA pathway reduces DAX-1 protein, the observations indicate that this signaling pathway generates positive signals to increase StAR gene transcription by reduction of the transcriptional repressor.

## 2.2 LH-induced positive signaling through AA pathway

In addition to the cAMP-PKA signaling pathway, LH induces positive signaling through AA pathway. Stimulation of Leydig cells with LH induced AA release in one minute (Cooke et al., 1991). Previous studies have described three mechanisms for the AA release by LH or hCG: 1) Binding of the trophic hormones to their receptors activates G protein. G protein directly activates phospholipase A2 (PLA2) that catalyzes AA release from phospholipids (Axelrod et al., 1988; Ronco et al., 2002); 2) G protein increases cAMP formation that induces AA release, possibly through the activation of PLA2 by PKA-phosphorylation (Piomelli & Greengard, 1991; Wang et al., 2002); 3) AA is released by the co-regulation of acyl-CoA synthetase 4 (ACS-4) and acyl-CoA thioesterase (Acot2). Trophic hormone stimulation

induces ACS-4, and activates Acot2 by cAMP-PKA-phosphorylation. Co-action of these two enzymes catalyzes AA release from arachidonyl CoA (Cano et al., 2006; Cornejo Maciel et al., 2005; Finkielstein et al., 1998; Maloberti et al., 2005; Maloberti et al., 2007; Paz et al., 1994). The LH-induced AA release is required for testosterone biosynthesis. It was observed that incubation of Leydig cells with AA significantly increased testosterone production (Didolkar & Sundaram, 1987, 1989). When the AA release was blocked using PLA2 inhibitors, the LH-induced testosterone production was significantly reduced although the inhibitors did not affect the cAMP formation (Abayasekara et al., 1990). Inhibiting AA metabolism in rat Leydig cells produce similar results (Cooke et al., 1984; Dix et al., 1984; Mele et al., 1997; Sullivan & Cooke, 1985). In addition, blocking AA release by knockdown of the gene expressions of ACS-4 and Acot2 significantly reduced steroidogenesis (Maloberti et al., 2005). The studies indicated that LH induces positive signaling through AA pathway to regulate testosterone biosynthesis in Leydig cells.

To understand how the released AA plays such an important role in LH-induced steroidogenesis in Leydig cells, its effect on mitochondrial cholesterol transfer was investigated. It was found that blocking AA release failed to affect steroidogenesis when Leydig cells were incubated with 22(R)hydroxycholesterol (Abayasekara et al., 1990; Dix et al., 1984; Mele et al., 1997), a water soluble substrate that is able to diffuse to the mitochondrial inner membrane. The studies suggest that AA enhance testosterone biosynthesis by increasing the mitochondrial cholesterol transfer. The observations were corroborated by the increases in StAR protein and steroidogenesis in the Leydig cells incubated with AA. In addition, blocking AA release using PLA2 inhibitors dramatically reduced LH- or cAMP-induced StAR promoter activity, StAR mRNA, StAR protein and steroidogenesis in Leydig cells. Importantly, the reduced StAR gene expression and steroidogenesis by PLA2 inhibitor were reversed by addition of AA in the cell cultures (Wang et al., 2000; Wang et al., 1999). It is clear that AA increases testosterone biosynthesis by enhancing StAR gene expression. Moreover, the results from EMSA show that AA enhanced the binding of nuclear protein(s) to the StAR promoter DNA sequences between -67 and -96 (Wang et al., 2003a). The observation indicates that AA acts on StAR promoter and regulates StAR gene transcription.

AA is metabolized mainly through three metabolic pathways, the lipoxygenase, epoxygenase and cyclooxygenase pathways, generating various metabolites. To determine which pathways are involved in the LH-induced StAR gene expression and steroidogenesis, MA-10 Leydig cells pre-loaded with  $^3\text{H}$ -AA were used to study the effects of cAMP on AA metabolism. The results from HPLC analysis of the cell extract showed that cAMP stimulation significantly increased two groups of AA metabolites: the lipoxygenase-generated metabolites, 5-hydroxyeicosatetraenoic acid (5-HETE) and 5-hydroperoxyeicosatetraenoic acid (5-HPETE) (Wang et al., 2003a); and the epoxygenase-generated metabolites, 5,6-epoxyeicosatrienoic acid (5,6-EET), 8,9-epoxyeicosatrienoic acid (8,9-EET), and 11,12-epoxyeicosatrienoic acid (11,12-EET) (Wang et al., 2006). The roles of these AA metabolites in StAR gene expression were investigated with Leydig cells. It was observed that addition of each of these metabolites into the cultures of Leydig cells significantly increased cAMP-induced StAR promoter activity, StAR mRNA, StAR protein and steroidogenesis. In addition, when activity of lipoxygenase or epoxygenase was inhibited, the cAMP-induced StAR gene expression and steroidogenesis were significantly reduced (Wang et al., 2000). The observations indicated that each of the metabolites enhanced StAR gene expression at the level of transcription. Although how these AA



metabolites act on StAR promoter is not clear, the studies showed that LH- or cAMP-released AA is converted to positive signals, 5-HPETE, 5-HETE, 5,6-EET, 8,9-EET and 11,12-EET through lipoxygenase and epoxygenase pathways, which enhance StAR gene expression and testosterone biosynthesis in Leydig cells.

### 2.3 LH-induced negative signaling in StAR gene expression

Whereas LH-released AA is metabolized to the positive signals by lipoxygenase and epoxygenase, it is converted to negative signals by cyclooxygenase (COX). It was found that inhibition of COX activity using an inhibitor significantly increased cAMP-induced StAR protein and steroid hormone production in MA-10 mouse Leydig cells. Similar increases were observed in StAR promoter activity and StAR mRNA levels. Although inhibition of COX activity significantly increased the cAMP-induced StAR gene expression, in the absence of cAMP the inhibitor alone did not increase StAR protein expression and steroid hormone production. The studies suggest that the COX inhibitor itself is not able to induce StAR gene expression, but rather reduce the negative signals, resulting in significant increases in the cAMP-induced StAR gene expression and steroidogenesis. There are two isoforms of COX enzymes, COX1 and COX2, in Leydig cells. To determine which isoform is responsible for the inhibitory effect on StAR gene expression, Leydig cells were treated with the selective COX1 inhibitor SC560 or COX2 inhibitor NS398, respectively. Whereas inhibition of COX1 activity did not change StAR protein level, inhibition of COX2 activity dramatically enhanced cAMP-induced StAR protein expression and steroid hormone production. Further studies observed that while COX2 inhibitor significantly increased StAR protein, it did not affect PKA activity. In addition, over-expression of COX2 reduced StAR promoter activity. The observations indicate that COX2, not COX1, converts the LH- or cAMP-released AA to negative signals that depress StAR gene transcription in Leydig cells (Wang et al., 2003b).

The signaling through cAMP-PKA pathway also generates negative signals to inhibit StAR gene expression and steroidogenesis in Leydig cells by increasing COX2 expression. The mechanism responsible for the cAMP-increased COX2 may involve the signaling through cAMP-PKA pathway in the activation of several transcription factors that regulate COX2 promoter activity. One is CREB that binds on the CRE site at -53 to -59 of human COX2 promoter sequences (Schroer et al., 2002). CREB activation by PKA increased its binding to the CRE site and induced COX2 gene transcription. While forskolin or cAMP activated PKA and increased COX2 promoter activity, inhibition of PKA activity reversed the increase in the promoter activity. Another transcription factor NF- $\kappa$ B plays an essential role in regulating COX2 gene expression (Arun et al., 2009). This transcription factor was activated by PKA-phosphorylation of NF- $\kappa$ B p65 sub-unit. Inhibition of its phosphorylation using H89 reduced COX2 gene transcription. The involvement of cAMP-PKA signaling pathway in the LH-induced COX2 expression was supported by the studies with Leydig cells. An earlier study reported that hCG stimulation of Leydig cells increased COX2 expression (Frungieri et al., 2006). This observation was corroborated by a latter study that reported a LH-increased COX2 protein in rat Leydig cells. The increase in COX2 was almost abolished by inhibition of PKA activity with its inhibitor H89, indicating that cAMP-PKA-phosphorylation is required for the LH-induced COX2 expression in Leydig cells (Chen et al., 2007b).

As LH stimulates testosterone biosynthesis, the increase in testosterone may feed back to induce COX2 expression in Leydig cells. A recent study reported that testosterone induced COX2 expression and prostaglandin F<sub>2a</sub> production in hamster Leydig cells (Matzkin et al.,

2009). These effects of testosterone were abolished by the anti-androgen bicalutamide. A testosterone-stimulated phosphorylation of the mitogen-activated protein kinase (MAPK) was also observed in the study. It is known that the activity of MAPK is important in COX2 gene expression (Chun & Surh, 2004), because MAPK is able to activate the transcription factors that regulate COX2 gene transcription.

The studies described above indicate that when LH stimulates positive signals through cAMP-PKA pathway and AA pathway, it also induces negative signals through these two pathways. Therefore, the total effect of LH on StAR gene expression and testosterone biosynthesis depends on the ratio and levels of the positive and negative signals. The alterations of the signals in Leydig cell aging affect StAR gene expression and testosterone biosynthesis.

#### **2.4 The age-related alterations of the positive and negative signals in StAR gene expression**

Previous studies have reported the multiple alterations or defects during male aging that are involved in the decline in testosterone biosynthesis (Wang & Stocco, 2005). Many of these alterations or defects in the aging process increase the negative signals and reduce the positive signals in StAR gene expression. These changes reduce the sensitivity of Leydig cells to trophic hormone stimulation and result in the declines in StAR gene expression and testosterone biosynthesis.

It was observed that the age-related alteration in hypothalamic-pituitary-adrenal axis attenuates the sensitivity of the feedback control of adrenal steroidogenesis, resulting in the increases in basal and stress-induced glucocorticoid levels (Hardy & Cooper, 2010). The inhibitory effect of glucocorticoid on testosterone biosynthesis was reported by previous studies with rat Leydig cells. This steroid hormone was shown being able to inhibit PLA2 activity and block LH-induced AA release (Abayasekara et al., 1990). Further studies observed that blocking AA release using dexamethasone dramatically reduced StAR protein and steroidogenesis in MA-10 mouse Leydig cells. Similar results were obtained in the analyses of StAR promoter activity and StAR mRNA levels. The reduced StAR gene expression and steroidogenesis were reversed by addition of AA to the cell cultures (Wang et al., 2000). The studies suggest that the age-related defect in hypothalamic-pituitary-adrenal axis result in reduction in the LH-induced positive signals through AA pathway.

The positive signaling through cAMP-PKA pathway is also reduced in Leydig cell aging. The previous studies with aged Leydig cells reported a defect in this signaling pathway that caused the decreases in LH-induced cAMP formation and PKA activity (Lin et al., 1980; Luo et al., 2005). The decrease in LH-induced cAMP formation may significantly contribute to the reduced sensitivity of aged Leydig cells to LH stimulation. The mechanism for this defect in aged Leydig cells has not been completely understood. It was observed that the number of the LH-binding sites and their binding affinity decreased in aged Leydig cells, but how the reduced LH-binding capacity affected the cAMP formation remains to be elucidated (Chen et al., 2002). In addition, defect of G protein was investigated. It is possible that inefficiency of G protein or defect in coupling among the signal cascades affects the LH-induced cAMP formation (Chen et al., 2004).

In the aging process, the levels of many biological factors in blood or testis are altered. These alterations may reduce the positive signaling through cAMP-PKA pathway and enhance the negative signaling through COX2 pathway. One group of the biological factors that increase in aging is the inflammatory cytokines, such as Interlukin-1 (IL-1), Transforming growth factor

(TGF)- $\beta$  and tumor necrosis factor (TNF) (Chung et al., 2001; Morley & Baumgartner, 2004). IL-1 $\beta$  was reported to be inhibitory in hCG-, cAMP-, and forskolin-induced testosterone production (Calkins et al., 1990). While it is able to reduce activities of steroidogenic enzymes (Hales, 1992; Lin et al., 1991), IL-1 $\beta$  induced COX2 expression (Chen et al., 2007b; Walch & Morris, 2002) and reduced StAR protein expression (Ogilvie et al., 1999). TGF- $\beta$  in testis increases with age (Jung et al., 2004). The increase in TGF- $\beta$  may significantly reduce trophic hormone-induced cAMP formation and testosterone production in Leydig cells (Avallet et al., 1987). TNF- $\alpha$  reduced hCG-stimulated cAMP formation in a concentration-dependent manner in MA-10 mouse Leydig cells (Budnik et al., 1999), which was associated with a reduction in StAR protein. In addition, it was reported that the transcription factor NF- $\kappa$ B increases in the aging process (Chung et al., 2009; Chung et al., 2006). This increase would contribute to the increase in COX2, because NF- $\kappa$ B plays an essential role in COX2 gene transcription. Another group of the biological factors is reactive oxidative species (ROS). An age-related increase in ROS was previously described (Chen et al., 2001b). While ROS may inhibit testosterone through different mechanisms, it is able to activate NF- $\kappa$ B, resulting in the increases in COX2 and various inflammatory factors (Chung et al., 2006).

The age-related increases in the biological factors, such as IL-1 $\beta$ , NF- $\kappa$ B and ROS, suggest an increase in COX2 expression in Leydig cell aging, because these factors induce COX2 as mentioned above. This was supported by the increase in COX2 mRNA level detected in aged Leydig cells of rats (Syntin et al., 2001). It is known that COX2 produces an inhibitory effect on StAR gene expression and steroidogenesis (Wang et al., 2003b). These observations suggest a possibility of involving an age-related increase of COX2 in the declines in StAR gene expression and testosterone production. This possibility was demonstrated by the studies with aged rats (Wang et al., 2005). It was found that from 3 to 30 months of age, the levels of COX2 protein in aged rat Leydig cells increased by 346% over that of young Leydig cells. The increase in COX2 was associated with the decreases in StAR protein, testosterone biosynthesis, and blood testosterone concentration in aged rats. Inhibition of COX2 activity with the selective COX2 inhibitor NS398 reversed the decreases in StAR protein and testosterone production in aged Leydig cells. In addition, while over-expression of COX2 reduced cAMP-stimulated StAR protein and steroidogenesis, co-incubation with NS398 reversed the reduced StAR protein and steroid hormone. These findings reveal a novel mechanism in the age-related declines in testosterone biosynthesis.

### **3. Mechanism for COX2-dependent inhibition of StAR gene expression and testosterone biosynthesis in Leydig cell aging**

The studies described above indicated an involvement of COX2 in the age-related declines in StAR gene expression and testosterone biosynthesis. However, the mechanism for the COX2-dependent inhibition of StAR gene expression in Leydig cells needs to be elucidated. While further investigations are needed for the elucidation, the studies in the recent years have significantly improved the understanding of the mechanism.

#### **3.1 AA distribution between COX2 and lipoxygenase metabolic pathways**

It is known that the LH-released AA is converted to positive signals through lipoxygenase and epoxygenase pathways (Wang et al., 2006; Wang et al., 2003a), and also negative signals through COX2 pathway (Wang et al., 2003b). This suggests that StAR gene expression and testosterone biosynthesis in Leydig cell aging are regulated by the activities of these AA

metabolic enzymes, because they control the ratio of the positive and negative signals. To prove this hypothesis, MA-10 mouse Leydig cells pre-loaded with  $^3\text{H-AA}$  were incubated with NS398 to inhibit their COX2 activities. AA metabolites were extracted from the cells and analyzed by HPLC. The HPLC profile of AA metabolites indicated that inhibition of COX2 activity increased AA metabolites produced through lipoxygenase pathway, with the levels of 5-HETE and 5-HPETE being significantly increased by the treatment. Incubation of rat Leydig cells with 5-HETE significantly increased testosterone production (Wang et al., 2005). It was previously reported that 5-HETE and 5-HPETE transduce positive signals to enhance StAR gene expression and steroidogenesis in Leydig cells (Wang et al., 2003a). The observations indicated that the distribution of AA between these two metabolic pathways is regulated by their enzyme activities. Thus, inhibition of COX2 activity changed the AA distribution, resulting in the increases in 5-HETE and 5-HPETE that enhanced StAR gene expression. It is possible that as COX2 expression increases in aged Leydig cells, more AA is metabolized through COX2 pathway to produce inhibitory metabolites that depress StAR gene expression and testosterone biosynthesis.

### **3.2 The signaling through COX2-prostaglandin F $2\alpha$ -receptor pathway in StAR gene expression**

In the COX2 metabolic pathway, COX2 catalyzes AA to prostaglandin(PG)H $2$  that is further metabolized to PGD $2$ , PGE $2$ , PGF $2\alpha$ , PGI $2$  and thromboxane A $2$  (TBX A $2$ ) by different prostaglandin synthases and thromboxane A synthase (TBXAS). Among these metabolites, PGF $2\alpha$  is involved in the COX2-dependent inhibition of testosterone biosynthesis. Previous studies reported the hCG-induced production of PGF $2\alpha$  (Haour et al., 1979), which acted as a negative signal to inhibit testosterone production in Leydig cells (Bartke et al., 1973; Saksena et al., 1973). The inhibitory effect of PGF $2\alpha$  was verified by several studies. In an *in vivo* study, the infusion of PGF $2\alpha$  to rats (250 $\mu\text{g}/\text{rat}$ ) for 4 hours reduced blood testosterone to 50%, whereas the inhibition of PGF $2\alpha$  production with indomethacin increased blood testosterone concentrations. When the decapsulated testis preparation was incubated with PGF $2\alpha$ , LH-induced testosterone production was reduced in a dose-dependent manner (Fuchs & Chantharaksri, 1981). The observations were corroborated by the additional studies on the inhibitory effects of PGF $2\alpha$  on Leydig cell steroidogenesis (Romanelli et al., 1995; Sawada et al., 1994). To find how PGF $2\alpha$  inhibits testosterone biosynthesis, the PGF $2\alpha$  receptors were located on hamster Leydig cells. Further investigation observed that incubation of the Leydig cells with 1  $\mu\text{M}$  of PGF $2\alpha$  for 10 to 60 minutes significantly reduced StAR protein. The study described a COX-2-dependent signaling pathway via COX2, PGF $2\alpha$  production, PGF $2\alpha$  receptors, and StAR protein in the regulation of hCG-induced testosterone biosynthesis (Frungeri et al., 2006). How this signaling pathway regulates StAR protein expression is not clear. To understand the mechanism, several regions in rat StAR promoter DNA sequences were detected to be responsive to PGF $2\alpha$ , including the bind sites of DAX-1, c-Fos and YY1. DAX-1 is described above as a transcriptional repressor. Injection of PGF $2\alpha$  for 2 hours resulted in the increase in ovarian DAX-1 protein. The increase in DAX-1 protein was associated with the 50% reduction in StAR mRNA (Sandhoff & McLean, 1999). The c-Fos binding sites were located at -85, -187 and -1561 of StAR promoter sequences. Administration of PGF $2\alpha$  increased c-Fos expression and reduced StAR gene transcription (Shea-Eaton et al., 2002). The YY1 binding sites were found in StAR promoter sequences at -1230/-1238, -1550/-1559 and -1651/-1660. PGF $2\alpha$  enhanced ovarian YY1 expression, resulting in a decrease in StAR gene expression (Liu et al., 2007; Nacklely et al.,

2002). However, whether PGF<sub>2</sub>α increases the expressions of these transcriptional repressors in Leydig cells remains to be clarified.

### 3.3 The signaling through COX2-TBXAS-TBX A2-receptor pathway in StAR gene expression

Following the studies on PGF<sub>2</sub>α, TBXAS activity was inhibited to examine the role of the COX2-TBXAS-TBX A2-receptor signaling pathway in Leydig cell steroidogenesis. Inhibition of TBXAS activity with the inhibitor furegrelate significantly enhanced the cAMP-induced steroidogenesis in MA-10 mouse Leydig cells. However, co-incubation of the cells with 22(R)hydroxycholesterol abolished the stimulatory effect of the inhibitor on steroidogenesis, suggesting that TBXAS produce an inhibitory effect on the mitochondrial cholesterol transfer. The results were confirmed by the increase in StAR protein in the cells treated with the TBXAS inhibitor (Wang et al., 2008). Following these observations, luciferase assays of StAR promoter activity and reverse transcription-polymerase chain reaction (RT-PCR) were performed to determine whether the TBXAS inhibitor acted on StAR gene transcription. It was found that inhibition of TBXAS activity significantly increased StAR promoter activity and StAR mRNA levels. To confirm the inhibitory effect of TBXAS on StAR gene expression, the TBXAS gene was silenced using RNA interference (RNAi). As TBXAS gene was silenced by RNAi, StAR mRNA, StAR protein and steroid hormone production in the cells were significantly increased. It is clear that the signaling through COX2 and TBXAS acts on StAR promoter and depresses StAR gene transcription. The study was continued to identify the transcription factor(s) that is affected by the activity of TBXAS. The results from the study reveal that inhibition of TBXAS activity reduced DAX-1 protein. When DAX-1 protein was reduced by inhibiting TBXAS activity, StAR gene transcription was significantly enhanced, suggesting that co-action of COX2 and TBXAS convert AA to inhibitory metabolite(s) that inhibits StAR gene transcription by regulating DAX-1 expression (Wang et al., 2008).

The first AA metabolite in the COX2-TBXAS pathway is TBX A2. The TBX A2 receptors were detected in several mouse Leydig cell lines and identified on the cell surface (Pandey et al., 2009). The specific binding of the receptor antagonist SQ29548 to the receptors on MA-10 mouse Leydig cells was demonstrated by binding assay and the binding competition between <sup>3</sup>H-SQ29548 and another receptor antagonist BM567. When the concentrations of BM567 increased from 0 to 10 μM, the <sup>3</sup>H-SQ29548 bound to the cells was reduced to 3%. The receptor antagonist SQ29548 was used to block the binding of TBX A2 to the receptors and to determine its effect on the Leydig cell steroidogenesis. It was observed that blocking the TBX A2 receptors on the Leydig cells incubated with 0.1 mM cAMP dramatically increased StAR protein in a concentration-dependent manner. The increase in StAR protein was paralleled with the increase in steroid hormone production, with progesterone production being increased from 35 to 208 pg/μg cellular protein as the concentrations of SQ29548 were increased from 0 to 25 μM. The results were verified using another receptor antagonist BM567 (Pandey et al., 2009). Since the COX2-dependent signaling is involved in the age-related declines in StAR gene expression and testosterone biosynthesis (Wang et al., 2005), aged Leydig cells were used to determine whether blocking the TBX A2 receptors is able to reversed the declines. When the TBX A2 receptors were blocked with SQ29548, StAR protein in the aged Leydig cells increased significantly. In concomitant with the increase in StAR protein, testosterone production by the aged Leydig cells also significantly increased from 86 to 146 pg/μg cellular protein (Pandey et al., 2009).

To understand how the receptor antagonists enhanced StAR protein expression and steroidogenesis, their effects on StAR gene transcription were investigated. Luciferase assays of StAR promoter activity indicated that blocking the TBX A2 receptors with the antagonists enhanced StAR promoter activity in a concentration-dependent manner in the MA-10 cells incubated with 0.1 mM cAMP. The increase in the promoter activity was associated with a similar increase in StAR mRNA level (Pandey et al., 2009). The observations indicated that blocking the TBX A2 receptor enhanced StAR gene transcription, similar to the observations on the cells treated with TBXAS inhibitor (Wang et al., 2008). Further study showed that blocking the TBX A2 receptors significantly reduced DAX-1 protein and increased StAR protein in MA-10 mouse Leydig cells. The results were confirmed with the Leydig cells isolated from aged rats. The aged Leydig cells expressed high levels of DAX-1 protein, but blocking the TBX A2 receptors dramatically reduced the DAX-1 protein, which was associated with the increases in StAR protein and testosterone production (Pandey et al., 2009). These observations indicated that the signaling through COX2-TBXAS-TBX A2-receptor inhibits StAR gene expression and testosterone biosynthesis by regulation of DAX-1 expression.

In addition, while sub-threshold levels of cAMP were unable to stimulate significant increases in StAR gene expression, interrupting the signaling through this pathway at any step, by inhibiting COX2 activity (Wang et al., 2005; Wang et al., 2003b) or TBXAS activity (Wang et al., 2008) or blocking the TBX A2 receptors (Pandey et al., 2009), reduced the threshold, with sub-threshold levels of cAMP being able to induce maximal levels of StAR protein and steroidogenesis. These studies indicated that the signaling through COX2-TBXAS-TBX A2-receptor pathway plays an important role in regulating sensitivity of Leydig cells to LH or cAMP stimulation. Therefore, when COX2 increases in Leydig cell aging, the increase in COX2 enhances the negative signaling through this pathway, which in turn reduces the sensitivity of Leydig cells and inhibits the LH-induced StAR gene expression and testosterone biosynthesis.

#### **4. Flavonoid intervention in the COX2-dependent inhibition of StAR gene expression and testosterone biosynthesis**

To study the possibility of delaying the decline in blood testosterone by intervention in the mechanism, aged rats were fed with increasing concentrations of a selective COX2 inhibitor mixed in their diet. After 30 days, StAR protein in their Leydig cells increased in a concentration-dependent manner. The blood testosterone concentrations increased up to 120% over control (Wang et al., 2005). The studies suggest a possibility of delaying the age-related declines in StAR protein and testosterone using COX2 inhibitors. However, long-term application of the COX2 inhibitors currently used in the clinical practice is limited by their side effects. Therefore, alternative approaches are needed for the health of aging males. In the recent years, steroidogenic effects of natural flavonoids have been studied with Leydig cells. Flavonoids are a group of the polyphenolic compounds that are widely distributed in various food and food supplements, especially in fruits and vegetables. Previous studies have reported the activities of flavonoids in anti-inflammation, anti-cancer, and anti-oxidation (Cardenas et al., 2006; Chen et al., 1990; Ferrandiz & Alcaraz, 1991). One of the important mechanisms for these activities is the inhibition of COX2 expression and blocking the COX2-dependent signaling by flavonoids, which enables flavonoids to enhance StAR gene expression and testosterone biosynthesis in Leydig cells. A group of flavonoids

has been identified, including chrysin, apigenin, luteolin, and quercetin (Fig. 1), to be able to enhance StAR gene expression and steroidogenesis in Leydig cells by blocking the COX2-dependent signaling.

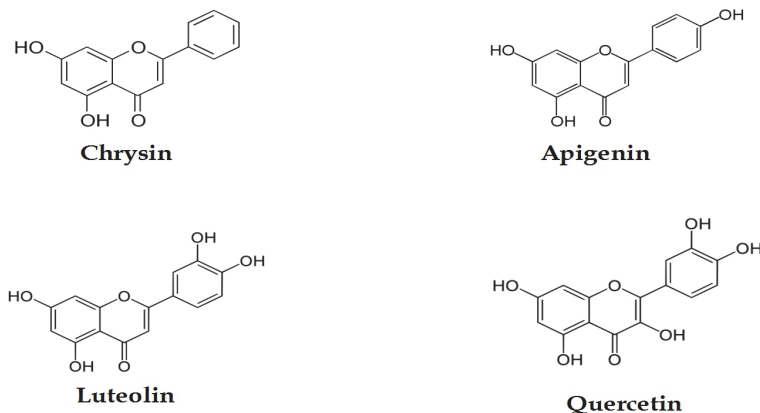


Fig. 1. Chemical structures of the flavonoids used in the experiments to enhance StAR gene expression and steroidogenesis in Leydig cells

#### 4.1 Chrysin

Chrysin is present in plants (Williams et al., 1997), honey and propolis (Gambelunghe et al., 2003; Jiang et al., 2008; Kassim et al., 2010). It blocks the COX2-dependent signaling principally by inhibition of COX2 expression. The reduction of COX2 by chrysin is due to its inhibitory effects on the activities of several transcription factors that regulate COX2 gene transcription in different tissues. Chrysin is able to inhibit the activity of NF- $\kappa$ B and reduce COX2 promoter activity (Ha et al., 2010; Li et al., 2010). In macrophages, chrysin suppressed lipopolysaccharide (LPS)-induced COX2 expression by inhibiting activity of nuclear factor for IL-6 (NF-IL6) (Woo et al., 2005). NF-IL6 is a member of the C/EBP family that binds to human COX2 promoter region at -124/-132 and induces COX-2 expression. Another member of C/EBP family is C/EBP $\beta$  that is activated by c-Jun N terminal kinase (JNK) (Cho et al., 2003). Chrysin inhibited JNK activation and reduced LPS-induced COX2 expression (Ha et al., 2010). In addition, the cytokines, such as IL-1 $\beta$  and TNF- $\alpha$ , are able to induce COX2 expression (Chen et al., 2001a; Ishikawa et al., 2005). It was observed that chrysin significantly reduced the LPS-released IL-1 $\beta$  and TNF- $\alpha$  (Ha et al., 2010; Romier et al., 2008). In addition to the inhibition of COX2 gene expression, chrysin and its derivatives are able to inhibit COX2 activity. A group of chrysin derivatives was synthesized by modification of its molecular structure. The modification significantly improves the activities of the derivatives in inhibiting COX2. Some of the chrysin derivatives are able to bind to COX2 molecule and selectively inhibit COX2 activity (Cho et al., 2004; Dao et al., 2004).

Chrysin has been described as a testosterone-increasing agent that blocks further testosterone metabolism by inhibiting aromatase activity (Dhawan et al., 2002; Kellis & Vickery, 1984). This testosterone-increasing activity of chrysin was confirmed in the recent studies with Leydig cells isolated from mouse and MA-10 mouse Leydig cell line, with the steroid hormone productions of the Leydig cells being significantly enhanced by chrysin

(Jana et al., 2008). However, it failed to induce the significant increase in steroid hormone when MA-10 Leydig cells were co-incubated with 22(R)hydroxycholesterol, suggesting that this flavonoid increase steroidogenesis mainly by improving the cholesterol transfer to the mitochondrial inner membrane. This was proved by the results from Western blot analyses that showed a significant increase in StAR protein in the Leydig cells treated with chrysin. The observations were corroborated by the significant increases in StAR promoter activity and StAR mRNA levels in the cells, indicating that chrysin acted on StAR promoter and enhanced StAR gene transcription. The transcription factors that act on StAR promoter were examined in the Leydig cells incubated with chrysin. It was found that this flavonoid significantly reduced the transcriptional repressor, DAX-1 protein (Jana et al., 2008). As DAX-1 protein and DAX-1 mRNA were reduced, StAR protein and steroid hormone production dramatically increased. Similar to the effect of COX2 inhibitors, chrysin did not affect PKA activity. In the absence of cAMP, this flavonoid alone was not able to increase StAR protein and steroidogenesis. However, in the presence of 10  $\mu$ m chrysin, sensitivity of Leydig cells dramatically increased, with sub-threshold levels of cAMP being able to induce maximal levels of StAR protein and steroidogenesis. The studies indicated that chrysin enhanced steroidogenesis in Leydig cells mainly by enhancing cAMP-induced StAR gene expression.

#### 4.2 Apigenin

Apigenin is present in various plants at different concentrations (Miean & Mohamed, 2001). It was found in parsley at high level (Nielsen et al., 1999). This flavonoid blocks COX2-dependent signaling at two separated steps, by inhibiting COX2 expression and blocking the TBX A2 receptors. It inhibits COX2 expression at the levels of transcription and translation. At the transcriptional level, apigenin inhibits activities of the transcription factors that are important for COX2 gene expression. Previous studies reported that incubation of mouse macrophages with apigenin reduced COX2 expression by inhibition of NF-kB activation, through a mechanism involving the apigenin-reduced degradation of the inhibitor kB (IkB) (Liang et al., 1999). This observation was enhanced by the study on the apigenin-reduced COX2 expression, in which apigenin-inhibited IkB $\alpha$  degradation resulted in reductions in NF-kB-binding to the promoter DNA and the TNF- $\alpha$ -induced COX2 expression (Shukla & Gupta, 2004). In addition, apigenin is able to inhibit the activation of MAPK. It is known that MAPK activation is essential for the activities of several transcription factors, such as CREB, NF-kB, and C/EBP, that induce COX2 expression in various tissues (Chun & Surh, 2004; Tsatsanis et al., 2006). The inhibitory effects of apigenin on MAPK activation significantly reduced COX2 gene transcription (Ha et al., 2008; Yi Lau & Leung, 2010). At the translational level, apigenin increased the localization to cytoplasm of two proteins, HuR and T-cell-restricted intracellular antigen 1-related protein (TIAR). HuR and TIAR then bound to the AU-rich elements in the 3'-untranslated region of COX2 mRNA and inhibited COX2 translation (Tong et al., 2007). In addition to its inhibitory effects on COX2 expression, apigenin acts as a natural antagonist of the TBX A2 receptors. Binding of apigenin to the receptors blocked the signaling through COX2-TBXAS-TBX A2-receptors pathway (Guerrero et al., 2007; Navarro-Nunez et al., 2008).

It was reported that blocking the signaling at any step of the COX2-TBXAS-TBX A2-receptors pathway significantly increased StAR gene expression and testosterone biosynthesis in Leydig cells (Pandey et al., 2009). Therefore, the effects of apigenin on steroidogenesis were studied using MA-10 mouse Leydig cell line and Leydig cells isolated



from mouse. The binding of apigenin to the TBX A2 receptors was examined. The binding competition between apigenin and the selective receptor antagonist  $^3\text{H}$ -SQ29548 indicated that this flavonoid specifically blocked the TBX A2 receptors on MA-10 mouse Leydig cells. Blocking the receptors with apigenin significantly increased StAR protein and testosterone production in mouse Leydig cells (Li et al., 2011). In MA-10 Leydig cells, apigenin induced concentration-dependent increases in StAR promoter activity, StAR mRNA, StAR protein and steroid hormone production. Further study indicated that this flavonoid enhanced StAR gene transcription by reduction of the transcriptional repressor, DAX-1 protein. While the mechanism for the reduction of DAX-1 protein in apigenin-treated cells is unknown, the results from the study suggest that minimal levels of PKA and protein kinase C (PKC) activities are essential for the effects of apigenin on DAX-1, StAR and steroidogenesis in Leydig cells (Li et al., 2011).

### 4.3 Luteolin

Luteolin is found in fruits and vegetables (Harnly et al., 2006). It was detected in bird chili (1035.0 mg/kg, in dry weight), belimbi leaves (464.5 mg/kg), onion leaves (391.0 mg/kg), belimbi fruit (202.0 mg/kg), dried asam gelugur (107.5 mg/kg), local celery (80.5 mg/kg), broccoli (74.5 mg/kg), carrot (37.5 mg/kg), limau purut leaves (30.5 mg/kg), French bean (11.0 mg/kg), and white radish (9.0 mg/kg) (Miean & Mohamed, 2001). Similar to the effects of apigenin, luteolin is able to inhibit COX2 expression and block the TBX A2 receptors. It was reported that luteolin reduced COX2 expression by inhibiting NF- $\kappa$ B activity (Chen et al., 2007a; Gutierrez-Venegas et al., 2006; Kim & Jobin, 2005), through the mechanism involving the luteolin-reduced I $\kappa$ B kinase (IKK) activity, I $\kappa$ B degradation, nuclear translocation of NF- $\kappa$ B p65 subunit, and NF- $\kappa$ B-binding to the promoter DNA. Luteolin may reduce COX2 expression by inhibiting activity of MAPK and subsequently reducing the activities of the transcription factors that are essential for COX2 transcription (Choi & Lee, 2010; Gutierrez-Venegas et al., 2006). It is known that COX2 expression is up-regulated by inflammatory cytokines, such as IL-1 $\beta$  and TNF- $\alpha$ . Luteolin was reported being able to reduce these cytokines, which may contribute to its inhibitory effect on COX2 expression (Wu et al., 2009). In addition, luteolin is also a natural antagonist of the TBX A2 receptors. It blocked the COX2-dependent signaling through COX2-TBXAS-TBX A2-receptor pathway by binding to the receptors (Guerrero et al., 2005; Guerrero et al., 2007).

The inhibitory effect of luteolin on COX2-dependent signaling suggests a possibility of using this flavonoid to enhance StAR gene expression and steroidogenesis in Leydig cells. This possibility was examined by the experiments with MA-10 mouse Leydig cells cultured for 6 hours in the medium containing increasing concentrations of luteolin and a cAMP analog (dbcAMP). StAR and DAX-1 proteins, steroid production, StAR mRNA and StAR promoter activity were analyzed as described in the previous study (Jana et al., 2008). As shown in Fig. 2A, the treatments with luteolin induced a concentration-dependent increase in steroid hormone production. Progesterone concentrations in culture medium were increased from 4.9 to 124.7ng/ml, as the levels of luteolin in the culture were increased from 0 to 12  $\mu$ M. In the presence of 22(R)hydroxycholesterol(22R), there was no significant difference in steroid production among the treatments, suggesting that luteolin increase steroidogenesis by improving mitochondrial cholesterol transfer in Leydig cells. The results were enhanced by the luteolin-increased StAR protein expression. Similar increases were observed in the analyses of StAR promoter activity and StAR mRNA levels (Fig. 2B), indicating a regulatory effect of luteolin on StAR gene transcription. In addition, a synergistic interaction between

luteolin and cAMP was observed (Fig. 2C), in which luteolin reduced the threshold of cAMP-induced StAR gene expression and increased the sensitivity of Leydig cells to cAMP stimulation. Further study indicated that luteolin enhanced StAR gene transcription by inhibiting DAX-1 expression (Fig. 2D), similar to the observation with apigenin.

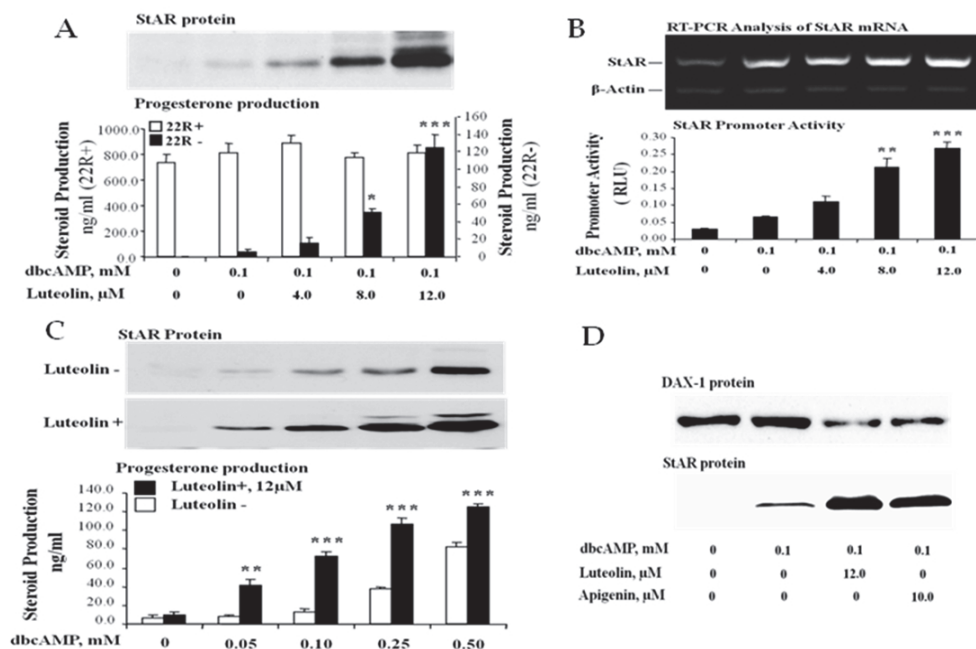


Fig. 2. Effect of luteolin on StAR gene expression and steroidogenesis in MA-10 mouse Leydig cells. MA-10 cells were incubated with luteolin for 6 hours, and then collected for analyses: A, StAR protein and steroidogenesis; B, StAR gene transcription; C, Interaction between luteolin and cAMP; D, Reduction in DAX-1 protein.

#### 4.4 Quercetin

Quercetin was reported as one of the major flavonoids in the plants (Miean & Mohamed, 2001), especially in onions (Slimestad et al., 2007). It blocks the COX2-dependent signaling by inhibiting COX2 expression and TBX A2 production. Similar to other flavonoids, quercetin depresses COX2 expression by inhibiting activities of NF-kB. It was observed that this flavonoid reduced the activity of NF-kB by inhibiting IKK/I $\kappa$ B signaling cascade (Garcia-Mediavilla et al., 2007), which in turn reduced NF-kB nuclear translocation, its binding to promoter, and COX2 expression (Crespo et al., 2008; Puangpraphant & de Mejia, 2009). Another signaling pathway regulating COX2 expression is PI3K-Akt pathway. This signaling pathway may activate CREB (Alique et al., 2011) or NF-kB (Yang et al., 2009), and induce COX2 expression. A recent study found that quercetin directly bound with PI3K to inhibit PI3K activity and Akt phosphorylation, resulting in a reduction in COX2 expression (Lee et al., 2010). In the LPS-induced COX2 expression, the members of MAPK, including extracellular signal related kinase (ERK), p38 and JNK, are involved in the LPS-induced

signaling. While LPS activates the MAPKs, quercetin inhibited the activation of each of them, and subsequently reduced I $\kappa$ B phosphorylation and COX2 expression (Gutierrez-Venegas et al., 2007). The observations were enhanced by the study on the inhibitory effects of quercetin on ROS-induced MAPK activation and COX2 expression (Huang et al., 2006). The inhibition of MAPKs by quercetin was associated with the reductions in inflammatory cytokines, such as IL-1 $\beta$  and TNF- $\alpha$  (Overman et al., 2011). In addition, quercetin is able to block the COX2-TBXAS-TBX A2-receptor signaling pathway by inhibition of TBX A2 formation (Garcia-Saura et al., 2005; Sheu et al., 2004).

The steroidogenic effect of quercetin was studied with MA-10 mouse Leydig cells. It was reported that quercetin increased StAR mRNA levels, StAR promoter activity and steroid hormone production (Chen et al., 2007c). The observations were corroborated by the results shown in Fig. 3, which indicated the quercetin-enhanced StAR gene transcription and translation in MA-10 cells cultured for 6 hours (Fig. 3 A and B). Following these studies, the transcription factors in StAR gene transcription were examined. It was found that the incubation of MA-10 Leydig cells with quercetin reduced DAX-1 protein, similar to the effects of other flavonoids. The reduction in DAX-1 protein was associated with a dramatic increase in StAR protein (Fig. 3C), suggesting that quercetin enhance StAR gene expression by reduction of DAX-1 expression.

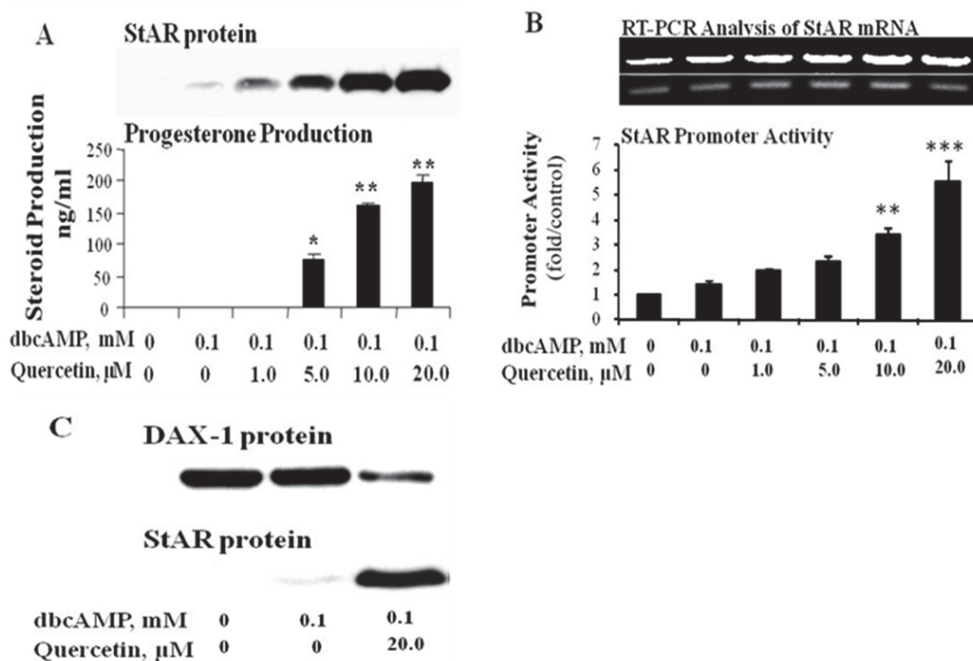


Fig. 3. Effect of quercetin on StAR gene expression and steroidogenesis in MA-10 mouse Leydig cells. MA-10 cells were incubated with quercetin for 6 hours, and then collected for analyses: A, StAR protein and steroidogenesis; B, StAR gene transcription; C, Reduction in DAX-1 protein

#### **4.5 Potential application of natural flavonoids in delaying the declines in StAR gene expression and testosterone biosynthesis**

The studies reviewed above suggest that flavonoids are potential resources for alternative medicine to delay the decline in StAR gene expression. Among the various compounds of natural flavonoids, many of them are able to reduce COX2 expression, such as Kaempferol (Garcia-Mediavilla et al., 2007), tectorigenin, tectoridin (Kim et al., 1999), wogonin (Chen et al., 2008) and Silibinin (Kim et al., 2009). Some of the flavonoids reduce the inflammatory factors that induce COX2 expression (Garcia-Lafuente et al., 2009; Khanna et al., 2007; Kim et al., 2004). Another group of flavonoids was reported as receptor antagonists that inhibit the COX2-dependent signaling by blocking the TBX A2-binding to the receptors (Navarro-Nunez et al., 2009). In addition to the COX2-dependent signaling, StAR gene expression is affected by the signaling through different pathways. For example, calcium influx through L-type calcium channels regulates the threshold of cAMP-induced StAR gene expression, so that blocking the L-type calcium channels reduced the threshold and enhanced StAR gene expression in Leydig cells (Pandey et al., 2010). There is a group of natural flavonoids that are able to block the L-type calcium channels, such as genistein (Belevych et al., 2002), daidzein (Yokoshiki et al., 1996), equol (Liew et al., 2003) and epigallocatechin-3-gallate (EGCG) (Kang et al., 2010). The steroidogenic effects of the flavonoids in this group were described (Yu et al., 2010). It is possible to identify different flavonoids that might enhance StAR gene expression and testosterone biosynthesis through different mechanisms.

The potential for application of natural flavonoids in delaying the decline in testosterone is supported by the animal studies on the flavonoid-enhanced testosterone production and reproductive function. A study with 2-year-old male rats reported that supplementation with chrysin improved their reproductive functions, with their sperm count, fertilization potential and litter size being increased when they were allowed to interact with female rats (Dhawan et al., 2002). It was also observed that lifetime exposure to the flavonoids, including daidzein, glycitein and genistein, increased serum and testicular testosterone concentrations of rats (McVey et al., 2004). The observation was enhanced by the study on the effects of catechins on testosterone biosynthesis in rats, in which injection with catechins or its derivatives increased testosterone in blood (Yu et al., 2010). A recent study reported the beneficial effects of quercetin in diabetic rats, with their sperm viability and motility being improved by the flavonoid. These effects of quercetin are associated with an increase in serum total testosterone (Khaki et al., 2010). In addition, some flavonoids in herbal medicine are able to enhance testosterone production in rats. For example, icariin, a flavonoid in the plants in *Epimedium* family, has been extracted from traditional Chinese medicine (Du et al., 2002). It was observed that supplementation of icariin to 15-month-old male rats significantly increased their blood testosterone levels (Zhang & Yang, 2006).

For application of natural flavonoids to improve the health of aging males, further studies, especially long-term animal studies and clinical studies, are needed. Two of the important considerations in the studies are mechanism and efficacy of the flavonoid-enhanced testosterone biosynthesis. The mechanisms reported for the inhibitory effects of flavonoids on COX2-dependent signaling are mostly based on the studies with the cells from various tissues. These mechanisms need to be verified with Leydig cells. In addition to the COX2-dependent signaling, other mechanisms might be involved in the flavonoid-enhanced StAR gene expression and testosterone production. Further studies on the mechanisms may improve the understanding of the steroidogenic effects of flavonoids. Regarding the efficacy, bioavailability is an important factor being discussed in the studies on flavonoids

(Setchell et al., 2001; Setchell et al., 2002). Specifically in the flavonoid-enhanced StAR gene expression and testosterone production, the following factors have been discussed recently: 1) Biphasic effects of flavonoids on StAR gene expression. As mentioned above, some of the flavonoids are able to block L-type calcium channels. It was reported that blocking L-type calcium channel produced biphasic effects on StAR gene expression in Leydig cells (Pandey et al., 2010). While these flavonoids might enhance StAR gene expression by reducing the transcriptional repressor DAX-1 protein, they might inhibit the positive effect of calcium-dependent signaling on StAR gene expression; 2) Inhibitory effects of flavonoids on steroidogenic enzymes. It was reported that some of the flavonoids are able to inhibit activities of the enzymes involved in testosterone biosynthesis (Figueiroa et al., 2009; Hu et al., 2010). These inhibitory effects may reduce the efficacy of flavonoid-enhanced testosterone production; 3) Levels of cAMP in Leydig cells. The levels of cAMP-PKA-phosphorylation are critical for the flavonoid-enhanced StAR gene expression in Leydig cells. As shown in Fig. 2C, in the absence of cAMP, flavonoid alone is not able to induce significant increases in StAR protein expression and steroid hormone production (Jana et al., 2008; Li et al., 2011). In the studies without exogenous cAMP, the endogenous cAMP becomes important for the steroidogenic effect of flavonoids. When the levels of endogenous cAMP or PKA activity in Leydig cells are not sufficient, flavonoid might not be able to induce significant increase in StAR gene expression. Therefore, minimal level of cAMP or PKA activity should be considered for the flavonoid-enhanced StAR gene expression and testosterone biosynthesis.

## 5. Summary

In summary, LH-stimulation of Leydig cells induces both positive and negative signals in the regulation of StAR gene expression and testosterone biosynthesis. In the aging process, the increase in COX2 enhances the negative signaling, resulting in the declines in StAR gene expression and testosterone biosynthesis in Leydig cells. It was found that some of the flavonoids are able to block the COX2-dependent signaling and enhance StAR gene expression. While further investigations are needed, the studies suggest a potential for application of the natural flavonoids in delaying the age-related declines in StAR gene expression and testosterone biosynthesis.

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# Role of Central Insulin-Like Growth Factor-1 Receptor Signalling in Ageing and Endocrine Regulation

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## 1. Introduction

Insulin-like growth factors (IGFs) are, besides other mechanisms, controlled by growth hormone (GH) secretion and there are at least two different IGFs: IGF-1 and IGF-2. IGFs regulate various cellular processes e.g. survival, differentiation and proliferation (McMorris et al. 1986; McMorris & Dubois-Dalcq 1988; Mozell & McMorris 1991).

Growth hormone, which induces IGF-1 secretion from the liver, is generated in the anterior pituitary and regulated by the hypothalamus via growth hormone releasing hormone (GHRH) and growth hormone inhibiting hormone (GHIH) (Jansson et al., 1985; Carlsson & Jansson 1990). IGFs in the brain are synthesised *de novo* or transported across the blood brain barrier (BBB) and seem to induce a variety of effects on the central nervous system (CNS). So far, the exact transport mechanisms of IGFs into the brain are not fully understood (Duffy, Pardridge, and Rosenfeld 1988).

In the rodent brain, IGF-1 is mainly expressed in neuron-dense regions like the hippocampus, striatum, thalamus, hypothalamus and olfactory bulb (Rotwein et al. 1988; Bondy et al. 1990; Garcia-Segura et al. 1991). Accordingly, the insulin-like growth factor-1 receptors (IGF-1Rs) are mainly found in the olfactory bulb, cerebellar cortex and granule cell layer of the dentate gyrus (Rotwein et al. 1988; Bondy et al. 1990; Bondy and Lee 1993; Werther et al. 1990). However, the function of IGF-1R mediated signals in the central nervous system (CNS) is still under investigation.

After binding of IGF-1 to the IGF-1R, a signalling cascade is activated which leads to recruitment and subsequently phosphorylation of intracellular adaptor proteins, the so called insulin receptor substrates (IRS) (Jacobs et al. 1983; Rubin et al., 1983; Sun et al. 1991; Lavan et al. 1997; Lavan et al., 1997). Via these adaptor proteins, the MAP kinase (MAPK, mitogen activated protein kinase) cascade, as well as the phosphatidylinositol(PI)3-kinase signalling pathway, is turned on leading to protein kinase B (PKB/AKT) activation, which phosphorylates forkhead box O transcription factors (FoxO) causing their nuclear export (Stokoe et al. 1997; Alessi et al. 1996; Partridge & Bruning 2008). FoxO mediated transcription is involved in cell proliferation, differentiation and oxidative stress defence (Clark et al. 1993; Brunet et al. 1999; Dijkers et al. 2000; Dijkers, Medema, Pals et al. 2000; Medema et al. 2000) suggesting a function of FoxO during development and cellular stress response.

Recently, the function of the IGF-1R signalling pathway in neurons has been investigated using different model organisms like worms and flies as well as conventional and conditional mutagenesis in mice. These experiments revealed possible functions of IGF-1 mediated signals in endocrine regulation, longevity, protein turnover and in the pathogenesis for ageing-associated diseases e.g. Alzheimer's Disease (AD). The current article discusses the mechanisms of regulation and the physiological as well as a possible pathophysiological role of IGF-1 mediated signals in the CNS.

## **2. The GH/insulin-like growth factor system**

### **2.1 The somatotrophic system**

Growth hormone (GH, somatotropin) is produced in the anterior pituitary by certain specified cells and regulated via the hypothalamus by the growth hormone releasing hormone (GHRH, somatoliberin), the growth hormone inhibiting hormone (GHIH, somatostatin), but also by suppression of the short loop negative feedback of GH itself (Jansson, Eden, and Isaksson 1985; Carlsson and Jansson 1990).

The GH-producing cells of the anterior pituitary, also called somatotropes or somatotroph, form 30-40 % of the anterior pituitary (Gage et al. 1996). These cells require transcription factors of the POU-family (Mullis 2010) including the homeoproteins Pit-1 and Prop-1 (prophet of Pit-1) for normal embryonic development and differentiation (Mullis 2005) as well as for GH expression. The expression of these transcription factors and therefore the differentiation of the somatotroph are controlled by GHRH producing neurons of the hypothalamus. Additionally, GH-release in the pituitary is inhibited by serum IGF-1 and enhanced by serum ghrelin (Wortley et al. 2005; Zigman et al. 2005) (Figure 1).

In the periphery, GH mainly influences growth and development via IGF-1. IGF-1 and IGF-2 can act endocrine, paracrine and autocrine (Cohick and Clemmons 1993; Stewart and Rotwein 1996; Mohan, Baylink, and Pettis 1996; Butler and LeRoith 2001). Additionally, there is a truncated form of IGF-1, Des(1-3)IGF-1, which lacks the tripeptide Gly-Pro-Glu at the N-terminus probably resulting from post-translational cleavage of IGF-1. Des(1-3)IGF-1 is mainly expressed in brain and active in lower concentrations than un-truncated IGF-1 (Ballard et al. 1996; Francis et al. 1988; Carlsson-Skwirut et al. 1989; Ogasawara et al. 1989). Studies on hypoxic-ischemic brain injuries in rats suggest that the tripeptide fragment has neuroprotective properties and influences neuronal activity (Guan et al. 1999).

Circulating IGF-1 is mainly produced in the liver, but both IGF-1 and IGF-2 are expressed in many non-hepatic tissues (Cohick and Clemmons 1993; Stewart and Rotwein 1996; Butler and LeRoith 2001). The most important effects of IGFs are cell proliferation and differentiation, skeletal growth and mineralisation as well as the development and function of the reproductive organs and the central nervous system (CNS) (Duan and Xu 2005).

GH is secreted pulsatile in rodents as well as in humans (Jansson, Eden, and Isaksson 1985; Frohman and Jansson 1986; Giustina and Veldhuis 1998) and follows a circadian pattern with one major peak after falling asleep. There is a sexual dimorphism in GH secretion, which is more dominant in rodents than in humans (Jansson, Eden, and Isaksson 1985; Tannenbaum and Martin 1976). In females, the GH secretory pattern seems to be less regular compared to males (Giustina and Veldhuis 1998), with higher basal interpulse GH levels, more frequent but lower amplitude pulses and a lower major nocturnal GH pulse (Jaffe et al. 1998; Jessup et al. 2003). Spontaneous and GHRH-stimulated GH secretion is suppressed more efficiently by IGF-1 in men than in women (Ohlsson et al. 2009) suggesting that also

sex steroids interfere with GH secretion (Veldhuis et al. 2008, 2009; Leung et al. 2004). Body growth is influenced more efficiently by GH if it is applied in a pulsatile fashion than applied continuously (Jansson, Eden, and Isaksson 1985; Clark et al. 1985). This might be due to a higher induction of IGF-1 expression in growth plates and skeletal muscles by pulsatile GH, whereas continuous GH secretion stimulates liver IGF-1 expression and serum IGF-1 levels at least as well as a pulsatile GH release (Isgaard et al. 1988; Bick et al. 1992). Therefore, liver-derived IGF-1 is not essential for body growth itself. This effect might be achieved by locally produced IGF-1 and/or other factors (Ohlsson et al. 2009).

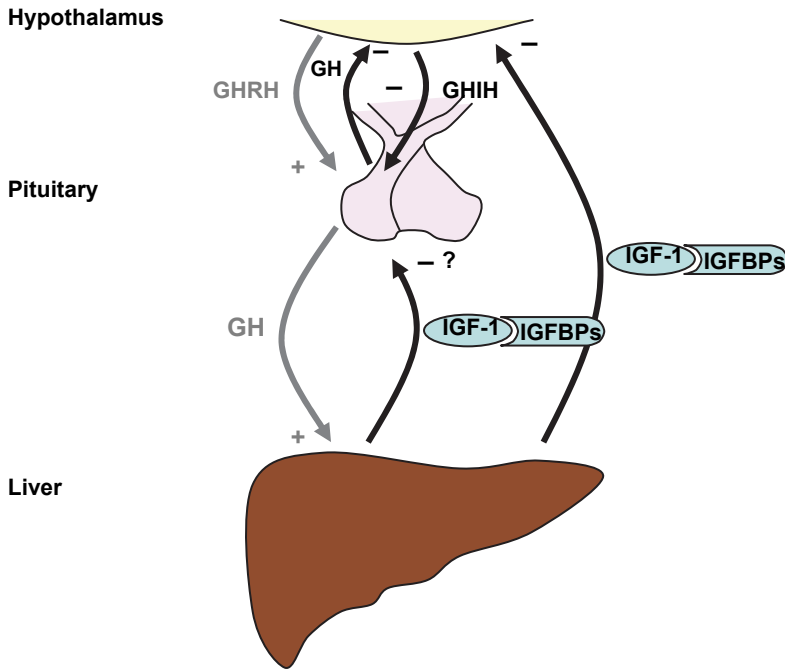


Fig. 1. GH/ IGF-1 system

Growth hormone releasing hormone (GHRH) and growth hormone inhibiting hormone (GHIH) are produced in neurons of the hypothalamus and transported via the hypothalamo-pituitary portal vein system to the anterior pituitary where they regulate growth hormone (GH) secretion and expression. GH stimulates insulin-like growth factor-1 (IGF-1) production in the liver. In addition, free IGF-1 serum levels are regulated by binding to the insulin-like growth factor binding proteins (IGFBPs). A short negative feedback loop of GH, as well as a negative feedback mechanism of IGF-1 on the hypothalamus and possibly on the anterior pituitary, determines serum IGF-1 levels.

Mouse models with tissue specific IGF-1 inactivation indicate that normal longitudinal bone growth is maintained by local bone-derived IGF-1 (Sjogren et al. 1999; Yakar et al. 1999). However, several other phenotypes, for example the one observed in the liver IGF-1

deficient (LID) mouse, suggest that locally derived IGF-1 cannot fully replace liver-derived IGF-1 function and vice versa.

A lacking negative feedback of liver-derived IGF-1 on GH secretion leads to a compensatory increase in serum GH levels in mice (Yakar et al. 1999; Sjogren et al. 1999) and also in humans (Veldhuis et al. 2001). Hence, the phenotype of the LID mice might either arise directly or is mediated by the elevated GH-levels. LID mice show an increased expression of pituitary GHRH- and ghrelin-receptors indicating that at least some action of up-regulating GH secretion influences the pituitary (Ohlsson et al. 2009). However, the target sites of IGF-1 for regulating GH-release remain unclear and might either be located in the hypothalamus and/ or the pituitary. Recently, a cell-specific knockout mouse in which the IGF-1 receptor (IGF-1R) was ablated from the somatotroph, the so called somatotroph IGF-1 receptor knockout (SIGFRKO) mouse, has been generated (Romero et al. 2010). The phenotype of the SIGFRKO mouse includes increased GH expression and secretion as well as increased serum IGF-1 levels (Romero et al. 2010). Feedback mechanisms in the hypothalamus resulted in decreased GHRH and increased GHIH mRNA levels (Romero et al. 2010). Furthermore, decreased growth hormone releasing hormone receptor (GHRH-R) expression was observed in the anterior pituitary (Romero et al. 2010). However, these changes were not able to reduce GH secretion in the SIGFRKO mouse indicating a role of IGF-1R signalling in the pituitary in addition to the hypothalamus in regulating GH secretion (Romero et al. 2010).

IGF-1 influences glucose metabolism directly, e.g. by inducing insulin-like effects on glucose-uptake in tissues expressing IGF-1-receptors, but also by suppressing the GH-release from the pituitary. GH is known to have diabetogenic effects (Yakar et al. 2004) by decreasing insulin-sensitivity in skeletal muscle, liver and fat. Consequently, LID mice show an impaired insulin-sensitivity. At the same time, those mice are protected against the increase in body fat mass that occurs in older age (Sjogren et al. 2001; Tang et al. 2005). This might be explained by elevated GH-levels in these mice (Ohlsson et al. 2009) as decreased GH secretion in both rodents and humans has been proposed to contribute to age-related obesity (Sonntag et al. 1980; Rudman et al. 1990).

The inactivation of IGF-1 or its receptor during early development reduces brain size including a reduction of the granule cell layer in the dentate gyrus and the number of oligodendrocytes and myelinated axons (Beck et al. 1995; Holzenberger et al. 2001; Vicario-Abejon et al. 2003) demonstrating the importance of IGF-1 signalling during brain development. LID mice show a milder phenotype with reduced exploratory activity (Svensson et al. 2005; Bohlooly et al. 2001), as well as impaired spatial learning and memory, suggesting that liver-derived IGF-1 enhances brain functions (Svensson et al. 2006). Circulating IGF-1 is essential for mediating exercise induced effects on the adult brain by promoting the numbers of newly generated neurons in the adult hippocampus, brain vessel growth, spatial learning and reducing anxiety (Trejo, Carro, and Torres-Aleman 2001; Trejo, Llorens-Martin, and Torres-Aleman 2008; Lopez-Lopez, LeRoith, and Torres-Aleman 2004). Additionally, liver-derived IGF-1 might enhance the clearance of brain amyloid- $\beta$  ( $A\beta$ ), whose aggregation is one of the hallmarks of AD (see 5.1).

## 2.2 IGF-binding proteins

Only a small amount of IGF-1 (~1 %) circulates “free” within the blood. The remaining 99 % are bound to IGF-binding proteins (IGFBPs). IGFBPs are a family of at least six proteins (IGFBP-1 to IGFBP-6), which bind IGF-1 with high affinities that are equal to or greater than

those of the IGF-1 receptor (Duan and Xu 2005). Additionally, there are several proteins with lower IGF-1 binding affinity, called IGFBP-related peptides (IGFBP-rPs), which have significant structural homologies with the amino(N)-terminal region of high-affinity IGFBPs (Rechler 1993; Kelley et al. 1996; Rajaram, Baylink, and Mohan 1997; Kim et al. 1997; Poretsky et al. 1999; Baxter 2000). So far, the functions of the IGFBP-rPs, also referred to as IGFBP-7 to -10, are not known (Mohan and Baylink 2002). Approximately 75-80 % of IGF is present as a 150 kDa complex, which consists of IGF-1/IGF-2 plus IGFBP-3 (70-75 %) or IGFBP-5 (5-10 %) and an acid-labile subunit (ALS) (Baxter, Meka, and Firth 2002). ALS is mainly produced in the liver (Baxter 1988; Baxter and Martin 1989; Baxter, Martin, and Beniac 1989) and its gene disruption leads to a reduction of circulating IGFs of 80 % (Boisclair et al. 2001). The ternary complex of IGF-1 or -2/ IGFBP-3 or -5/ ALS extends the half-life of IGFs to 15-20 h, compared to a half-life of 20-30 min for free IGF-1 in circulation (Guler, Zapf, and Froesch 1987) suggesting a reservoir function of this complex, which cannot cross the vascular endothelial barrier (Rajaram, Baylink, and Mohan 1997; Baxter 2000). 20-25 % of the IGFs bind to IGFBP-1,-2,-4 or -6 to form a complex, which is able to cross vascular endothelium (Baxter 2000; Rajaram, Baylink, and Mohan 1997). Thus, the endocrine actions of IGF-1 in serum are regulated by the IGFBPs determining how much IGF is bio-available to the local tissues.

Furthermore, IGFBPs are located in the extracellular matrix or on the cell surface where they either inhibit (mainly IGFBP-4 and -6) or potentiate (mainly IGFBP-3 and -5) IGF-1 binding to its receptors (Rajaram, Baylink, and Mohan 1997; Mohan et al. 1995; Qin et al. 1998; Jones and Clemmons 1995; Firth and Baxter 2002; Rechler and Clemmons 1998). Functions of the IGFBPs might vary indicated by the inhibiting or potentiating effect of IGFBP-1, -2, -3 and -5 on IGF-1 action depending on the experimental conditions (Yin, Xu, and Duan 2004).

The actions of IGFBPs are, in turn, modulated by IGFBP proteases that are further dependent on activators and inhibitors (Mohan et al. 2002). Those IGFBP proteases are able to cleave IGFBPs, thereby reducing their affinity for IGF-1. Some of them are relatively specific for a given IGFBP. For example, pregnancy-associated plasma protein-A (PAPP-A), which is produced by a variety of cell types, cleaves specifically IGFBP-4 (Lawrence et al. 1999; Byun et al. 2001; Conover et al. 2001), whereas complement C1s (Busby et al. 2000) and a disintegrin and metalloprotease-like (ADAM)-9 (Mohan et al. 2002) were identified to be relatively specific for IGFBP-5. Additionally, a number of other serum proteases were shown to be capable of cleaving IGFBPs, e.g. plasmin, cathepsin D and prostate specific antigen (Conover 1995; Fowlkes et al. 1995; Rajah et al. 1995; Rajaram, Baylink, and Mohan 1997; Maile and Holly 1999). Therefore, the binding affinity and hence the bio-availability of IGF-1 is regulated via the degradation of IGFBPs through the IGFBP protease system.

IGFBPs also have IGF independent functions, for example IGFBP-2 and -3 can induce direct cellular effects (Firth and Baxter 2002; Oh et al. 1993; Yamanaka et al. 1999; Schutt et al. 2004) and it has been shown that IGFBP-3 at least partially mediates these effects by binding to the cell surface, possibly to specific receptors (Mohseni-Zadeh and Binoux 1997; Rechler and Clemmons 1998). IGFBP-2, -3 and -5 contain sequences for nuclear localisation (Schutt et al. 2004; Radulescu 1994; Schedlich et al. 1998; Hoeflich et al. 2004) and might as well influence gene expression.

### **2.3 The insulin- and insulin-like growth factor-1 signalling pathway**

Insulin and IGF-1 receptors are receptor tyrosine kinases. Receptor tyrosine kinases contain a membrane-bound domain with tyrosine kinase activity which phosphorylates tyrosine-residues of downstream signalling proteins. Typical members of the receptor tyrosine kinase

family are the epidermal growth factor receptor (EGFR), the nerve growth factor receptor (NGFR) and the insulin receptor (IR). The IR was discovered in 1974 and its tyrosine kinase was found in 1982 (Kasuga, Karlsson, and Kahn 1982; Kasuga et al. 1982). Later, in addition to the IR, the insulin-like growth factor-1 receptor (IGF-1R) was discovered as a tyrosine kinase activity containing receptor (Jacobs et al. 1983; Rubin, Shia, and Pilch 1983).

The IR and IGF-1R are heterotetrameric structures. The different subunits are linked by disulfide bonds. The  $\alpha$ -subunits are exclusively localised extracellular (Van Obberghen et al. 1981; Ullrich et al. 1986). The  $\beta$ -subunits consist of a short extracellular part, a transmembrane domain and an intracellular part with ATP-binding motifs, autophosphorylation sites and tyrosine-specific protein kinase activity, which is activated after binding of insulin or IGF-1 to their receptors (Chou et al. 1987).

The insulin receptor gene consists of 22 exons and 21 introns (Seino et al. 1989). Alternative splicing of exon 11, which codes for 12 amino acids, causes two different isoforms: A, which lacks the 12 amino acids, and B. This alternative splicing does not exist for the IGF-1 receptor, because this receptor contains no equivalent to exon 11 of the IR. The A- and B-isoforms both bind to insulin with similar affinity (McClain 1991). The A-isoform has a higher affinity to IGF-1 (Yamaguchi et al. 1991) and IGF-2 (Frasca et al. 1999) compared to the B-isoform. The A-isoform is expressed in hematopoietic cells, fetal tissue and the adult nervous system. The B-isoform of the IR is mainly present in liver, adipose tissue and muscle (Seino and Bell 1989; Moller et al. 1989; Goldstein and Kahn 1989; Mosthaf et al. 1990). The selective binding of insulin or IGF-1 is also dependent on the assembly of the receptors (Pandini et al. 2002). The hybrid of IGF-1 receptor and the A-isoform of the IR binds IGF-1, IGF-2 and insulin with similar affinity. The hybrid of the IGF-1R and the B-isoform of the IR only binds IGF-1 (Louvi, Accili, and Efstratiadis 1997).

### 2.3.1 Insulin receptor substrates

The binding of IGF-1 or IGF-2 to the IGF-1R causes a conformational change of the receptor, which induces autophosphorylation. This leads to the recruitment of insulin receptor substrates (IRS) to the autophosphorylated receptor tyrosine kinase, which in turn phosphorylates the tyrosine residues of the IRS proteins (Figure 2). The IRS protein family consists at least of four proteins, IRS-1 to IRS-4 (Sun et al. 1991; Lavan et al. 1997; Lavan, Lane, and Lienhard 1997).

The IRS proteins show different expression patterns. IRS-1 and -2 are ubiquitously expressed, but IRS-3 is only present in rodent adipose tissue. IRS-4 mainly occurs in thymus, hypothalamus, kidney and heart. All IRS proteins share the same structural characteristics and have similar functions (Giovannone et al. 2000; Schubert et al. 2003).

The four IRS family members contain an N-terminal pleckstrin homology (PH) domain, a phosphotyrosine-binding (PTB) domain and a C-terminal tail containing multiple tyrosine phosphorylation sites. The phosphotyrosine motifs of the IRS proteins are binding sites for Src homology(SH)2 domain-containing proteins (Yenush and White 1997). The PH domain binds to lipids and with high affinity to phosphoinositides (Fruman, Rameh, and Cantley 1999). The PTB domain of IRS binds to phosphotyrosine residues of other proteins including the IR and IGF-1R. In more detail, the PTB domain binds to the phosphorylated NPXP motif at the juxtamembrane domain of the receptor after binding of insulin or IGF-1. Following binding to this motif, the IRS proteins are tyrosinephosphorylated (Cheatham and Kahn 1995; White 2002).



Only IRS-2 contains a domain which binds to the phosphorylated kinase regulatory loop of the  $\beta$ -subunit of the IR. This domain is called the KLRB domain (Sawka-Verhelle et al. 1997; Sawka-Verhelle et al. 1996). However, the physiological function of the KLRB domain of IRS-2 remains unclear.

Insulin induces tyrosine and serine phosphorylation of IRS-1 (Gual, Le Marchand-Brustel, and Tanti 2005). These phosphorylations lead to specific regulation of downstream signalling. The phosphorylation of serine residues of IRS-1 contribute to positive or negative regulation of IRS-1 action (Weigert et al. 2005; Weigert et al. 2008). Important for this regulation are the particular phosphorylation sites (Herschkovitz et al. 2007) as well as the timing of phosphorylation (Weigert et al. 2005; Weigert et al. 2008). Currently, the serine phosphorylation sites with positive effect on IRS-1 action are regarded to be phosphorylated at first to support IRS-1 activity protecting from phosphorylation at residues with inhibitory effect (Weigert et al. 2005; Weigert et al. 2008; Gual, Le Marchand-Brustel, and Tanti 2005; Luo et al. 2007). In addition, serine phosphorylation with activating effect might prevent the association of IRS-1 with tyrosine phosphatases (Luo et al. 2005). The serine residues with inhibitory effect are located near the PTB domain. These residues are phosphorylated later than the residues with positive effect upon insulin stimulation or other signals. The phosphorylation of serine sites near the PTB domain causes disruption of the binding between IRS-1 and the IR followed by degradation of IRS-1. The phosphorylation of inhibitory serine residues in the C-terminus of IRS-1 disturb their interaction with the phosphatidylinositide (PI)3-kinase (Figure 2) (Gual, Le Marchand-Brustel, and Tanti 2005; Boura-Halfon and Zick 2009). Serine sites with inhibitory effect are phosphorylated by serine kinases like the mammalian target of rapamycin (mTor), PKCzeta and p70S6 (S6K) kinase (Boura-Halfon and Zick 2009; Herschkovitz et al. 2007; Gual et al. 2003). Insulin and IGF-1 resistance might be induced via kinases like c-Jun N-terminal kinase (JNK), mTor/S6K, inhibitory- $\kappa$ B kinase  $\beta$  (IKK $\beta$ ), SIK-2 and extracellular signal regulated kinases (ERK) promoting the phosphorylation of the inhibitory sites of the IRS proteins (Boura-Halfon and Zick 2009; Herschkovitz et al. 2007). The IRS-2 serine phosphorylation sites are still under investigation. It is known that JNK phosphorylates Thr348 of IRS-2 which is located near the PTB domain (Solinas et al. 2006) and might cause disruption of the binding between IRS-2 and the receptor. Furthermore, JNK phosphorylates Ser488 of IRS-2 promoting the phosphorylation at Ser484 by glycogen synthase kinase (GSK)-3 $\beta$  and thereby inhibiting the signalling pathway (Sharfi and Eldar-Finkelman 2008).

### 2.3.2 PI3K signalling

The mammalian phosphatidylinositide(PI)3-kinases are subdivided into three classes, class I-III. Class I is further divided into Ia and Ib (Vanhaesebroeck et al. 2005). These classes catalyse the phosphorylation of the 3'hydroxyl position of phosphatidyl-myo-inositol lipids. The PI3K of the insulin and IGF-1 signalling pathway belongs to the class Ia kinases (Fruman, Meyers, and Cantley 1998). These kinases display a heterodimeric structure containing a catalytic subunit of 110 kDa. This subunit is non-covalently associated with a 50-, 55- or 85 kDa regulatory subunit. Following activation of the insulin receptor and IRS binding, the PI3K is recruited to the membrane via the p85 regulatory subunit. Other recruited factors are the growth factor receptor binding protein (GRB)-2 and the SH2-Phosphatase(SHP)2 (Figure 2).

The activated PI3K, in turn, phosphorylates phosphatidylinositide-diphosphate (PI<sub>4,5</sub>P) to produce phosphatidylinositide-triphosphate (PI<sub>3,4,5</sub>P). This event is reversible by PTEN (the

phosphatase and tensin homolog deleted on chromosome ten). The generation of  $PI_{3,4,5}P$  causes activation of the downstream signalling proteins like phosphoinositide-dependent protein kinase (PDK) and protein kinase B (PKB, AKT). PDK is present in two isoforms, PDK-1 and PDK-2. PDK-1 phosphorylates AKT at Thr308, which partially activates AKT. To completely activate AKT, phosphorylation of Ser473 is necessary (Alessi et al. 1996; Lawlor and Alessi 2001; Stokoe et al. 1997). AKT is a serine/threonine kinase with a size of 57 kDa. It contains a PH domain and there are three isoforms, AKT-1, AKT-2 and AKT-3. These isoforms display a conserved domain structure: a kinase domain, a PH-domain at the N- as well as a regulatory subunit at the C-terminus (Hresko, Murata, and Mueckler 2003). AKT phosphorylates tuberlin 2 (TSC-2). TSC-1 and -2 form a heterodimer with GTPase activity that inhibits the GTPase RHEB (RAS homolog enriched in brain). The phosphorylation via AKT causes the accumulation of the RHEB-GTP complex which activates mTOR (Astrinidis and Henske 2005; Hay and Sonenberg 2004). Furthermore, S6K is activated by phosphorylation of PDK-1 and mTOR (Figure 2). The regulation of protein synthesis via IGF-1 occurs through controlling the intrinsic activity and/or binding properties of specific translation initiation and elongation factors called eIFs and eEFs. mTOR phosphorylates 4E-BP (4E binding protein). This causes the release of eIF4E (eukaryotic initiation factor 4E) to form an active complex, which promotes translation initiation and also activation of S6K. S6K phosphorylates the eEF2 (eukaryotic elongation factor 2) kinase which releases eEF2 and initiates elongation (Figure 2) (Nojima et al. 2003; Oshiro et al. 2004).

Other proteins, which are regulated via IR and IGF-1R signalling, are the glycogen synthase kinase(GSK)-3 $\beta$ , a major tau kinase, and BAD (Bcl-2/Bcl-X-associated death promoter), a proapoptotic factor. These proteins are inactivated via the IR/IGF-1R signalling cascade (Song, Ouyang, and Bao 2005). BAD interacts with the apoptosis suppressors Bcl-2 and more intense with Bcl-X<sub>L</sub> (Yang et al. 1995). BAD directly binds to Bcl-X<sub>L</sub> with its BH3 homology domain (Zha et al. 1997). This interaction is regulated by the phosphorylation state of BAD. Therefore, the IGF-1R signalling is a potent inhibitor of neuronal apoptosis (Schubert et al. 2003).

### 2.3.3 Forkhead box O transcription factor

AKT phosphorylates the Forkhead box O transcription factors (FoxOs). This induces the binding to 14-3-3 and nuclear exclusion of FoxOs and thereby inactivation of FoxO-mediated transcription. FoxOs regulate transcription of genes, which are involved in apoptosis, metabolism, growth, ageing and development (Partridge and Bruning 2008).

The mammalian FoxO protein family consists of 4 members: FoxO1, FoxO3a, FoxO4 and FoxO6. These transcription factors contain a conserved DNA binding domain, the forkhead domain (FKHR) (Clark et al. 1993). FoxO1 and FoxO3a are ubiquitously expressed, whereas FoxO6 is exclusively found in the brain, and FoxO4 has yet not been detected in the brain (Furuyama et al. 2000; Jacobs et al. 2003). The expression pattern of the different FoxOs in the adult mouse brain is distinct. FoxO1 is predominantly expressed in the striatum, dentate gyrus and ventral hippocampus and FoxO3a in the cortex, cerebellum and hippocampus. FoxO6 is expressed in amygdala, hippocampus and cingulate cortex (Hoekman et al. 2006).

The FoxO transcription factors are regulated by post-translational modifications. One major modification is the phosphorylation of the FoxOs. FoxO1 is phosphorylated by AKT at Thr24, Ser256 and Ser319 (Biggs et al. 1999; Brunet et al. 1999; Kops et al. 1999; Rena et al. 1999; Tang et al. 1999). Phosphorylation triggers binding to 14-3-3 and subsequently

translocation out of the nucleus terminating FoxO mediated transcription (Figure 2) (Brunet et al. 1999). Additionally, FoxOs are phosphorylated by other kinases depending on the stimulus (Huang and Tindall 2007). Furthermore, FoxOs are regulated by ubiquitylation. Ubiquitylation is dependent on phosphorylation of Ser256 of FoxO1 via AKT (Huang et al. 2005). FoxO1 and FoxO3a need to be polyubiquitylated for degradation. In contrast, FoxO4 requires monoubiquitylation to be degraded (van der Horst et al. 2006). Another regulatory mechanism for FoxO transcription is acetylation. CBP and p300 with their associated proteins, for example CBP- and p300-associated factor (PCAF), display intrinsic histone acetyl-transferase activity. These proteins promote transcription via histone acetylation and they directly regulate transcription via acetylation of particular transcription factors (Li et al. 2002). It has been shown that CBP acetylates FoxO transcription factors and inhibits their action (Daitoku et al. 2004). Silent information regulator 1 (SIRT1) is a nicotinamide adenine dinucleotide(NAD)-dependent histone deacetylase, which forms a complex with acetylated FoxOs upon stress stimuli and deacetylates the transcription factors (Brunet et al. 2004; Kitamura et al. 2005) to regulate FoxO mediated transcription.

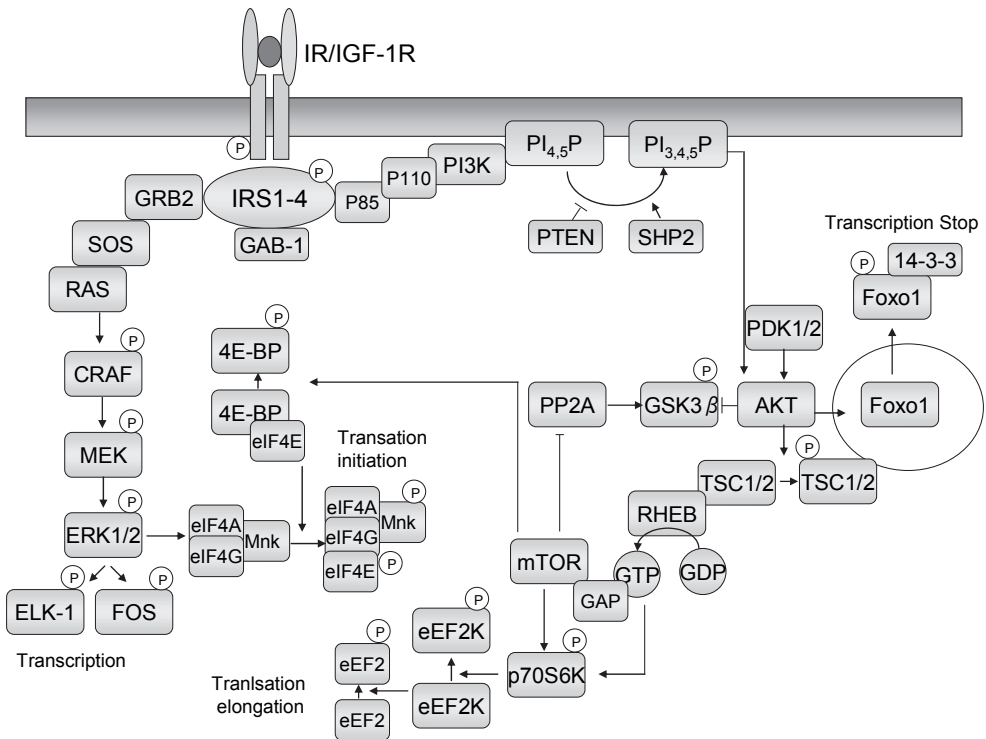


Fig. 2. IR/IGF-R signalling

The binding of Insulin or IGF-1 to the IR/IGF-1R causes autophosphorylation and activation of the receptor. Insulin receptor substrates (1-4) are recruited to the activated receptor and IRS phosphorylation results in activation of the MAP kinase (MAPK, mitogen activated protein kinase) and phosphatidylinositide(PI)3-kinase (PI3K) pathway.

### 3. IGF-1 signalling in the brain

#### 3.1 IGF-1's transport across the blood brain barrier

In the brain, present IGFs arise either by de novo synthesis or by transport from the blood into the brain. So far, the exact mechanisms of how IGFs cross the blood-brain barrier (BBB) are not fully understood. Early *in vitro* studies by Duffy and colleagues on isolated human brain capillaries showed that the affinity of IGF-2 to isolated human brain capillaries was approximately twofold higher than the affinity of IGF-1 binding and binding of both IGFs was nonsaturable over the range of 1 to 200 ng/ml IGF suggesting that there is a very efficient endocytosis mechanism (Duffy, Pardridge, and Rosenfeld 1988). Insulin displaced binding of IGF-1 (50% inhibited by 2 µg/ml Insulin) and IGF-2 (50% inhibited by 0,5 µg/ml Insulin). Furthermore, binding was largely inhibited by adding human serum, which was assumed to be due to the presence of IGFFBPs (Duffy, Pardridge, and Rosenfeld 1988). Subsequent studies suggested that there might be a high capacity transport system across the BBB, which is influenced by the IGFFBPs (Pan and Kastin 2000). Tores-Aleman and co-workers found that via the choroid plexus epithelium, circulating IGF-1 is transported into the CSF through a mechanism involving the multicargo protein transporter low-density lipoprotein receptor related protein 2 (LRP2), and concluded that increasing levels of IGF-1 in the CSF, as a result of increasing IGF-1 levels in the serum, might be explained via this transport mechanism (Carro et al. 2000; Carro et al. 2005). LRP1, another membrane cargo-transporter, was suggested as targeting platform for the circulating IGF-1/IGFBP-3/ALS-complex (Nishijima et al. 2010). LRP1 is abundantly expressed in brain endothelium and seems to be a cellular receptor for IGFBP-3 (Huang et al. 2003). Recently, it has been demonstrated that neuronal activity increases the permeability of the BBB for IGF-1 through neurovascular coupling (Nishijima et al. 2010). Therefore, serum IGF-1 might influence brain processes like synaptic plasticity and cognition. Furthermore, neurovascular coupling was proposed to locally change cerebral blood flow leading to activation of matrix metalloproteinase 9 (MMP9) through diverse mediators (arachidonic acid derivatives, ATP, etc.). MMP9 is an IGFBP-3 cleaving protease, which is released in response to neuronal activity (Michaluk and Kaczmarek 2007) and might link neuronal activation to the transport of serum IGF-1 via a LRP1 depending mechanism across the BBB (Nishijima et al. 2010). Hence, neurovascular coupling might result in neurotrophic coupling explaining the neuroprotective effects of physical as well as mental exercise and active social life on brain function (Carro et al. 2000; Fratiglioni, Paillard-Borg, and Winblad 2004).

#### 3.2 Brain IGF receptors

IGF-1 and to less extent IGF-2 bind and therefore activate the type 1 IGF-receptor (IGF-1R) (Rubin and Baserga 1995; White and Kahn 1994; LeRoith 2000). Several IGF-1R subtypes have been reported, for example the hybrid IGF-1Rs, which are hybrid dimers of the insulin-receptor (IR) and the IGF-1R (see 2.3) binding insulin as well as IGFs with similar affinity (Soos and Siddle 1989; Soos et al. 1990; Siddle et al. 1994; Moxham, Duronio, and Jacobs 1989; Pandini et al. 2002). The physiological significance of these receptor subtypes is still unclear (Russo et al. 2005).

Additionally, there is a type 2 IGF-receptor (IGF-2R) with a short cytoplasmic domain lacking a tyrosine kinase harbouring a higher affinity for IGF-2 than the IGF-1R (Sakano et al. 1991; Oh et al. 1991; Dore, Kar, and Quirion 1997; Braulke 1999; Kiess et al. 1994). This IGF-2R is a cation-independent mannose-6 phosphate (M6P) receptor binding not only IGF-

2 but M6P-containing ligands. This receptor functions in the mediation of endocytosis and lysosomal enzyme trafficking and regulation of apoptotic/ mitogenic effects (Morgan et al. 1987; von Figura and Hasilik 1986; Ghahary et al. 2000). Recent studies have demonstrated that IGF-2 influences memory enhancement via the IGF-2R suggesting that the IGF-2R might transmit some intracellular signalling (Chen et al. 2011).

### 3.3 IGF receptor expression in the brain

IGF-1R mRNA is widely expressed in the developing CNS and persists at high levels in the mature brain especially in neuron-rich regions such as the olfactory bulb, the granule cell layer of the dentate gyrus and cerebellar cortex (Rotwein et al. 1988; Bondy et al. 1990; Bondy and Lee 1993; Werther et al. 1990). Glia cells have a lower IGF-1R expression than neurons and therefore regions mainly containing those cells, like white matter zones, show low IGF-1R mRNA levels (Bondy and Lee 1993). Some neurons, which are still in the process of developing, express increasing IGF-1R mRNA levels postnatal until they have reached maturity, for example Purkinje cells (Bondy et al. 1992). In the adult brain, high expression levels of IGF-1R mRNA are also found in the choroid plexus, meninges and vascular sheaths (Bondy et al. 1992; Bohannon et al. 1988; Werther et al. 1989; Matsuo et al. 1989; Marks, Porte, and Baskin 1991).

The IGF-2R is abundantly expressed in the CNS, especially in the pyramidal cell layers of the hippocampus, the granule cell layer of the dentate gyrus, olfactory bulb, choroid plexus as well as in the microvasculature, retina, pituitary, brainstem and spinal cord (Hawkes and Kar 2004; Couce, Weatherington, and McGinty 1992; Wilczak et al. 2000; Valentino, Ocrant, and Rosenfeld 1990).

In addition to the IGF-1R and the IGF-2R, IR and IGF-1R hybrids are expressed in the brain, through which IGFs and insulin induce intracellular signalling. IRs are mainly expressed in regions that are linked to olfaction, appetite and autonomic functions, such as the olfactory bulb, limbic system and hypothalamus (Werther et al. 1987; Unger, Livingston, and Moss 1991) and seem to play a key-role in controlling feeding, body weight and reproduction (Bruning et al. 2000). Furthermore, IR expression is also present in remarkable concentration in the choroid plexus, circumventricular organs and brain microvessels (van Houten and Posner 1979, 1981; Werther et al. 1987) suggesting the IRs might contribute to the transport of insulin and possibly IGF-1 across the BBB.

## 4. IGF-1 action in the developing brain

### 4.1 Brain growth and myelination

Different mouse models indicated the essential actions of IGF-1 signalling for normal brain development. Homozygous IGF-1 knockout mice (IGF-1<sup>-/-</sup>), homozygous IGF-2 knockout mice (IGF-2<sup>-/-</sup>) as well as doubly deficient mutants are viable but have small brains (Beck et al. 1995; Liu et al. 1993). The majority of homozygous IGF-1<sup>-/-</sup> mice die perinatally and those which survive (< 5 %) demonstrate severe growth retardation with reduction in brain weight of 38 % distributed evenly over all major brain areas (Beck et al. 1995). However, certain cell-types and brain-regions were especially affected by the IGF-1 gene disruption such as white matter, striatum and hippocampus (Beck et al. 1995). The reduction of white matter was due to a net loss of axons and an additional shift from myelinated to unmyelinated fibres indicating the role of IGF-1 in axonal growth and/or maturation and its effect on the amount of oligodendrocytes and axon-myelination (Beck et al. 1995). These findings are in line with many *in vitro* studies, which demonstrated a stimulating effect of

IGF-1 on oligodendrocyte survival, development and proliferation (McMorris et al. 1986; McMorris and Dubois-Dalcq 1988; Mozell and McMorris 1991). In addition, formation of hippocampal granule cells and striatal parvalbumin-containing neurons was reduced in IGF-1<sup>-/-</sup> mice and therefore seems to require IGF-1 (Beck et al. 1995). Most dentate granule neurons as well as the myelination of axons are generated during postnatal development (Altman and Bayer 1990, 1990; Morell et al. 1972; Matthieu, Widmer, and Herschkowitz 1973) suggesting that IGF-1 plays a more important role during late embryonic and postnatal development. Intrauterine development was analysed in detail in single and combined homozygous knockout mice for IGF-1 and IGF-2 suggesting that before E13.5 the IGF-1R exclusively mediates IGF-2 actions and later, with increasing IGF-1 expression, the IGF-1R interacts with both IGFs (Baker et al. 1993). IGF-2 was found to be upregulated in IGF-1<sup>-/-</sup> mice suggesting that IGF-2 may partially compensate for the loss of IGF-1 expression and function (Ye et al. 2002).

Homozygous IGF-1R knockout mice (IGF-1R<sup>-/-</sup>) die at birth and have smaller brains (Liu et al. 1993). A similar growth retardation of the CNS was observed in transgenic mice overexpressing human IGF-binding protein-1 (hIGFBP-1) suggesting reduced IGF-1 action via IGF-1Rs (D'Ercole et al. 1994). Conversely, transgenic mice overexpressing human IGF-1 (hIGF-1) show increased brain growth and myelination (Carson et al. 1993; Ye, Carson, and D'Ercole 1995). In both mouse models, the transgenic hIGFBP-1 and hIGF-1 mouse, the cerebral cortex, hippocampus and diencephalon were the most affected brain regions (Ye, Carson, and D'Ercole 1995). Myelination was increased in hIGF-1 and reduced in hIGFBP-1 transgenic mice as well as the number of oligodendrocytes and the expression of myelin-specific proteins, respectively (Ye, Carson, and D'Ercole 1995). During development, the increase of myelin protein expression in the cerebral cortex of hIGF-1 transgenic mice correlates with the hIGF-1 transgene mRNA levels (Ye, Carson, and D'Ercole 1995). In conclusion, IGF-1 plays a crucial role in regulating neuronal growth and differentiation (Werther et al. 1998), but also enhances oligodendrocyte survival and myelination (Beck et al. 1995).

Several studies suggested that transcriptional programs control the development of axons or dendrites including their growth and branching (Jan and Jan 2003; Goldberg 2004; Polleux, Ince-Dunn, and Ghosh 2007). Recently, the role of FoxO-transcription factors, major downstream-targets of IIS (Insulin/IGF-1 signalling pathway), within the developing brain and their action of conducting IGF-1 signalling started to become clearer. FoxO proteins were found to be key-regulators of neuronal polarity in the mammalian brain and to trigger differentiation from immature neurons to post-mitotic neurons with specified axon- and dendrite-formations (de la Torre-Ubieta et al. 2010). In addition, protein kinase Pak1 was identified as direct target of FoxO-transcription factors linking FoxO-dependent transcription in the nucleus to an enzyme that promotes axonal polarity by controlling actin and microtubule dynamics (Edwards et al. 1999; Wittmann, Bokoch, and Waterman-Storer 2004) as well as dendritic spine morphogenesis and synapse differentiation (Hayashi et al. 2004; Hayashi et al. 2007; Nikolic 2008). Apart from Pak1, FoxO knockdown neurons demonstrated a number of downregulated polarity genes, such as Par6, R-Ras, APC and CRMP2 (de la Torre-Ubieta et al. 2010). Additionally, FoxO3a was shown to regulate homeostasis of neuronal stem cells (NSCs) both *in vitro* and *in vivo* by controlling a set of genes that determines cell cycle re-entry and optimal oxygen and glucose metabolism (Renault et al. 2009). However, the effect of FoxO3a deficiency in NSCs only became apparent in adult animals, as NSCs were shown to influence learning, memory and mood

(Zhang et al. 2008). Therefore, NSC homeostasis regulated by FoxO3a might influence the decline of cognitive function and possibly the onset of neurodegenerative diseases (de la Torre-Ubieta et al. 2010).

#### 4.2 Neuroendocrine regulation

Embryonic brain IGF-1R and therefore the action of the IGF-1 pathway in the developing brain was found to play a crucial role in determining somatotrophic plasticity and hence postnatal GH and IGF-1 signalling. These observations were made in a brain specific IGF-1R knockout mouse model (bIGF-1RKO<sup>-/-</sup>; bIGF-1RKO<sup>+/-</sup>) by the group of Holzenberger (Kappeler et al. 2008). Whereas homozygous mutants (bIGF-1RKO<sup>-/-</sup>) showed severe growth retardation and were infertile, heterozygous mutants (bIGF-1RKO<sup>+/-</sup>) were healthy and had an increased mean lifespan compared to controls (Kappeler et al. 2008). In bIGF-1RKO<sup>+/-</sup> mutants, serum IGF-1 and GH were lowered and pituitaries, like most other organs, were smaller compared to controls (Kappeler et al. 2008). As IGF-1Rs are only diminished in the brain but not in the anterior pituitary in this mouse model, the observed phenotype was proposed to result from alterations in GH-regulatory neurons of the hypothalamus. Hypothalamic GHRH expression was found to be significantly lower, as well as the Pit-1 mRNA levels. bIGF-1RKO<sup>+/-</sup> mice had preserved gonado- and thyrotropic functions but were growth retarded with a body length 5 % shorter than controls and a body weight of about 90% of controls at the age of 90 days (Kappeler et al. 2008). Interestingly, weight gain with age was slightly higher in adult bIGF-1RKO<sup>+/-</sup> mice than in controls, and female mutants finally reached the same body weight than controls (Kappeler et al. 2008). The gain of weight was most probably due to an enlargement of subcutaneous adipose tissue (AT), as there was less or no increase in visceral AT (Kappeler et al. 2008). In line with GH-deficient mouse models (Berryman et al. 2004; Berryman et al. 2006), the bIGF-1RKO<sup>+/-</sup> mice showed impaired glucose homeostasis and fat metabolism. Heterozygous inactivation of IGF-1R had no detectable effects on behaviour or other brain functions apart from the somatotrophic deficit. Compared to control littermates, bIGF-1RKO<sup>+/-</sup> mice had a significantly longer mean lifespan. However maximum lifespan was unchanged probably due to increased late-life mortality caused by hyperglycemia and dyslipidemia (Kappeler et al. 2008). Early dietary restriction showed a similar neuroendocrine response as the brain specific IGF-1R knockout (Kappeler et al. 2008), indicating a connection between nutrition, somatotrophic hormones, growth and their determination of lifespan.

Similar observations, in which morbidity in later life was determined during prenatal development, were made in humans. Long-term studies have been conducted on the Dutch famine birth cohort as well as on the Chinese famine cohort. Babies exposed in utero to calory restriction in late or mid gestation were growth retarded and showed impaired glucose tolerance in later life (Ravelli et al. 1998; Painter, Roseboom, and Bleker 2005). However, babies exposed in early gestation were not smaller than controls but demonstrated the most striking consequences of in utero undernutrition, namely a three-fold increase in coronary heart disease, hyper-/dyslipidemia and more frequent obesity (Painter, Roseboom, and Bleker 2005; Ravelli et al. 1999; Roseboom, van der Meulen, Osmond, Barker, Ravelli, Schroeder-Tanka et al. 2000; Roseboom, van der Meulen, Osmond, Barker, Ravelli, and Bleker 2000). Interestingly, mortality rates at older (50+) ages are significantly higher in the exposed group, and residual life expectancy at age of 50 was reduced by approximately 3 years (Lindeboom, Portrait, and van den Berg 2010), which is in line with the increased late-life mortality observed in the bIGF-1R<sup>+/-</sup> mice. In the cohort of

the Chinese famine, fetal and infant exposure to undernutrition also resulted in increased risk for metabolic syndrome (Li et al. 2010; Li et al. 2011; Yang et al. 2008; Luo et al. 2006). In summary, IGF-1 signalling during early development is defined by the number of brain IGF-1Rs or their sensitivity and determines endocrine as well as metabolic function in later life possibly playing a key-role in pathogenesis of age-associated diseases.

IGF-1 function in the developing brain	IGF-1 function in the adult brain
<ul style="list-style-type: none"> <li>• growth and differentiation of neurons</li> <li>• neuronal polarity and synapse formation</li> <li>• growth of oligodendrocytes</li> <li>• enhancement of myelination</li> <li>• neuroendocrine regulation of metabolism</li> </ul>	<ul style="list-style-type: none"> <li>• preservation of neuronal plasticity, for example in the olfactory bulb</li> <li>• brain vessel growth</li> <li>• spatial learning and other cognitive functions</li> <li>• neuroprotective in ischemic or traumatic injuries and several neurological disorders</li> <li>• reduced signalling extends lifespan</li> </ul>

Table 1. Function of IGF-1 in the brain

## 5. IGF-1 in Alzheimer's disease

Alzheimer's disease (AD) is a chronic and progressive neurodegenerative disease and the most common form of dementia leading to the loss of cognitive abilities and finally to death (Citron 2002; Cole et al. 2007).

AD was first described by Alois Alzheimer, a German physician, in 1906 (Alzheimer et al. 1995). The disease is characterised by  $\beta$ -amyloid accumulation, formation of extracellular amyloid plaques as well as neurofibrillary tangles. The  $\beta$ -amyloid plaques mainly contain aggregated amyloid- $\beta$  (A $\beta$ ) peptides (Masters et al. 1985). In contrast, the main components of neurofibrillary tangles are hyperphosphorylated and aggregated tau proteins (Ross et al., 2005). The aggregation of A $\beta$  is thought to be the molecular basis of neurodegeneration in AD (Masters et al. 1985).

### 5.1 Tau

The tau proteins consist of a N-terminal projection domain, a short tail sequence and a C-terminal domain with microtubule-binding (MTB) repeats. Six isoforms of tau are known in the human brain. These isoforms emerge from alternative splicing of exons 2, 3 and 10. Exon 2 and 3 encode N-terminal parts of tau and exon 10 codes for an additional MTB repeat. Thus, tau can present three or four MTB repeats (Ballatore, Lee, and Trojanowski 2007; Goedert and Spillantini 2006). Tau is predominantly located in the axons of neurons (Hirokawa et al. 1996) and is to less extent found in dendrites (Ittner et al. 2010). The function of tau is yet not completely understood, but it might influence the stabilisation of microtubules and regulation of axonal transport (Gotz, Ittner, and Kins 2006). Tau is phosphorylated at several sites via kinases like glycogen synthase kinase 3 (GSK-3 $\beta$ ), cyclin-dependent kinase 5 (Cdk5), c-Jun N-



terminal kinase (JNK) and ERK1/2 (Robertson et al. 1993; Hanger et al. 1992; Flaherty et al. 2000; Cho and Johnson 2004; Stoothoff and Johnson 2005). Abnormal high phosphorylation is called "hyperphosphorylation". Hyperphosphorylated tau proteins form so called paired helical filaments, which are characteristic for AD. The degradation of tau is inhibited by phosphorylation at the caspase cleavage sites. It has been shown that the mutation of Ser422, which causes a stable phosphorylation at this site, prevents caspase cleavage (Guillozet-Bongaarts et al. 2006). GSK-3 $\beta$  is one of the major tau kinases and is inactivated upon phosphorylation of Akt at Ser9 connecting insulin and IGF-1 signalling to tau phosphorylation. The major tau phosphatase in human brain is PP2A (Sontag et al. 1996), which is as well regulated via the IR/IGF-1R pathway suggesting that IR/IGF-1R signalling maintains an equilibrium of phosphorylation and dephosphorylation of tau (Liu et al. 2008; Millward, Zolnierowicz, and Hemmings 1999).

## 5.2 Amyloid- $\beta$

A $\beta$  is generated by proteolytic cleavage of the amyloid precursor protein (APP), a type-1 integral membrane protein. APP was first described and cloned in 1987 (Kang et al. 1987; Tanzi et al. 1987; Goldgaber et al. 1987; Robakis et al. 1987). The APP gene is located on chromosome 21. Hence, patients with trisomy 21 show a higher risk to develop Alzheimer's disease, because of the additional APP allele. Accordingly, the duplication of the isolated APP gene causes cerebral amyloid angiopathy and amyloidosis suggesting that increased APP expression itself is sufficient to cause Alzheimer-like pathology (Rovelet-Lecrux et al. 2006; Sleegers et al. 2006). Another risk factor for AD are mutations of the APP gene (Vassar 2004; Bertram and Tanzi 2005). APP contains a N-terminal extracellular domain and a shorter C-terminal cytoplasmic domain. Alternative splicing of the APP gene results in different isoforms of APP which are distinguishable by length. APP with 751 and 770 (APP751 and APP770) amino acids mainly occur in non-neuronal tissue. APP695 is mainly localised in neurons (Kang and Muller-Hill 1990). The function of APP and the APP-like proteins (APLP) is not clear yet. These proteins are possibly involved in cell adhesion, apoptosis and axonal transport.

The  $\beta$ -secretase BACE1 ( $\beta$ -site APP-cleaving enzyme) plays an essential role in the production of A $\beta$ . It cleaves APP at Asp<sup>+1</sup> at the N-terminus. The resulting fragments are called APPs $\beta$  and the C-terminal fragment C99. Upon cleavage of C99 by the  $\gamma$ -secretase, a complex formed by presenilin, nicastrin, Aph-1 and Pen-2, A $\beta$  peptides (4 kDa) and the APP intracellular domain (AICD) with a size of 6 kDa are generated. A $\beta$ -peptides mainly occur in two variants: A $\beta$ 40 which ends at residue 40 and A $\beta$ 42 ending at residue 42 after cleavage. Predominantly, the A $\beta$ 42 is prone to aggregate and forms toxic oligomers. Furthermore, APP is cleaved by the  $\alpha$ -secretases ADAM10 (a disintegrin and metalloproteinase-like 10) or TACE (tumour necrosis factor-alpha convertase). This results in the C-terminal fragment C83 and APPs $\alpha$ . The cleavage of APP by  $\alpha$ - or  $\beta$ -secretase is dependent on the competition between both enzymes. In case the  $\beta$ -secretase cleavage of APP increases,  $\alpha$ -secretase processing decreases and vice versa (Vassar et al. 1999; Skovronsky et al. 2000) (Figure 3).

In a healthy brain, there is more production of A $\beta$ 40 (~90 %) than there is of A $\beta$ 42 (~5-10 %) (Walsh and Selkoe 2007). The accumulation of A $\beta$ 42 is an important step in the formation of amyloid plaques (Iwatsubo et al. 1994). The A $\beta$ 42:A $\beta$ 40 ratio is a diagnostic tool for APP processing and development of AD (Haass and Selkoe 2007).

In addition to age-associated A $\beta$ 42 accumulation, mutations in presenilin 1, presenilin 2 and the APP gene lead to familiar early-onset AD (Tabaton and Tamagno 2007; Sherrington et al.

1996; Tanzi et al. 1992; Schellenberg et al. 1992; Van Broeckhoven et al. 1992; St George-Hyslop et al. 1992; Rogaeve et al. 1995).

The toxic effect of A $\beta$  is not fully understood yet, but might be induced via generation of ion channels, membrane disruption, oxidative stress, induction of apoptosis and inflammation (Hardy and Selkoe 2002; Nakagawa et al. 2000; Soto 2003; Roberson and Mucke 2006). The A $\beta$  aggregation process produces different intermediates. A $\beta$  monomers are soluble and amphipathic with an  $\alpha$ -helical conformation and kink regions in water-alcohol mixture (Coles et al. 1998; Crescenzi et al. 2002). A $\beta$ 40 displays a random coil structure in aqueous solution (Zhang et al. 2000) and A $\beta$ 42 shows  $\beta$ -sheet structure at physiological conditions (Barrow and Zagorski 1991). A $\beta$  dimers are located intracellular *in vivo* and show a hydrophobic core (Roher et al. 1996). Small A $\beta$  oligomers are highly cytotoxic compared to mature A $\beta$  fibrils (Dahlgren et al. 2002; McLean et al. 1999; Cleary et al. 2005; Lesne et al. 2006). The so called A $\beta$ -derived diffusible ligands (ADDLs) show no fibrillar structure and are neurotoxic in a size of about 17 to 42 kDa (Chromy et al. 2003; Klein, Stine, and Teplow 2004; Lambert et al. 1998). The levels of ADDLs are linked to cognitive impairments in AD (Georganopoulou et al. 2005). A $\beta$  protofibrils are the precursors of A $\beta$  fibrils. These protofibrils are present as rod-like and flexible structures. The dyes Congo red and thioflavin T bind to the core of the protofibrils, which indicates a high level of  $\beta$ -sheets (Harper et al. 1999; Arimon et al. 2005; Harper et al. 1997; Kheterpal et al. 2003; Walsh et al. 1997; Williams et al. 2005). A $\beta$  fibrils are insoluble, thermodynamically stable aggregates containing repeats of  $\beta$ -sheets (Ross and Poirier 2005). They also bind Congo red and thioflavin T (Klunk, Jacob, and Mason 1999; LeVine 1999). The amyloid plaques are extracellular aggregates of insoluble A $\beta$  fibrils (Muller-Hill and Beyreuther 1989). These plaques are surrounded by activated microglia, astrocytes and dystrophic dendrites (Selkoe 2004).

Different clinical studies revealed an association of AD and type 2 diabetes (Janson et al. 2004; Ott et al. 1999; Stewart and Liolitsa 1999). A connection of glucose intolerance, impairment of insulin secretion and the risk to develop AD was recently discovered (Ott et al. 1996; Luchsinger et al. 2004; Ronnema et al. 2008). Furthermore, AD patients develop more frequently impaired glucose tolerance and type 2 diabetes (Janson et al. 2004) indicating that IR/IGF-1R signalling might influence AD pathogenesis.

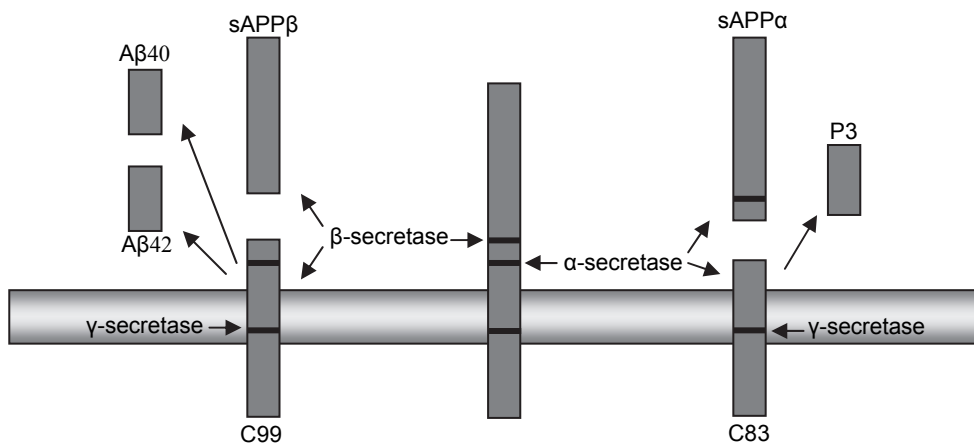


Fig. 3. Processing of amyloid precursor protein (APP).

APP might be cleaved via the  $\alpha$ -,  $\beta$ - or  $\gamma$ -secretase.  $\alpha$ -secretase cleavage generates a membrane bound C83 fragment and sAPP $\alpha$ . In case C83 is proteolytically cleaved by the  $\gamma$ -secretase the P3 fragment occurs.  $\beta$ -secretase processing produces the fragments sAPP $\beta$  and C99. In case of a simultaneous or subsequent cleavage via  $\beta$ - and  $\gamma$ -secretase the A $\beta$ 40 or A $\beta$ 42 peptides are released.

### 5.3 IR, IGF-1R signalling and Alzheimer's disease

The IR and IGF-1R signalling pathway is disturbed in the central nervous system (CNS) of AD patients (Frolich et al. 1998; Frolich et al. 1999; Moloney et al. 2010). Analysis of the mRNA level of insulin and the IR showed a decrease of about 80% in AD patients. Additionally, the expression of the IGF-1R was reduced in AD brains compared to controls (Moloney et al. 2010; Rivera et al. 2005). In contrast, the IGF-1 serum levels of AD patients are increased indicating IGF-1 resistance in AD (Rivera et al. 2005; Vardy et al. 2007). Furthermore, IRS-1 and -2 expression is reduced in AD brains and phosphorylation of IRS-1 at Ser312 and Ser616 is increased, which decreases IRS-1 action characterising AD as "brain type" diabetes (Pilcher 2006). Thus, brains of AD patients are insulin and IGF-1 resistant. Whether these changes are cause or consequence of neurodegeneration is a matter of debate. IGF-1 knockout mice display increase of tau phosphorylation at Ser396 and Ser202 while the tau protein level was not influenced (Cheng et al. 2005). In NIRKO mice, the brain-specific IR knockout mice, tau was hyperphosphorylated at Thr231 (Schubert et al. 2004), whereas IRS-2 knockout mice showed hyperphosphorylation at Ser202 (Schubert et al. 2003). The different phosphorylation patterns of tau in different insulin and IGF-1 resistant mouse models indicate that additional factors may play a role for tau phosphorylation in these models (Freude et al. 2009).

Tg2576 mice express the Swedish mutation of APP (APP<sup>sw</sup>) and are an established mouse model for analysing amyloid pathology (Vassar et al. 1999; De Strooper 2003; Harada et al. 2006). IRS-2 (IRS-2<sup>-/-</sup>) or neuron specific IGF-1R knockout (nIGF-1R<sup>-/-</sup>) in Tg2576 mice protects these mice from premature death and decreases A $\beta$ -accumulation (Freude et al. 2009).

BACE-1 and Presenilin-1/-2, which cleave APP and generate neurotoxic A $\beta$ 42, are possible targets for AD treatment since  $\beta$ -secretase cleavage is the rate limiting step of A $\beta$  generation. During ageing, the expression of the neurotrophin receptor tyrosine kinase receptor A (TrkA) and the p75 neurotrophin receptor (p75<sup>NTR</sup>) changes considerably. Whereas TrkA receptor expression decreases, the p75 neurotrophin receptor increases with age. Human neuroblastoma cells SHSY5Y and primary cultured neurons showed a switch from TrkA to p75<sup>NTR</sup> expression after treatment with IGF-1 (Costantini, Scrabble, and Puglielli 2006). This increases BACE-1 activity via hydrolysis of sphingomyelin and release of ceramide stabilising BACE-1 (Puglielli 2008; Puglielli et al. 2003). It has been shown that embryonic hippocampal neurons treated with A $\beta$ 42 as ligand of p75<sup>NTR</sup> cause cell death. Neurons, which are deficient in p75<sup>NTR</sup> and also treated with A $\beta$ 42, show less cell death. This may represent the molecular mechanism linking IR and IGF-1R signalling pathway to ageing and neurodegeneration (Sotthibundhu et al. 2008).

In *Caenorhabditis elegans* the knockdown of DAF-2, the orthologue of mammalian IR and IGF-1R, reduces A $\beta$ 42 toxicity (Cohen et al. 2006). This reduced A $\beta$ 42 toxicity results from the activity of the downstream transcription factors DAF-16, the orthologue of mammalian FoxO1 and 3a as well as heat shock transcription factor-1 (HSF-1) (Hsu, Murphy, and Kenyon 2003; Birkenkamp and Coffey 2003; Cohen et al. 2006). The

detoxification of A $\beta$ 42 by decreased DAF-2 signalling involves two possible mechanisms. First, HSF-1 regulates disaggregation of toxic oligomers followed by degradation of the resulting fragments. Second, DAF-16 regulates the formation of aggregates with high molecular weight and low toxicity, which are built from aggregates with low molecular weight but high toxicity (A $\beta$  hyperaggregation) (Cohen et al. 2006). Recently, A $\beta$  hyperaggregation has been identified as a mechanism of A $\beta$  detoxification in an IGF-1 resistant mouse model of AD (Cohen et al. 2009).

Several ways of A $\beta$  clearance from the brain have been discovered contributing to A $\beta$  detoxification. A $\beta$  clearance is achieved via transport over the blood brain barrier, enzymatic degradation and phagocytosis by microglia. Recently, several enzymes have been discovered which degrade A $\beta$ , e.g. insulin degrading enzyme (IDE), endothelin converting enzyme (ECE), neprilysin, and angiotensin converting enzyme (ACE). The expression of IDE is activated by IR and IGF-1R signalling (Zhao et al. 2004). Transport across the blood brain barrier (BBB) is mediated by distinct receptors. This transport is achieved via binding to the low-density lipoprotein receptor related protein (LRP). The binding of A $\beta$  to LRP occurs directly or in complex with APOE (apolipoprotein E) and/or  $\alpha$ 2-macroglobulin ( $\alpha$ 2M). After crossing the BBB, A $\beta$  is transported to peripheral tissues for degradation, for example the liver (Tanzi, Moir, and Wagner 2004). It has been proposed that high IGF-1 levels cause degradation or clearance of A $\beta$ . Tg2576 mice present decreased IGF-1 levels compared to wild type mice. The treatment with IGF-1 yield to increased transport of A $\beta$  from the brain, possibly via the choroid plexus (Carro et al. 2002). Accordingly, the inactivation of the IGF-1R signalling in the choroid plexus caused AD-like pathology (Carro et al. 2006). In contrast, studies in rats and Tg2576 mice using acute, subchronic and chronic IGF-1 treatment found no changes in tau phosphorylation and A $\beta$  concentrations (Lanz et al. 2008). Possibly, chronic peripheral treatment with IGF-1 causes downregulation of the IGF-1R signalling pathway as it has been shown for a cohort of individuals with high serum IGF-1 level but low IGF-1R signalling (Suh et al. 2008). This might explain the conflicting results of the different studies. AD mouse models with induced insulin resistance via high fat diet displayed an exacerbation of amyloid pathology (Ho et al. 2004).

The different studies dealing with IR/IGF-1R signalling and AD prove a connection between this signalling pathways and AD pathology. However, the exact molecular mechanisms need to be elucidated.

## 6. IGF-1 in the brain and ageing

IR/IGF-1R signalling is a conserved pathway functioning in development, fertility (Liang et al. 2003; Tatar, Bartke, and Antebi 2003; Broughton et al. 2005), growth (Butler and Le Roith 2001; Ikeya et al. 2002) and metabolic regulation (Saltiel and Kahn 2001). Decreased IR/IGF-1R signalling causes lifespan extension in model organisms like *Drosophila melanogaster*, *Caenorhabditis elegans* and *Mus musculus* (Clancy et al. 2001; Holzenberger et al. 2003; Tatar et al. 2001).

The life expectancy of humans has been increased upon environmental amelioration (Wilmoth 2000). However, this is accompanied by an increase of age associated disorders. Therefore, the study of the molecular mechanism of ageing might lead to identification of disease modifying pathways. One of these pathways is the IR/IGF-1R signalling cascade. Studies investigating temporal or spatial restricted changes of the IR/IGF-1R signalling pathway in different model organisms, using the RU486-induced GAL4/UAS (upstream

activation sequence) in *Drosophila*, as well as RNAi (RNA interference) incorporation via feeding of bacteria to *C. elegans* and Cre/loxP system in mice (Roman et al. 2001; Sauer 1998), have been performed.

The CNS is responsible for endocrine release of insulin-like peptides which activate the IR/IGF-1R signalling cascade and subsequently shorten lifespan (Ikeya et al. 2002; Broughton et al. 2005). Confusingly, acute increase of IR/IGF-1R signalling is neuroprotective but reduction of the signalling causes lifespan extension (Bateman and McNeill 2006; Chrysis et al. 2001).

### 6.1 IGF-1 signalling in *C. elegans*

The impact of IR/IGF-1R-like signalling (IIS) pathway on lifespan was first discovered in *C. elegans*. IIS in *C. elegans* is similar to the pathway in mammals (Taguchi and White 2008). It is activated through binding of insulin-like peptides (INS) to DAF-2 (abnormal Dauer formation-2), the IR/IGF-1R in worms (Kenyon et al. 1993). Upon stimulation of DAF-2 an insulin receptor substrate 1 orthologue (IST-1) is recruited to DAF-2 and AGE-1 (AGEing alteration-1), orthologue to human p110. P110, the catalytic subunit of PI3K, promotes the generation of phosphatidylinositol-triphosphate (PI<sub>3,4,5</sub>P) which then activates AKT kinase family members (Morris, Tissenbaum, and Ruvkun 1996; Kops et al. 1999; Paradis and Ruvkun 1998).

The mutations of DAF-2 and AGE-1 cause lifespan extension in a DAF-16 dependent manner which is a forkhead transcription factor in worms (Kenyon et al. 1993; Lin et al. 1997; Ogg et al. 1997; Morris, Tissenbaum, and Ruvkun 1996) and homologue to the mammalian FoxO1 and FoxO3a.

AGE-1 and DAF-2 mutant worms show enhanced thermotaxis learning behaviour during ageing. This might be due to resistance to neuronal diseases and oxidative stress (Murakami 2007).

The insulin-like peptides (INS) are found throughout the whole body of *C. elegans* (Pierce et al. 2001; Li, Kennedy, and Ruvkun 2003). Specific sensory neurons regulate lifespan through DAF-16 (Alcedo and Kenyon 2004). The ablation of olfactory neurons causes lifespan extension being only partially dependent on DAF-16 indicating an involvement of other factors. The regulation of INS is not well analysed, but the sensory neurons seem to be the major source of INS for peripheral tissues (Alcedo and Kenyon 2004).

A study using tissue-specific expression of AGE-1 and DAF-16 revealed that wild-type AGE-1 recovers the extended lifespan of AGE-1 mutants. This was shown for wild-type AGE-1 expressed in neurons and the intestine. In addition, wild-type DAF-16 rescues reduced lifespan of DAF-16/AGE-1 double mutants when expressed in neurons or intestine. Moreover, wild-type DAF-16 exerts its full effect when expressed in neurons and the intestine exclusively. However, DAF-16 expression showed only minor effects when expressed in other tissues (Broughton and Partridge 2009; Iser, Gami, and Wolkow 2007).

### 6.2 IGF-1 signalling in *Drosophila melanogaster*

*D. melanogaster* possesses endocrine tissues, which are similar to mammals (Toivonen and Partridge 2009). The median neurosecretory cells (mNSCs) of *Drosophila* are localised in the pars intercerebralis. mNSCs generate three of the seven *Drosophila* insulin-like peptides (DILPs). These mNSCs are functionally similar to  $\beta$ -cells of the pancreas which produce

insulin. These neurosecretory cells show an analogue development to the mammalian anterior pituitary (Wang et al. 2007). The ablation of mNSCs causes lifespan extension, because of the depletion of DILP-2, -3 and -5 (Broughton et al. 2005). In a further study using a dominant-negative form of p53 expressed in the CNS of flies, lifespan extension was induced (Bauer et al. 2005; Slee, O'Connor, and Lu 2004). This correlates with decreased DILP-2 expression and reduced PI3K activity in the periphery (Bauer et al. 2007).

In addition to ablation of mNSCs, mutation of the insulin-like receptor (InR) or of its substrate Chico extends lifespan (Clancy et al. 2001; Tu, Epstein, and Tatar 2002; Tatar et al. 2001). The activation of the InR causes phosphorylation of dFOXO, which is equivalent to nematode DAF-16 and mammalian FoxO1 and FoxO3a (Puig et al. 2003; Junger et al. 2003). dFOXO activation in the pericerebral fat body has been shown to regulate ageing. This activation of dFOXO reduces the expression of DLP-2 in mNSCs followed by downregulation of the InR signalling pathway in peripheral fat tissue (Hwangbo et al. 2004). The JNK signalling pathway in neurons enhances stress resistance and lifespan extension partially via dFOXO (Essers et al. 2004; Wang, Bohmann, and Jasper 2005). Oxidative stress induces JNK signalling and subsequently promotes the nuclear localisation of dFOXO and induces expression of antioxidant proteins (Huang and Tindall 2007; Kops et al. 2002). Neurons are prone to oxidative stress based on the high production of ROS and low amount of antioxidant proteins (Lin and Beal 2006). Peroxiredoxin abolishes hydroperoxide using thioredoxin as hydrogen donor, which decreases ROS (reactive oxygen species) (Lim et al. 1993). Mammals express six peroxiredoxins (I-VI). Peroxiredoxin II is solely expressed in the brain (Jin et al. 2005). Jafrac1, the homologue of the human Peroxiredoxin II (hPrxII), is a downstream protein of JNK signalling and target gene of dFOXO in neurons. Jafrac1 reduces reactive oxygen species (ROS) and extends lifespan (Lee et al. 2009) linking decreased IIS to increased clearance of ROS as possible mechanism for lifespan extension.

### 6.3 IGF-1 signalling regulates mammalian lifespan

In mice, it is well known that adult body size is an indicator of GH/IGF-1 actions and negatively correlated to longevity (Rollo 2002; Miller et al. 2002). Furthermore, IGF-1 plasma levels are negatively correlated with median lifespan (Yuan et al. 2009). Up to date, several mouse models of healthy ageing and longevity have been identified and analysed. Most of those long-lived mice have alterations within the IIS, GH or mTOR signalling pathway, indicating their key-role in influencing the process of ageing itself (Broughton and Partridge 2009; Kenyon 2010; Kenyon 2005; Piper et al. 2008; Bluher, Kahn, and Kahn 2003; Kappeler et al. 2008; Selman et al. 2008).

Highest impact on lifespan extension seems to have the ablation of GH signalling, as mutants with impaired GH action demonstrate higher increase of median and maximal lifespan than those with mutations that directly impact IGF-1 signalling (Coschigano et al. 2000; Coschigano et al. 2003; Brown-Borg et al. 1996; Flurkey et al. 2001). Examples for altered GH signalling are the Ames dwarf mice (Prop1<sup>def/def</sup>), Snell dwarf mice (Pit-1<sup>def/def</sup>) and Laron dwarf mice (GHR<sup>-/-</sup>). As the transcription factors Prop1 and Pit-1 are also essential for induction of thyroidea stimulating hormone (TSH) and prolactin, Ames dwarf mice and Snell dwarf mice are not only GH deficient but in addition produce less thyroidea stimulating hormone (TSH) and prolactin. Therefore, it can not be excluded that some of the effects seen in Ames and Snell dwarf mice might arise through deletion of TSH and prolactin. Laron dwarf mice (GHRKO, GHR<sup>-/-</sup>) are only resistant to GH signalling, and their longevity phenotype has been reproducible in different laboratories on different genetic

backgrounds and diets (Coschigano et al. 2003; Bartke and Brown-Borg 2004; Bonkowski et al. 2006).

As described above, GH action is mainly, but not completely, mediated by IGF-1 and circulating levels of IGF-1 are drastically reduced in GH-deficient Ames and Snell dwarf mice as well as in GH-resistant Laron dwarf mice (Brown-Borg et al. 1996; Coschigano et al. 2000; Flurkey et al. 2001). This reduction is primarily due to decreased expression of IGF-1 in the liver, whereas locally derived IGF-1 is, if at all, less affected (Bartke 2011). Normal expression of IGF-1 in the brain, especially in the hippocampus, might explain the maintenance of cognitive function in the long-lived mutants of the GH/IGF-1 system (Kinney et al. 2001). Besides reduced circulating IGF-1, lower insulin levels and enhanced insulin sensitivity are among the most prominent endocrine features shared by Ames dwarf, Snell dwarf and GHRKO mice (Bartke 2006) and have been found to be beneficial for ageing and survival (Bartke 2011). Additionally, increased adiponectin levels, which are associated with improved insulin sensitivity, anti-inflammatory and anti-atherogenic effects, were observed in all three mouse lines (Berryman et al. 2004; Wang et al. 2006). GH-deficient and -resistant mice were also found to have other phenotypic characteristics through which lifespan is thought to be increased, such as resistance to oxidative and other cytotoxic stresses (Bartke et al. 2001; Murakami, Salmon, and Miller 2003), increased activity of antioxidant enzymes (Hauck et al. 2002; Bartke et al. 2001; Romanick, Rakoczy, and Brown-Borg 2004), reduced body temperature (Hunter et al. 1999; Hauck et al. 2001) and reduced susceptibility to cancer (Yang et al. 1996; Deitel et al. 2002; Ikeno et al. 2003).

Another model for extended healthy lifespan in mammals is the fat-specific insulin receptor knockout (FIRKO) mouse (Bluher, Kahn, and Kahn 2003). These mutant mice maintain low body fat despite normal food intake, have normal lipid metabolism and demonstrate improved glucose tolerance and insulin sensitivity throughout life (Bluher, Kahn, and Kahn 2003). The underlying mechanisms of this model are not well understood and apart from impaired IR signalling, altered inflammatory state and mitochondrial activity in these animals are discussed.

A remarkable increase of mean and maximal lifespan has been found in female and male mice lacking PAPP-A. PAPP-A is a metalloproteinase that cleaves inhibitory IGF-BPs (as described above), thereby increasing local bioavailability of IGF-1 without altering IGF-1 expression (Lawrence et al. 1999). Conversely, deletion of PAPP-A in PAPP-A<sup>-/-</sup> mice results in reduced local IGF-1 levels, and thus PAPP-A<sup>-/-</sup> mice are born as dwarfs (Conover et al. 2004). In these mutants, extension of lifespan was not associated with impaired glucose or fat metabolism and serum IGF-1, insulin and GH levels were unaltered (Conover and Bale 2007). However, these mice have markedly reduced incidence of spontaneous tumors (Conover and Bale 2007) and were found to be resistant to develop experimentally induced neointimal hyperplasia and atherosclerosis (Harrington, Simari, and Conover 2007; Resch, Simari, and Conover 2006).

As described above, homozygous deletion of the IGF-1R or IGF-1 is lethal or produces severe developmental defects, however extended lifespan was reported in mice with heterozygous deletion of the IGF-1R (IGF-1R<sup>+/-</sup>) (Holzenberger et al. 2003). IGF-1R<sup>+/-</sup> mice have upregulated serum IGF-1 levels, which might indicate an endocrine compensatory response to the reduced numbers of IGF-1R, and these mice do not develop dwarfism (Holzenberger et al. 2003). Those mutants have no alterations in energy metabolism, physical activity, fertility and reproduction but display greater resistance to oxidative stress (Holzenberger et al. 2003) probably causing the observed lifespan extension, which is

predominantly seen in females. As described earlier, the selective reduction of IGF-1 signalling in the brain of bIGF1R<sup>+/-</sup> mice leads to an increase in median but not maximal lifespan (Kappeler et al. 2008).

The knockout models for IRS-proteins provide evidence that interference with IGF-1/insulin signalling downstream of the IGF-1 and insulin receptor influences lifespan in mice. Female IRS-1 knockout (IRS-1<sup>-/-</sup>) mice were reported to be long-lived and resistant to a number of conditions related to neurological and neuromuscular, immune, skin and bone disease (Selman et al. 2008). In these animals, body weight including fat mass was reduced, but endocrine function of the pituitary as well as circulating IGF-1 levels were preserved (Selman et al. 2008). Additionally, female IRS-1<sup>-/-</sup> mice were hyperinsulinemic and IGF-1/insulin resistant and rectal temperature was significantly elevated (Selman et al. 2008). All these characteristics are in contrast to the long-lived GH-deficient and GH-resistant mice, however female IRS-1<sup>-/-</sup> mutants have similar alterations in expression of genes involved in oxidative stress defence and DNA repair (Selman et al. 2008). In the same study, no significant increase of lifespan was found for male IRS-1<sup>-/-</sup> mice, as well as for IRS-1<sup>+/-</sup> and IRS-2<sup>+/-</sup> mice of both sexes, and IRS-2<sup>-/-</sup> mice were even short-lived (Selman et al. 2008).

IRS-2<sup>+/-</sup> mice were found to be long-lived, slightly larger and more insulin sensitive and glucose tolerant than wild-type mice (Taguchi, Wartschow, and White 2007). However, the animals studied by Selman et al. (2008) were fed a 'standard' rodent diet with a fat content of 5 %, while those studied by Taguchi et al. (2007) were on a 'high energy' diet with a fat content of 9 %, hence indicating that the IRS-2<sup>+/-</sup> mice might be protected from the harming effects of a high-fat diet (Bartke 2008). In addition, Taguchi et al. (2007) reported that brain specific alterations of IRS-2 in bIRS-2<sup>+/-</sup> and bIRS-2<sup>-/-</sup> mice result in increased lifespan despite glucose and insulin resistance (Taguchi, Wartschow, and White 2007). Further work will be needed to clarify and reconcile the observations made in those two laboratories (Bartke 2008).

Regardless the criticism on the reproducibility of the observations made by Holzenberger (Kappeler et al. 2008) and Taguchi (2007), they provide evidence that selective disruption of the IIS pathway in neurons might not only extend lifespan in *C.elegans* and *Drosophila* (Wolkow et al. 2000; Kenyon 2005) but also in mammals. These findings may indirectly be verified by other studies that demonstrate a protection from AD pathology in a transgenic AD mouse model via deletion of IGF-1R or IRS-2 (Cohen et al. 2009; Freude et al. 2009; Killick et al. 2009).

#### **6.4 IGF-1 signalling and its relevance to human longevity**

The IIS pathway is highly conserved throughout evolution from nematodes and flies to mammals. In the last years, an increasing body of data suggests that this pathway is important for human longevity as well.

GH secretion and IGF-1 levels decrease with age (Rudman et al. 1981), which contributes to ageing-associated changes in body composition such as increased adipose tissue and reduced lean body mass leading to impaired insulin sensitivity and cardiovascular disease. The same observations are made in patients with GH deficiency (Khan et al. 2001) and mutation in the GHR gene: Low serum IGF-1 levels, short stature, obesity, glucose intolerance and possible mental retardation but no decrease in life expectancy (Rincon, Rudin, and Barzilai 2005). Conversely, pathologic GH excess in acromegaly leads to reduced life expectancy due to cardiovascular disease, diabetes and malignancies (Orme et al. 1998). Additionally, body height has been identified as a cancer risk in a number of large



population studies (Bartke 2011) and serum IGF-1 levels correlate with cancer mortality in elderly men (Major et al. 2010). Therefore, the decline of GH secretion in the process of ageing might not simply reflect a progressive failure of the hypothalamus-somatotrope axis, but might be protective for the development of insulin resistance and cancer (Bartke 2003; Shechter et al. 2007). GH/IGF-1 therapy in elderly and GH deficient patients improves the ratio of lean and fat body mass, the lipid profile, protein synthesis, bone density, immune functions and memory (Khan et al. 2001), but certainly bears adverse side-effects, as GH is known to be diabetogenic and IGF-1 might increase the risk of cancer (Juul 1999; Khan et al. 2001; Pollak, Schernhammer, and Hankinson 2004; Major et al. 2010).

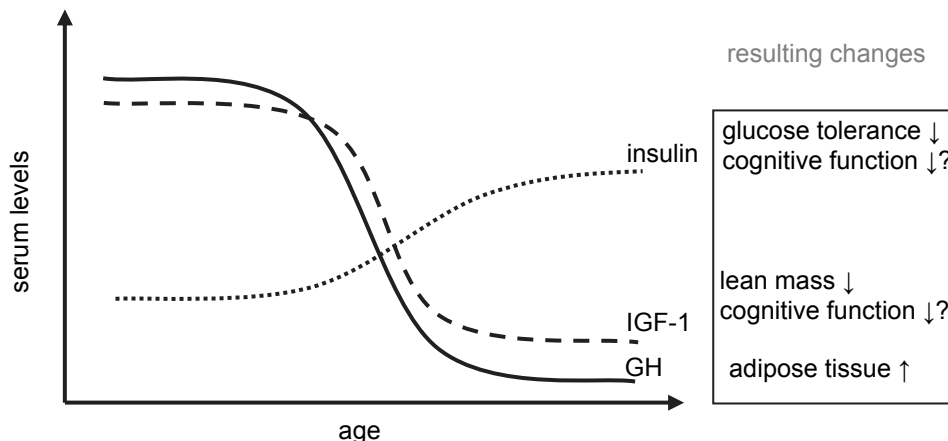


Fig. 4. GH/IGF-1 in ageing

Serum levels of growth hormone (GH) and insulin-like growth factor-1 (IGF-1) decrease during ageing contributing to increased fat mass and decreased lean mass and might influence cognitive function negatively. Serum insulin levels increase with age indicating age-associated insulin insensitivity possibly leading to impaired glucose tolerance and diabetes.

Recently, a study on a cohort of Ecuadorian patients carrying mutations in the GHR gene was published after 22 years of monitoring. Patients demonstrated severe GH resistance and reduced circulating IGF-1 and IGF-2 levels resulting in short stature (Guevara-Aguirre et al. 2011). No extension in lifespan was found in this cohort, which is in contrast to the GHR deficient mouse model (Guevara-Aguirre et al. 2011). However, causes of death were different in unaffected relatives compared to GHR deficient subjects, which died much more frequently from accidents, alcohol-related diseases and convulsive disorders, which might be the reason for unchanged lifespan (Guevara-Aguirre et al. 2011). Cancer accounted for 17 % and diabetes for 5 % of all diseases in non-affected relatives, whereas only one nonlethal malignancy and no cases of diabetes, probably due to improved insulin sensitivity, were reported for the individuals with GHR mutations (Guevara-Aguirre et al. 2011). Importantly, GHR deficient subjects appear to have no increased mortality from vascular diseases compared to their relatives (30 % of deaths verses 33 % of deaths in the non-affected relatives), however the proportion of strokes (3 % verses 12 %) and cardiac disease (27 % verses 21 %) was slightly different (Guevara-Aguirre et al. 2011).

In a cohort of Ashkenazi Jewish centenarians, a gender-specific increase in serum IGF-1 associated with smaller stature in female offspring was found to be due to heterozygous mutations in the IGF-1R gene (Suh et al. 2008). These rare mutations were significantly more common in female centenarians and overrepresented compared to controls (Suh et al. 2008). These observations are in line with the IGF-1R<sup>+/-</sup> mouse model, in which female mice show growth retardation and extended lifespan (Holzenberger et al. 2003) but IGF-1 resistance.

Further studies on human centenarians, mainly screening selectively genes of the IIS pathway, identified certain genes in the insulin/IGF-1 pathway to be important for human longevity. Several cohorts with different genetic background have reported an association of FOXO3A single nucleotide polymorphisms (SNPs) with longevity (Willcox et al. 2008; Flachsbart et al. 2009; Anselmi et al. 2009; Pawlikowska et al. 2009; Li et al. 2009). Interestingly, the study on long-lived Han Chinese revealed a SNP of FOXO1 associated with female longevity exclusively (Li et al. 2009).

Furthermore, in the Leiden 85-plus Study an association of a certain FOXO1 haplotype with higher HbA1c levels, higher prevalence of diabetes, myocardial infarction as well as increased mortality was observed (Kuningas et al. 2007). In addition, a certain haplotype of the FOXO3A gene revealed increased risks for stroke and mortality (Kuningas et al. 2007). These findings indicate that alterations within the FOXO genes might be causative involved in age-associated diseases and regulation of lifespan (Flachsbart et al. 2009).

## 7. Conclusion

Recent studies in different model organisms, including *C. elegans*, *Drosophila melanogaster* and *Mus musculus* have generated piling knowledge about the IIS pathway and its relevance for healthy ageing and longevity. The data obtained in these model organisms have been translated to humans and lead to the identification of the IIS pathway as regulator for human lifespan and ageing.

The GH and IGF-1 signalling pathway plays a key-role in regulating growth and metabolism, and alterations within this pathway have crucial effects on health and lifespan. Model-organisms with reduced GH or IGF-1 signalling are frequently long-lived or show an increased mean lifespan. Interestingly, serum GH and IGF-1 levels decrease with age, and this might be interpreted not solely as progressive failure of the hypothalamus-somatotrope axis but rather as protection for insulin resistance and malignancies.

IGF-1R signalling is required for normal brain development, and acute IGF-1 action might enhance cognitive functions and ameliorates ischemic or traumatic brain injuries. Additionally, recent studies demonstrate that IGF-1R signalling or the number of IGF-1Rs in the brain during early development determines metabolism and possibly age-associated diseases indicating a role of IGF-1 mediated signals as neuroendocrine regulator of health and lifespan.

Foxo-transcription factors have been identified as main downstream-target of IIS and seem to be essential for activating gene transcription that mediates longevity. Furthermore, genomic screening of centenarians with different genetic background found matching SNPs or haplotypes in the FOXO3A gene suggesting a key-role of FOXO3A in influencing lifespan not only in model organisms but also in humans.

However, the role of IIS in AD still raises questions, as the impaired signalling might be cause or consequence of neurodegeneration.

Therapeutic approaches altering the IIS pathway might not only improve treatment of neurodegenerative disorders but provide a possibility to prevent ageing-associated diseases in the future.

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## **Part 6**

# **Endocrinology of Stress**





# The Endocrine Response to Stress - A Comparative View

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## 1. Introduction

### 1.1 The stress concept

The word stress is used extensively to name a situation of tension that can be applied to living organisms such as animals or plants but also to ecosystems or in geological phenomena. Nevertheless, the concept of biological stress is closely connected to the historic development of the meaning of this word by Hans Selye after his short paper in *Nature* (Selye, 1936), following a first approach by Walter Cannon who restricted the physiological changes of stress and injuries to the effects of catecholamines and the adrenal medulla. Other key contributions of Selye to the stress associated concepts were the word stressor, meaning the agent causing stress effects and the non-specificity of the neuroendocrine response, even after positive or negative stressors (Szabo, 1998). Being such a general and widely used concept, the term stress has received many definitions, some of them trying to characterize the phenomenon, others focusing the elicited response and others even including the types of stressors, i.e. symbolic or real (physical, chemical, pathogenic). Nevertheless, in all of them some key elements are included: A source or stressor, the non-specific reaction and the neuroendocrine response.

As a relevant physiological mechanism, the stress response by itself is not inherently bad. For example, glucocorticoids are released in animals in response to situations that are not normally regarded as stressful, including courtship, copulation and hunting. In addition, hormones which increase during stress periods, are also part of the reproductive process and induce hormonal cascades causing parturition in some species (Möestl and Palme, 2002). As an example, brine shrimp *Artemia* exposed under gnotobiotic conditions to a non-lethal heat shock increases the expression of Heat Shock Protein-70 (HSP-70), thus inducing a non-specific molecular stress response. When *Artemia* was challenged with pathogenic bacteria, *Vibrio campbellii* and *Vibrio proteolyticus*, a cross-protection against pathogens was observed if an appropriate combination of heat application and recovery treatment was applied (Sung et al., 2007).

### 1.2 Stress, a general phenomenon in living organisms

The overall stress response, meaning the array of reactions generated in most body compartments as a result of the threat of the stressor, is common to all organisms. The fight-or-flight response and the fear reactions can be seen as a common behavior from

invertebrates to man and the necessary energetic supply and coordination of neural circuits for these reactions are also common patterns among animals along the phylogenetic tree. Nonetheless, evolutive ancient organisms are also sharing some of the basic responses that are seen in lower vertebrates and mammals, for instance, heat shock protein expression or the increase of antioxidant enzymes.

Stress responses have been also described in microorganisms. It has been shown that mitogen-activated protein kinase (MAPK) cascades play an important role in transducing environmental threats to transcriptional machinery by means of phosphorylation and regulation of several key transcription factors (Karin, 1998). Although it is well known that these cascades are activated by hormones or cytokines in vertebrates, stressors such as anoxia, osmotic shock or radiation may also induce this activation. In bacteria such responses have been detected as well as the heat shock response. Thus, in *Escherichia coli* it has been shown that transcription factors recognize specific heat-shock promoters, and that in most bacteria the control of major Heat-Shock genes is highly regulated (Segal and Ron, 1998). In fungi, it has been observed that stress originated from nutrient deprivation causes debilitation of fungal propagules, autolysis, inhibition of spores and loss of pathogenic aggressiveness (Hyakumachi and Arora 1998). This is another common aspect of the stress reaction response, i.e. the consequences derived from energy depletion that can be mirrored with the higher vertebrate stress responses. Thus, all these changes are related to a reorganization of metabolic and energetic pathways to face the effects of the stressor, a mechanism that is essentially the same type of stress response in higher vertebrates and mammals.

The stress concept has also been applied to plants when facing unfavorable environment or constriction of nutrients, water or inadequate soil conditions and a significant amount of scientific literature is available concerning plant stress, mostly related to unfavorable or changing environmental conditions. In plants, a multitude of stressors with different modes of action elicit very similar non-specific responses, besides those specific ones related to the particular stressor. Larcher (1987) defined the stress response in plants as the state in which increasing demands made upon a plant lead to an initial destabilization of functions followed by normalization and improved resistance. Lichtenthaler (1984) extended the stress concept in plants by including regeneration and differentiating between eustress (activating and stimulating positive elements for plant development) and distress (severe stress that affects negatively the plant, causing damage), concepts that have been used also in animals. Plants have a centralized system of stress response that enables to develop physiological responses and this has been shown to work under low-resource environments such as deserts, shaded understory or infertile soils. This response includes slow growth, low synthetic rate (photosynthesis) and low nutrient uptake. As in animals, these responses are driven by hormones and include changes in their balance, for instance producing more abscisic acid and often cytokinins. Thus, there is now strong evidence that the plant hormone abscisic acid (ABA) plays an important role in the regulation of drought stress, since a plant can use the ABA signalling mechanism and other chemical signals to adjust the amount of water loss in response to changes in both the rhizospheric and the aerial environment. This hormone will work as an equivalent of renal hormones of animals after environmental stress. Another example is the biotic stress that induces changes in hormone synthesis and hormonal signalling cascades in the auxin, gibberellin, ABA, ethylene and jasmonate pathways, and in addition modifying plant defense mechanisms, some of them associated to the ubiquitin/proteasome system, a similar pathway that is found in vertebrates (Wilkinson and Davies, 2002).

## 2. The stress response

The stress response in vertebrates has been defined following the model proposed in mammals by Selye. Together with the conserved cellular response, some other common elements such as the non-specific neuroendocrine activation and the interaction between regulatory systems, are also observed in non-mammalian vertebrates.

### 2.1 Molecular and cellular responses

Cells encounter a range of physiological and environmental stresses that require adaptive changes in gene expression. Stress conditions include ultraviolet (UV) irradiation, temperature changes, nutrient limitation, oxidative stress, hypoxia and exposure to various drugs or toxins. Exposure of cells to stress elicits adaptive responses that require the coordinated expression of stress-response genes, which affect cell survival, apoptosis, cell-cycle progression and differentiation (Holcik and Sonenberg 2005). The cell stress response assesses and counteracts stress-induced damage, temporarily increases tolerance to such damage, and/or removes terminally damaged cells by programmed cell death (apoptosis). The capacity of the response depends on the proteome expressed in a cell at a particular time and is therefore species- and cell type-dependent (Kultz, 2005).

One of the major cell stressors is the oxygen radical. Although the concept of endogenous oxidants was at first controversial, the identification of superoxide dismutase (SOD), an enzyme whose main function is the removal of superoxide anions, involved that oxygen radicals are main stressors for the cells and that removal mechanisms are stimulated to reduce adverse effects. In terms of aging it was also proposed that higher metabolism and elevated use of oxygen involve protein and cell damage and overall life reduction at longer term. Given that mitochondria produce most of the energy in the cell, and correspondingly consume the bulk of intracellular oxygen, the free-radical theory of ageing supports the hypothesis that the higher the metabolic rate of an organism, the greater the production of reactive oxygen species (ROS) and hence the shorter the life span. In this way, studies on reactive oxygen species related to aging have determined a number of key molecules that are regulating common mechanisms in organisms, from invertebrates to humans. For instance, work done in a model organism, the worm *Caenorhabditis elegans* has determined that the forkhead transcription factor, DAF-16, seems to be in a central position to integrate a variety of signals induced by stress and the nutritional status, such as MAPK pathways (through JNK-1), insulin (through DAF-2) and steroid hormone signalling through DAF-12 (Dawson & Dawson 2003). Moreover, insulin and insulin/IGF signalling may not only be involved in the regulation of oxidative stress response and longevity, but also it is quite likely that they may have a role in other degenerative disease mechanisms involving cellular stress. (Baumeister et al., 2006).

However, in some species the strict correlation between metabolic rate and life span is not maintained and this would depend on the production rate of reactive oxygen species combined with the prevention mechanisms that avoid this production. This is particularly true for birds and primates, who tend to live longer than would be predicted by their metabolic rates. Careful analysis of oxidative production rates demonstrated that at a given metabolic rate, mitochondria from these species tend to produce fewer ROS. (Finkel and Holbrooke, 2000). This indicates that ROS production rather than metabolic rate provides the strongest correlation with overall longevity. Another consequence, in terms of stress regulation in cells, is that under situations of metabolic stress, mitochondrial oxidant

products seem to function as signalling molecules (Finkel and Holbrooke, 2000). Therefore, stress episodes involving a rise in intracellular oxidant levels have three important effects: damage to various cell components, triggering of the activation of specific signalling pathways and development of mechanisms reducing ROS production.

Another of the cell responses to stress is selective translation as it has been shown that this event commonly occurs during cellular stress and during apoptosis. Moreover, global translation is reduced in response to most types of cellular stress. This results in savings of cellular energy, which is mainly consumed in the process of translation, estimated as an average of about 50% of the cellular energetic resources. Remarkably, the stress-induced attenuation of global translation is often accompanied by a switch towards the upregulation of the selective translation of proteins that are required for cell survival under stress (Holcik and Sonenberg 2005).

Another key aspect of the cell response is the modulation of major pathways of energy metabolism, closely linked to the oxidative burst in stressed cells. Selected enzymes related to energetic pathways contribute strongly to the control of key pathways such as glycolysis, pentose phosphate pathway, and the citrate cycle. Induction of these enzymes during stress may be necessary for generating reducing equivalents (NADH, NADPH) that are needed for cellular antioxidant systems. In this way, a number of key enzymes such as the enzyme Enolase have been shown to be highly induced under different types of potential stressors. Thus, in vertebrates Enolase is expressed in most tissues as a response to either physical or chemical stressors, pathogens or immune stimuli and even under pathogenic states such as cancer. As it is a key enzyme of the glycolytic pathway, the role of this enzyme appears to be relevant in switching the energetic flows under any type of stress source (Ribas et al., 2004).

Regarding cellular responses to stressors, heat shock proteins (Hsp) are considered one of the main cell mechanisms responding against stressors. Stressors disturb cellular homeostasis and induce a corresponding homeostatic response of these proteins that in most cases have the characteristic of being permanent until environmental conditions change again, and that are triggered by stressor-specific sensors that monitor specific environmental variables. Hsp are a defined set of proteins that are conserved along evolution and perform protective cellular functions such as cell cycle control, protein chaperoning and repair, DNA and chromatin stabilization and repair, or removal of damaged proteins. Thus, from a number of 300 common proteins that have been identified in many organisms from human to yeast or bacteria, about 40 of them are involved in the cell response to stress. This response can occur via stressor-specific interactions, post-translational modifications, and fragmentation of stress proteins resulting in an induction of a common set of stress proteins triggered by molecular damage or oxidative accumulation (Kültz, 2005). In combination with the DNA repair machinery, molecular chaperones are required to recognize unfolded proteins and either target them for removal, stop their aggregation, or assist in their refolding into the native, functional state. These proteins are extensively utilized as bioindicators of environmental stress in many different types of organisms.

## **2.2 The integrated physiological response**

The stress response system in vertebrates has both central nervous system (CNS) and peripheral components. The central tissue components of the stress system in mammals are located in the hypothalamus and the Locus ceruleus in the brainstem, and include a number of endocrine messengers with a principal role in mediating the neuroendocrine stress response, being the CRH (Corticotrophin Releasing Hormone) the key peptide in this

activation. The roles of the related peptides, the urocortins, in stress responses are beginning to be understood in mammals, but very little is known about their expression and function in non mammalian species. Also, CRH and urocortins are expressed throughout the body where they may play diverse, but as yet poorly characterized, roles in tissue development and homeostasis (Denver, 2009). Another hypothalamic peptide, TRH (Thyrotropin Releasing Hormone) also has such activation properties in fish. These hypothalamic factors stimulate the release of the Adrenocorticotrophic Hormone (ACTH) from the pituitary which in turn induces production and release of the major stress steroid, cortisol, by interrenal cells located within the head kidney (Tort, 2010).

The components of the stress system include the peripheral limbs of the hypothalamic-pituitary-adrenal (HPA) axis; the efferent sympathetic-adrenomedullary system; and components of the parasympathetic system. Given the potential for the convergence of these mechanisms, specifically in the nervous system, it may be suggested that interactions between the two systems would be produced already at the initial stages of corticosteroid signalling (Riedemann et al., 2010). In emergency situations, the hypothalamus is activated and stimulates the sympathetic nervous system and the adrenal medulla which releases adrenaline and noradrenalin, and the adrenal cortex which releases GCs. The effects are global, and the nerves as well as hormones stimulate the systems needed for fight-or-flight (it is usually a short duration stress response). These peripheral components may be limited to these former elements, or one could include other hormonal axes and structures that are stimulated by the neuroendocrine agents and that exert important actions on the whole metabolism and the overall energetics. These other peripheral components are considered below.

### 3. Comparative endocrine response to stress

Endocrine changes after stress are clearly observed in vertebrates where a common endocrine pattern can be recognized (see table 1). Nevertheless, invertebrates also experience changes in hormones after stressors. For instance, in crustaceans, the hyperglycemic hormone (CHH) from the lobster, *Homarus americanus* increases in hemolymph following emersion. Significant levels of hemolymph CHH have been also measured in lobsters that had been eyestalk-ablated and it has been observed that these animals continued to produce CHH, even though the main source of CHH had been removed, probably produced in other portions of the central nervous system (Chang et al., 1999). As another example, in arthropods, proteins of the hemocyanin gene family are involved in major physiological processes, including aerobic respiration, the innate immune response, and molting. Members of this hemocyanin family, cryptocyanin, and phenoloxidase, are multi-subunit molecules that assemble into hexamers and higher aggregates. The hemocyanin hexamer heterogeneity is maintained as a mechanism of selection for functional diversity under environmental stress when changing developmental and environmental conditions (Terwilliger et al., 2006), a similar response that can be observed in vertebrates related to specific endocrine or immune molecules.

#### 3.1 Mammals

The stress response in the brain starts in the parvocellular neurons of corticotropin-releasing hormone (CRH); the arginine vasopressin (AVP) neurons of the paraventricular nuclei (PVN) of the hypothalamus; the CRH neurons of the paraventricular and parabrachial

	Fish	Amphibians	Reptiles	Birds	Mammals
Pituitary anatomy	Bigger pars intermedia Smaller median eminence	Bigger pars intermedia Smaller ventral lobe	Longer pars nervosa Bigger pars intermedia	Bigger ventral lobe Bigger median eminence Smaller pars intermedia	Bigger ventral lobe
Adrenal anatomy	Cells scattered in the head part of the kidney	Cells inside the kidney in islets (urodele) or strands (anuran)	Cells in lobular structures close to the gonads	Cells in lobular structures at top of the kidneys	Cells in lobular or irregular structures at top of the kidneys
Catecholamine	Adrenaline	Adrenaline	Adrenaline	Adrenaline and Noradrenalin	Adrenaline
Corticosteroid	Cortisol	Corticosterone	Corticosterone	Corticosterone	Cortisol or Corticosterone

Table 1. Details of the pituitary and adrenal anatomy and the main secretion, catecholamines and corticosteroids in vertebrates

nuclei of the medulla and the Locus ceruleus (LC); and other mostly noradrenergic (NE) cell groups in the medulla and pons (LC/NE system). The principal effectors include CRH, AVP, the proopiomelanocortin-derived peptides (POMC)  $\alpha$ -melanocyte-stimulating hormone (MSH) and  $\beta$ -endorphin, the glucocorticoids (GC) and the catecholamines noradrenalin and adrenaline. Signalling by CRH-like peptides is mediated by at least two distinct G protein-coupled receptors and modulated by a secreted binding protein. These neuropeptides function as hypophysiotropins and as neurotransmitters/ neuromodulators, influencing stress-related behaviors, such as anxiety and fear. In addition to modulating HPA activity and behavioral stress responses, CRH-like peptides are implicated in timing key life history transitions, such as metamorphosis in amphibians and birth in mammals. CRH-like peptides and signalling components are also expressed outside of the central nervous system where they have diverse physiological functions (Denver, 2009).

In mammals, the adrenal gland, also known as the suprarenal gland, is a two-paired gland, yellow- or orange-colored, triangle-shaped, located over the superior part of the kidney, one on each side. The adrenals are mainly responsible for regulating the response to stress through the synthesis of catecholamines and corticosteroid hormones. In other vertebrates, this gland is also associated with kidneys but its anatomical arrangement is different, as it will be described later on this chapter. In mammals, the adrenals have a distinct outer-cortex and inner-medulla arrangement, which is characteristic of this group of vertebrates. The adrenal medulla occupies the central part of each adrenal gland and accounts for 22% of the gland weight; the remaining 78% of the weight corresponds to the adrenal cortex and adrenal capsule that surrounds the gland. Both the adrenal medulla and cortex receive regulatory input from the nervous system (Binkley, 1995).

The central region (medulla) of the adrenal gland, have specific cells called chromaffin cells, which are considered modified postganglionic cells of the sympathetic nervous system. These chromaffin cells secrete catecholamines, adrenaline (or epinephrine), noradrenalin (or norepinephrine) and to a lesser extent, dopamine. The mammalian adrenal cortex can be divided into three layers: zona glomerulosa, zona fasciculata and zona reticularis; all three layers produce corticosterone, but otherwise, the layers are specialized as regards their main

hormone products. Briefly, the zona glomerulosa, which represent about 15% of the adrenal weight, is the outer layer and is specialized in production of aldosterone. The zona fasciculata is the central and widest layer of the adrenal cortex (around 50% of adrenal weight) and secretes cortisol and androgens. Finally, the zona reticularis, represents 10% of the adrenal weight, secrete cortisol and androgens. These two last zones respond to ACTH stimulation.

In an initial alarm phase, catecholamines are released into the circulatory system, where they circulate in a free form or conjugated to blood proteins; in blood, catecholamines have a short half-life of about 3 to 4 minutes. Its biosynthetic pathway of synthesis is highly conserved throughout the animal kingdom with the rate-limiting step controlled by the enzyme tyrosine hydroxylase (Perry and Capaldo, 2010). Both catecholamines have a large number of actions, most of which contribute to the sympathetic fight-or-flight response. The best well known functions of adrenaline and noradrenaline are their actions in the cardiovascular system, the stimulation of glycogenolysis in skeletal and cardiac muscle, mobilizing glucose in those tissues, which aimed at reducing the detrimental effects of stressors on physiological function or optimizing physiological processes during periods of increased energetic demand. In the more highly evolved vertebrates, such as mammals and birds, the acute humoral adrenergic stress response effectively complements the neuronal regulation of physiological systems via the sympathetic division of the autonomic nervous system (Perry and Capaldo, 2010).

Cells of adrenal cortex respond to the HPA axis and mediate the stress response through production of steroid hormones, namely mineralocorticoids and GCs. The HPA axis involves the release of corticotropin releasing hormone (CRH) and Vasopressin from hypothalamic PVN, and CRH is the main responsible for stimulating adrenocorticotrophic hormone (ACTH) release from the anterior pituitary gland. ACTH arrives at the target cells in the adrenal cortex where it stimulates the synthesis of GCs. In humans, monkeys, sheep and cats cortisol is predominantly secreted; whereas rodents mainly secrete corticosterone. Some other mammals, such as dogs, secrete a mixture of cortisol and corticosterone in a similar ratio (Boonstra, 2004). GCs act at multiple sites within the body in an attempt to maintain homeostasis, but because of the potential damaging effects of chronic exposure to GCs, the HPA axis is tightly regulated through classic negative feedback loops. Thus, cortisol feeds back on hypothalamus and pituitary to cause rapid inhibition of CRH release. Under acute stress conditions, feedback mechanisms are efficient and the system returns to normal, resulting in short-term effects on body processes. Under conditions of chronic stress, feedback signals are weak and the system remains activated for longer periods, resulting in effects on body processes that can be long term and detrimental (Boonstra et al., 2004).

After short-term stressors, cortisol affects the intermediary metabolism by stimulating gluconeogenesis in liver, increasing glycogen formation and increasing the availability of substrates derived from proteins and fats. All these actions tend to produce hyperglycemia, increasing the availability of quick energy to muscle and nervous tissue. Chronic or repeated exposure to a stressor, characterized by lower but prolonged endocrine secretions, often involve longer recovery periods and much higher energetic and performance costs, due to both stressor persistence and the derived effects of the stress response effectors, i.e., GCs. These negative effects include alterations at several physiological levels, from impaired growth and reproductive capacity to immune suppression (Tort, 2010).

### 3.2 Birds

In birds adrenal glands may be a single median structure or two separate organs, in close contact with each other, lying just cranial to the kidneys and gonads on either side of the aorta and caudal vena cava. The adrenals are small, ovoid in form, with a yellow or orange color, and are surrounded by a loose connective capsule. In contrast to mammals, the cortex is not well differentiated from the medulla. Clusters of chromaffin cells are mixed with blood vessels and the interrenal steroidogenic cells are radially arranged in the subcapsular zone and in the inner part of the gland (reviewed by Ghosh et al., 2001).

Adrenaline and noradrenalin are secreted by the medullary part of the gland and unlike in mammals, the enzyme PNMT exists in all adrenomedullary chromaffin cells, so it cannot be considered a marker in differentiating adrenaline from noradrenalin cells. PNMT exists in its active form only in the adrenaline-storing cells (Ghosh et al., 2001). The chromaffin cells are usually mixed and arranged without any preferential location, but unlike most other vertebrate groups, there is strong inter-specific variation in the noradrenalin/adrenaline cell ratio (Varano, 1980). This ratio seems to bear a distinct relation to avian phylogeny; thus, less evolved birds have more noradrenalin, while recently evolved birds have more adrenaline. In these terms, birds occupy an intermediate evolutionary position having noradrenalin/adrenaline ratios around 1/1 (Ghosh, 1977; Varano, 1980).

The most important glucocorticoid in birds is corticosterone, which is secreted by the cortical part of the gland and has both glucocorticoid and mineralocorticoid activity, having a more important role than aldosterone in electrolyte balance. In general, birds have low basal circulating corticosteroids levels, but these levels increase during stress-related responses as occurs in mammals (Boonstra et al., 2004). The acute short action stress response (fight or flight) is activated within seconds by the sympathetic nervous system which induces release of catecholamines from chromaffin cells of the adrenal medulla into the general circulation and release of CRH primarily from the hypothalamus, which induces ACTH secretion from the anterior pituitary and, in response to ACTH, release of corticosterone into the general circulation within minutes.

Ghosh and collaborators (2001) demonstrated that catecholamines cause hepatic and muscle glycogenolysis which can lead to hyperglycemia, as it occurs in mammals. The avian adrenal chromaffin tissue is influenced by the steroidogenic cells; GCs increase noradrenalin content in the chick and adrenaline content in the pigeon adrenal glands, with a synergistic action requiring activity of the splanchnic nerve (Ghosh et al., 2001). Compared to mammals, less is known about the effects of catecholamines on bird metabolism. Starvation experiments on birds of different food habits demonstrated a simultaneous depletion and release of catecholamines and glycogen.

### 3.3 Reptiles

In reptiles, the adrenal glands are discrete bodies; yellow/red in color, separated from the kidneys, and in close relationship with the gonads, lying in its dorsal part. The only exception to this pattern is in the chelonians where the adrenal gland is in close contact with the ventral surface of the kidney, as in anuran amphibians. Chelonians have dorsoventrally glands which lie against the kidney. Ventrally, they are covered by peritoneum that extends forward to form the mesorchium or mesovarium of the adjacent gonads. Snakes and lizards have adrenal glands incorporated into the mesorchium or mesovarium, close to their respective gonads. They are elongated in snakes and usually globular in lizards. The right gland is attached to the caudal vena cava (reviewed by Perry and Capaldo, 2010).



The chromaffin and the steroidogenic tissues are associated, but there is a substantial variation in the degree of mixing that depends on the considered group. Thus, in Crocodylians and Chelonians, chromaffin and steroidogenic tissues are largely intermingled. In Rincoccephalian lizards, most of the chromaffin tissue forms a dorsal mass that send extensions between the interrenal cells; moreover, many clusters of chromaffin cells are present within the steroidogenic parenchyma, and some clusters of chromaffin cells are present on the ventral surface of the gland. Most Squamata lizards or iguanas show a similar distribution, with the exception of the ventral chromaffin tissue, usually absent in these reptiles (reviewed by Perry and Capaldo, 2010). Moreover, the distribution of the two tissues presents a great variability, which is correlated to the phylogeny of the species. Generally, a high degree of separation between the two tissues is typical of ancient species, whereas a trend toward the close association between them is typical of more recent species (Laforgia et al., 1991).

The distribution of noradrenalin and adrenaline cells is different among groups of reptiles. Usually, the islets intermingled in the steroidogenic parenchyma are occupied only by adrenaline cells, whereas noradrenalin and adrenaline cells are present in the chromaffin superficial tissue. In reptiles, the ratio between noradrenalin/adrenaline reflects the degree of separation between steroidogenic and chromaffin tissues. Thus, high values of this ratio, and therefore a high number of noradrenalin cells, correspond to a high degree of separation of the two tissues whereas low values of this ratio, correspond to a high degree of mixing between them. The reason for this correspondence, as it has been clarified by Perry and Capaldo (2010), is that in reptiles and mammals, the enzyme PNMT, that catalyses the methylation of noradrenalin into adrenaline, is activated by GCs. Therefore, when the two tissues are spatially separated, GC delivery to the chromaffin cells is likely to be impeded, whereas, when the degree of integration is high, GC delivery to the chromaffin cells is likely to be facilitated.

In response to stress, reptiles produce different patterns of catecholamine release, which can increase or decrease according to the type of stressor. For example, restraint stress in alligators causes a decrease in plasma adrenaline and noradrenalin levels at 24 h, and an increase in noradrenalin levels by 48 h. The role of catecholamines in regulating reptilian metabolism is poorly studied although it is clear that adrenaline causes hyperglycemia and promotes glycogenolysis in liver and glycogen deposition (Norris, 2007).

Corticosterone is the major adrenal corticosteroid secreted by reptiles (see Tyrrel and Cree, 1998) but the patterns of basal corticosterone secretion vary considerably among them. However, almost all studies concerning the HPA modulation by stressors show that reptiles respond to stressors by increasing plasma levels of corticosterone (Tyrrel and Cree, 1998; Moore and Jessop, 2003). Nonetheless, it is evident a variation in the rate, duration and magnitude of the adrenocortical response to the same type of stressor, reflecting a change in the sensitivity of the HPA axis to stressors (Wingfield and Romero, 2001). A number of studies have demonstrated that differences in physiological state among individuals, such as body condition, reproductive state, disease status, age, sex, genotypic variation, and social status can result in adrenocortical modulation (Dunlap and Schall, 1995). In addition, differences in external environment such as variation in rainfall, temperature, food availability, and humidity or general habitat quality can result in individual adrenocortical modulation between populations (Moore et al., 2001).

### 3.4 Amphibians

The adrenal glands of amphibians are found in close association with the kidneys, although their exact location varies with the species. Like reptiles and birds, the adrenal gland

appears homogenous on cut surface, and histologically it is comprised of intermingled cortical and medullary elements, rather than having the clear delineation between cortex and medulla seen in mammalian species. Thus, in amphibians the chromaffin cells are closely associated with the steroidogenic cells; additionally, chromaffin cells can be found in the extra-adrenal chromaffin tissue, within the paravertebral sympathetic ganglia and in the abdominal para-aortic region, but their precise role at these sites is not well understood. The arrangement of adrenal chromaffin cells is markedly different in anurans and urodeles (Accordi, 1991; Chimenti and Accordi, 2008). In urodeles, the steroidogenic tissue forms numerous small bodies, partially embedded in the ventral surface of the kidney with groups of chromaffin cells interspersed (reviewed by Perry and Capaldo, 2010). In anurans, noradrenalin and adrenaline cells are usually intermingled, with no preferential distribution. The adrenal gland produces both catecholamines and corticosteroids (Wright 2001).

Amphibian chromaffin cells generally receive a scarce nerve supply and because of the tight intermingling between steroidogenic and chromaffin tissues, regulation of the chromaffin cell activity is likely achieved by paracrine interactions between the two tissues (Capaldo et al., 2008a, b). Amphibian chromaffin cells are regulated by multiple factors, including humoral agents carried from the blood stream, neurotransmitters and neuropeptides.

It is known that ACTH is one of the main regulators of steroidogenic tissue activity in amphibians (Norris, 2007; Sicard and Vaudry, 2000) and that corticosterone, the main corticosteroid secreted by amphibians, generally increase as a response to stress (reviewed by Moore et al., 2003). However despite the existence of adrenocortical modulation, there is little understanding of what physiological mechanisms operate within the HPA axis to enable modulation of corticosterone release during stress both in amphibians and in reptiles (Moore et al., 2001). A limited number of studies have documented the effects of stressors on the catecholamine release in amphibians. But, in general, stress situations such as hypoxia and forced exercise (Romero et al., 2004) elicit an increase in plasma catecholamines. Elevated catecholamine concentrations cause hyperglycemia via its stimulatory action on glycogenolysis in both liver and muscle (Norris, 2007).

Adaptation to an unfavorable environment can take place under very difficult conditions, as demonstrated in desert amphibians: tadpoles of these species can accelerate metamorphosis as their pond dries, with earlier birth of a more mature product, as long as an unfavorable larval environment is not experienced too early or too severely. Significant changes in the hormonal patterns are found under these circumstances. Similar adaptive phenomena are observed in humans involving elevation of corticosteroid secretion when fetal adverse circumstances are too severe. However, there is very likely a price to pay for this immediate beneficial effect on survival. Hippocampal damage could explain the high rate of learning disabilities and a number of diseases in adulthood (Amiel-Tison et al., 2004).

### 3.5 Fish

Although few studies have been undertaken in fish regarding the mechanisms and the neurophysiology of stress detection by the central nervous system (CNS) and sensory organs, the scheme of stress induced neuroactivation is similar than in other vertebrates. Stressors are detected and first processed in the CNS and then through neuroendocrine axis. In most species the Corticotropin Releasing Hormone or CRH-like activity has been found in the preoptic nucleus of the hypothalamus. The CRH travels to the pituitary where it

stimulates the corticotrope cells to release ACTH but in fish, it also stimulates the melanotropic cells to release  $\alpha$ -Melanocyte-stimulating-hormone ( $\alpha$ -MSH) and  $\beta$ -endorphin. Similarly, other peptides as TRH activates ACTH release and the HPI cascade. In the interrenal tissue of fish, ACTH is the main responsible for the cortisol release but other agents, like Angiotensin, Urotensin and Atrial Natriuretic Factor (ANF), may interact with ACTH in the cortisol secretion. It has also been shown that melanotroph products ( $\alpha$ -MSH and  $\beta$ -Endorphin) may be important regulators of the corticosteroidogenesis and cortisol secretion.

In fish there is close association between adrenocortical and chromaffin tissue except in elasmobranch fishes where the tissues are completely separated and the adrenocortical tissue comprise a discrete gland which lies dorsally between the two posterior lobes of the kidney. The arrangement of chromaffin cells varies among the various groups of fishes along an evolutionary timeline. For example, in hagfish (*Myxine*) and lampreys (*Lampetra*), chromaffin cells are located within the heart (reviewed by Perry and Capaldo, 2010), as well as in the great veins returning blood to the heart (Gallo and Civinini, 2003). In teleost fish, the chromaffin cells are located within the walls of the posterior cardinal vein and in close association with the lymphoid tissue of the kidney. In general, chromaffin cells are often observed either singly or clustered into groups of several cells. The association of chromaffin cells with the steroidogenic interrenal cells may vary amongst different teleost species (Perry and Capaldo, 2010). Noradrenalin and adrenaline are the main catecholamines produced by fish chromaffin cells as it occurs in the other vertebrate groups. Noradrenalin and adrenaline producing cells can be distinguished on the basis of morphological characteristics. In teleost fish as in the other groups of fishes, the aminergic chromaffin and interrenal steroidogenic tissues form a diffuse organ at the anterior part of the kidney (called the head kidney), which is situated around the posterior cardinal vein and their branches (Gallo and Civini, 2004). Thus, the head kidney is the homolog to the adrenal gland, and is an organ of particular importance due to its cellular diversity and multiple functions associated (e.g. phagocytosis, hematopoiesis, catecholamine secretion by chromaffin cells, and cortisol secretion by interrenal cells).

Catecholamines, predominantly adrenaline, are released from chromaffin cells (Reid et al., 1998), immediately after stress. Once in the circulation, these hormones diminish the detrimental consequences frequently associated with stressful situations as in the other groups of vertebrates. Similarly, one of the primary roles of catecholamines is to modulate cardiovascular and respiratory function in order to maintain adequate levels of oxygen in the blood and, therefore, sufficient supply to the tissues mobilizing energy stores for the increased energy demands that often accompany stress (Perry and Wood, 1989; Randall and Perry, 1992 in Reid et al., 1998).

Activation of the hypothalamo-pituitary axis and release of ACTH into the circulation by the pituitary is also an integral part of the primary stress response of fish. ACTH stimulates the interrenal cells embedded in the kidney to synthesize and release cortisol into circulation for distribution to target tissues (Barton, 2002; Tort, 2010). The release of cortisol in teleostean and other bony fishes is delayed relative to catecholamine release. In fish, high plasma cortisol levels have a wide range of metabolic effects including, the modulation of carbohydrate metabolism through gluconeogenesis, increases in protein turnover, regulation of amino acid metabolism, ammonia output, glutamine synthetase and aminotransferase activity, and increased lipolysis (reviewed in Mommsen et al., 1999). Cortisol modulates the inflammatory response (Mackenzie et al., 2006; Aluru and Vijayan,

2009) and a range of other immune system responses (Maule and VanDerKooi, 1999), and appears to attenuate the cellular heat shock protein response to thermal insult (Ackerman et al., 2000; Basu et al., 2001 in Pankhurst 2010). Corticosteroid hormones also play a key role in osmoregulation (reviewed in McCormick, 1995), and are critical for controlling metabolism, hydromineral balance, and the overall stress response. Among corticosteroid hormones that have been well characterized in most vertebrate groups, the identity of one of the earliest vertebrate corticosteroid hormones, 11-deoxycortisol has been recently found in lampreys, a member of the agnathans that evolved more than 500 million years ago. This corticosteroid is regulated by the hypothalamus-pituitary axis and responds to acute stress (Close et al., 2010). This indicates that a complex and highly specific corticosteroid signalling pathway evolved at least 500 million years ago with the arrival of the earliest vertebrate, although it is assumed that this molecule may be derived also from an early ancestral corticosteroid receptor molecule (Thornton and Carroll, 2011).

Regarding the nuclear glucocorticoid receptor (GR), found in all vertebrates, it is known that in fish regulates cell growth, bone density, metabolism and modulates the cardiovascular system. Besides cortisol, also significant levels of 11-deoxycortisol and 11-deoxycorticosterone occur and these ligands bind to GRs and Mineralocorticoid receptors (MRs). It has been shown in fish that teleosts may have one or two different GR genes (Acerete et al., 2007; Bury et al. 2003). This discrepancy likely results from the fish specific genome duplication event. One of these two GR genes has two different transcripts that are generated by alternative splicing (Bury et al. 2003; Greenwood et al. 2003). Most interestingly, the three different GR forms in fish are differentially expressed *in vivo* and show different transactivational capacities, but only slightly different affinity for their ligand. As both genes and different splice variants are transcriptionally active, it is suggested that they both play an important and probably different role in the fish physiology (Bury et al. 2003, Greenwood et al. 2003). This multiple corticoid receptors in fish and a more complex signalling mechanism by related steroids provides an interesting model for comparative GR function (Stolte et al., 2006).

#### **4. Consequences of the neuroendocrine activation**

Response involving the sympatico-chromaffin axis, involves the activation of chromaffin tissue by specific neurons, resulting in the release of catecholamines, adrenalin and noradrenalin, which in turn induce the generation of large amounts of energy to meet the energetic needs of this active reaction. This is a very rapid response among all vertebrates and mainly involves the cardiovascular system. Regarding the HPA axis, a longer time is needed, since an endocrine cascade is activated and therefore the effects will be produced when the final hormone in the cascade, a corticosteroid has been released. Looking at the knowledge on comparative animal physiology, it can be sustained that the proteins, gene structures, and signalling pathways of the HPA axis are present in the earliest vertebrates and have been maintained by natural selection because of their critical adaptive roles. In all vertebrates so far studied, the HPA axis is activated as a response to stressors and is controlled centrally by peptides of the CRH family. It can be assumed that, irrespective of the behavioral or physiological outcomes, acute and chronic elevations of corticosteroid secretion initiate metabolic alterations and biochemical processes. Because of the dichotomy in the effects of acute and chronic GC responses, studies generally focus on either acute or chronic elevations of GCs (elevated basal values). When basal GC concentrations are

elevated, a common consequence is a weakening of the further acute GC response, probably through negative feedback mechanisms in the hypothalamic-pituitary-adrenal axis. This is an endocrine vicious circle: a chronically high baseline can provoke pathologies, and a weak acute response is ineffective at handling short-term stressors (Creel, 2001).

Since the energetic cost of the stress situation is high, it is clear that other hormone axes, mostly devoted to the metabolic support will have an influence on the immune function. Thus, the growth hormone and the somatotrophic axis have also been shown to affect immune processes, and opioids and thyroid hormones have also been shown to modulate immune responses. The activation of catecholamines and corticosteroids induce a wide number of changes, in particular because these molecules have receptors in most tissues. Therefore, many if not all of the hormones involved in stress responses possess, in addition to their direct effects, induce pleiotropic or collateral consequences that may or may not reinforce direct or primary effect. These other effects can mediate the mechanisms that might affect other unrelated adaptive needs, as for example to modulate the responses of the cardiovascular system, osmotic equilibrium, disease resistance mechanisms and immunocompetence, energetic metabolism and reproduction (see Figure 1).

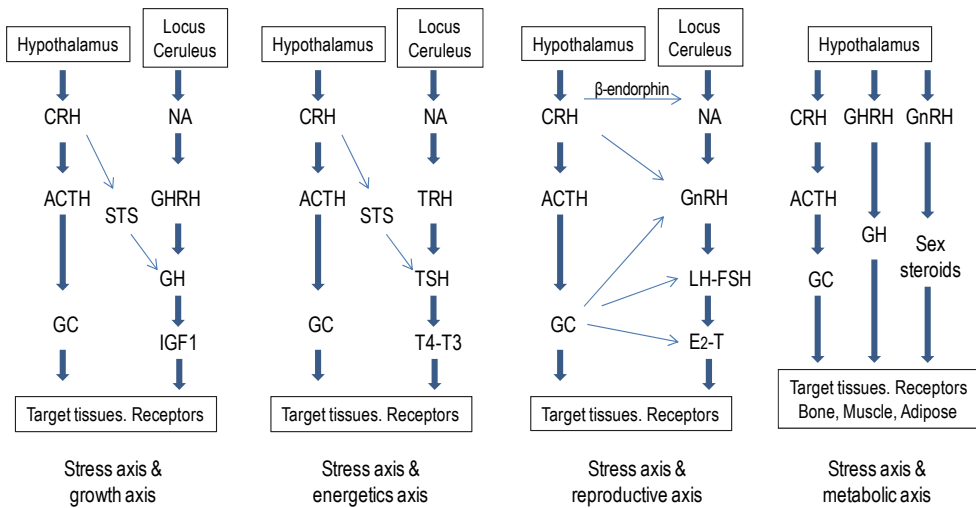


Fig. 1. Schematic diagrams of the endocrine interactions between the stress axis and growth, reproduction, energetics and metabolism

In terms of energetics and growth effects, stress generally shows depressive effects, inhibiting feeding behavior in all vertebrates. The effects of CRH on food intake are evolutionarily ancient, as this peptide inhibits feeding already in fishes, and the same effect is observed in birds and mammals. The effects of melanocortins on food intake have not been as extensively studied, but available evidence suggests that the anorexic role of neuronal melanocortins has been conserved. Data from mammals suggest an important role for hypothalamic neuropeptides, in particular the melanocortins and CRH-like peptides, in mediating stress-induced inhibition of feeding. Although there is evidence that CRH and the melanocortins influence hypothalamic circuitry controlling food intake, these peptides may have a more primitive role in modulating visuomotor pathways involved in the recognition

and acquisition of food. (Carr, 2002). Stress rapidly reduces visually guided prey-catching behavior in toads, an effect that can be mimicked by administration of CRH, while corticosterone and isoproterenol are without effect. Melanocortins also reduce prey oriented turning movements in amphibians and, in addition, facilitate the acquisition of habituation to a moving prey. The effects of these neuropeptides are rapid, occurring within 30 min after administration. By modulating visual and motor processing circuits these neuropeptides may help animals make appropriate behavioral decisions during stress episodes.

Many studies in the last decade have shown that stress can affect immune system both in mammals and lower vertebrates (Verburg-Van Kemenade et al., 2009; Dhabhar et al., 2009), although most of the reports are related to the effects of corticosteroids, and describe generally suppressive effects. Studies with human, murine and rat immune cells showed the immunosuppressive effects of elevated GC levels following stressful circumstances. GCs suppress Th1 cellular immunity and mediate a Th2 shift by suppressing production of T helper cells type 1/(Th1; tumor necrosis factor- $\alpha$ , interferon- $\gamma$ , interleukin-2 and interleukin-12 cytokines and inducing production of T helper cells type 1 (Elenkov 2004). Suppressive effects have been shown also in fish, where administration of cortisol induce a reduction of cytokine expression in cultured macrophages (Mackenzie et al., 2006; Castillo et al, 2009). Husbandry stressors applied to different species of fish result in a reduction of immunocompetence, showing decreased activity of immune response mechanisms (Montero et al., 1999), and reduction in efficiency after a combination of stress and pathogen treatment (Mauri et al., 2011).

Nevertheless, there are also episodes in which enhancement of immune function is observed, for instance during the immediate reaction, and this may depend on several considerations. One is the time-course, since acute or short-term stress often enhances innate and adaptive immune responses whereas chronic or long-term stress normally suppress or dysregulate immune function (Dhabhar et al., 2009; Tort, 2011). Another is the body compartment at which we are looking. Thus, skin is enriched with immune cells during acute stress, showing immuno-enhancement, while circulating blood may show depletion of leukocytes. A third factor may be the energetic situation of the animal since the demand of resources after stress are increased and they may be insufficient for other needs than facing the coping with stress itself. Whether there is an excess of demands and a shortage of energy resources, the immune system can be suppressed. Nevertheless, these arguments based on conservation of energy have been invoked to explain potential adaptive benefits of stress-induced immuno-suppression, but in one hand, some mechanisms for immuno-suppression expend, rather than conserve, energy. On the other hand, it can be also observed that while some immune responses are depressed or delayed, others are present without any decrease in efficiency, for instance, reducing the number of lymphocytes but increasing granulocytes. Therefore, the hypothesis is rather a temporal reorganization of immune resources than a pure suppression response. Maladaptive implications are present when stress becomes chronic; a situation that is unusual in nature and that evolution has yet to resolve (Dhabhar et al., 2009).

Stressors have been shown to reduce reproductive performance and, in lower vertebrates, even impair completely the reproductive process (Pottinger, 1999; Pankhurst 2009; Tort, 2011). In fact, the Hypothalamic-Pituitary-Gonadal axis and the Hypothalamic-Pituitary-Adrenal/Interrenal axis share a number of mediators, mainly steroid hormones. Therefore, stress and the activation of HPA axis affects the production of steroids. Interestingly, the

reproductive system can become resistant to inhibition by GCs in some reproductive contexts. For example, if GCs allocate resources away from reproduction, and thereby reduce individual fitness by impairing successful production of offspring, the benefit of the reproductive system ignoring the GC signal may outweigh the cost of not responding to the stressor. In salmon species and several marsupials, death occurs shortly after breeding. The proximate cause of death is the extremely high levels of GCs that catabolize essential proteins (Wingfield & Romero, 2001). Reproduction in these animals clearly continues despite elevated GCs. Furthermore, GCs do not inhibit reproduction in many short-lived species and in older individuals, and in dominant individuals in some species where the dominant individual has a limited period with access to mates (Wingfield & Sapolsky, 2003). Consequently, susceptibility to GC-induced inhibition of reproduction is highly specific depending on the importance of continuing reproduction in the presence of stress, which may vary depending upon age, sex or stage of the breeding cycle and of course, the species (Romero and Butler, 2007).

In the regulation of the stress response by steroid hormones it has been assumed that the production and regulation of steroid hormones has been viewed as a multi-organ process involving glucocorticoids and sex steroids. However, active steroids can also be synthesized locally in target tissues, either from circulating inactive precursors or *de novo* from cholesterol. This may be the case in the brain for neurosteroids and in the immune system. Furthermore, recent evidence suggests that other steroid hormones LH, GnRH, ACTH and CRH are expressed locally in target tissues, potentially providing a mechanism for local regulation of neurosteroid and immunosteroid synthesis. The balance between systemic and local steroid signals depends critically on life history stage, species adaptations, and the costs of systemic signals. Thus, individual tissues and organs may become capable of autonomously synthesizing and modulating local steroid signals, perhaps interacting with the HPG and HPA axes and the overall response to stress (Schmidt et al., 2008).

## 5. Integration and regulation

The stress response is characterized by the interplaying of several centers, mechanisms and systems, in order to reestablish the homeostatic conditions. Although it has been known for long time that the stress response is initiated by the activation of the Sympathetic-Chromaffin (SC) and the hypothalamus-pituitary-interrenal (HPI) axes, it was also known from the beginning that other regulatory systems are involved from the initial stages in the building of the stress response. Thus, it was already described by Selye that some pathological components were usually included in the events related to the General Adaptation Syndrome, such as gastrointestinal ulcers and thymolymphatic atrophy, clear signs of immunosuppressed status. Later on, an increasing number of evidences obtained during the last decades, both in lower and higher vertebrates, indicate that the stress response includes also the immune system. Even at the early life stages, the neuroimmunoendocrine interaction is active under stress. Hence, an important number of interconnections are established after stress, not only between the nervous and the endocrine system, but also between the endocrine and immune system, thus constituting a complex network of transmitters between the three regulatory systems. Although this aspect has not been extensively studied in all vertebrates (Verburg-Van Kemenade et al., 2009), it seems apparent that in all of them the nervous, endocrine and immune systems do not operate independently but rather they are part of the repertoire of the physiological

responses available to react in front of particular circumstances out of the normal physiological range, i.e.: disease, exercise, extreme environmental changes. Communication between these three physiological systems has had less attention in lower vertebrates compared to mammals, mainly because the amount of effort dedicated to such species is less and because of the lack of specific biochemical tools. However, such functional connections are present in all vertebrates and therefore this interconnection network may be an early mechanism in the evolution (see figure 2).

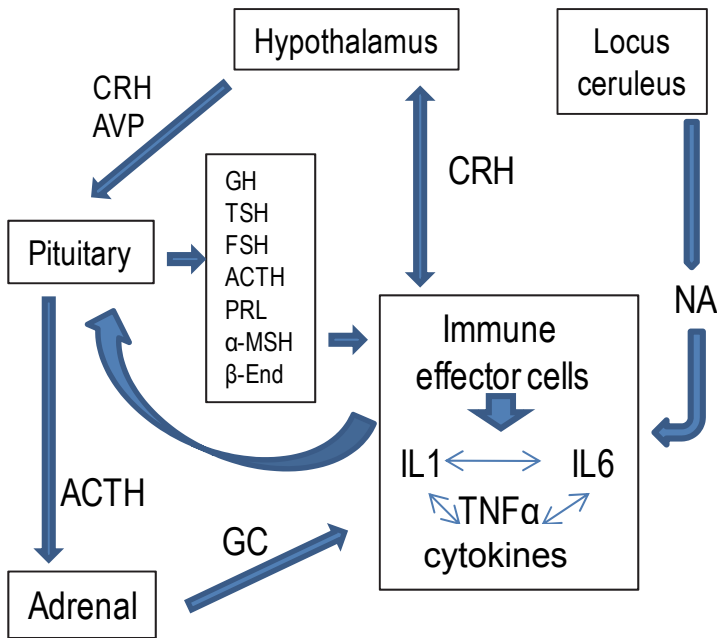


Fig. 2. Stress axis and main neuroimmunoendocrine interactions

These interactions start already at the central levels and there are multiple sites of interaction among the various components of the stress system. Neural control of stress is a complex process that requires the integration of information regarding both actual and potential outcomes. The organization of stress pathways further suggests that inputs on the physiological status of the animal can contribute to the eventual endocrine or autonomic response to the stressor. The majority of the pathways initiating physiological stress responses seem to be made at the level of limbic structures, which communicate information to subcortical sites positioned to interface with ongoing homeostatic feedback (Ulrich-Lai and Herman, 2009). The physical separation of autonomic and HPA stress effector circuits promotes some degree of independence of the two stress-modulatory cascades, allowing for appropriate tuning of neural and hormonal responses to specific demand characteristics of the actual or anticipated event. However, these two physiological systems also work together, both in terms of overlap in their underlying neural circuitry and in terms of their physiological functions. Charmandari et al., (2005) reviewed some of these connections. Reciprocal neural connections exist between the CRH and noradrenergic neurons of the



central stress system, with CRH and noradrenalin stimulating each other primarily through CRH type 1 and  $\alpha 1$ -noradrenergic receptors, respectively. Autoregulatory negative feedback loops are also present in both the PVN, CRH and brainstem noradrenergic neurons, with collateral fibers inhibiting CRH and catecholamine secretion via presynaptic CRH and  $\alpha 2$ -noradrenergic receptors, respectively. Both the CRH and the noradrenergic neurons also receive stimulatory innervations from the serotonergic and cholinergic systems, and inhibitory input from the  $\gamma$ -aminobutyric acid (GABA)-benzodiazepine (BZD) and opioid peptide neuronal systems of the brain, as well as from the end-product of the HPA axis, the GCs (Charmandari et al., 2005). It is likely that dysfunctions of information processing across these circuits, resulting from environmental adversity and/or genetic factors, generate alterations that can culminate in maladaptation.

In terms of the bidirectional relationships between endocrine and immune systems many of the hormones assessed so far can have an influence on immune agents or mechanisms, and even more some of them play significant roles in the regulation of the immune response. This is the case of the hypothalamic CRH and the adrenal hormone cortisol. The other axis related to stress, the sympathetico-chromaffin axis also influences immune response through catecholamines. In the reverse direction, the majority of evidence indicates that either direct or indirect stimulation of hypothalamic CRH secretion is the primary means by which cytokines (IL-1, IL-6, and TNF- $\alpha$ ) activate the HPA axis. Thus, the neural pathways which IL-1 have been proposed to influence the neuroendocrine hypothalamus in mammals are numerous and diverse, including the inhibitory effects of either inhibitors of prostaglandin synthesis or disruption of catecholaminergic input into the hypothalamus (Turnbull and Rivier, 1999)

Moreover, a reduced number of family molecules appear consistently in the interconnection pathways in all vertebrates. For example, immunocytes can release Proopiomelanocortin (POMC)-derived peptides which have been shown to be involved in the regulation of the immune system. The Growth Hormone can stimulate the activity of the immune system. Sympathetic neurotransmitters can modulate the respiratory burst activity of trout phagocytes. Melanotropins, Melanocyte Stimulating Hormone (MSH), and Melanocyte Concentrating Hormone, (MCH) exert relevant stimulatory effects of the immune system. In mammalian species POMC is produced not only by neuroendocrine tissues, but also in lymphoid cells, which reinforces the neuroimmunoendocrine connection. More work has to be done to ascertain the regulatory mechanisms, and in particular, the role of the receptors and the role of putative paracrine mechanisms that may explain at some extent the interconnection mechanisms between regulatory systems.

This may be the case of organs such the head kidney in fish, as it concentrates several secretory cells belonging to either immune or endocrine systems. It should be emphasized that in fish the head kidney or pronephros is an important centre of the endocrine response. In fact this organ becomes key player in the organization of the integrated response to stressors. Head kidney plays a substantial endocrine role in the secretion of the two main hormones of the hormonal axes, cortisol from interrenal cells, the major fish glucocorticoid and mineralocorticoid, and catecholamines, released by chromaffin cells which are components of the sympathetic nervous system releasing, and receiving specific connections from the central nervous system [30]. Moreover, not only the neural and the endocrine system meet in this organ, but also the immune and hematopoietic systems are found in the pronephros. Thus, this organ is the homologous to the bone marrow of higher vertebrates which involves that the production of lymphocytes, monocytes and neutrophils is localized

in this organ. It is concluded, then, that the head kidney deals with a number of key physiological responses in fish species. Therefore, fish represent an interesting comparative model for the study of the stress effects on the nervous, endocrine and immune systems, due to the structure and composition of the head kidney (Tort, 2010). In summary, the concept of neuroimmunoendocrine connection identifying specific tissues with specific systems (lymphoid tissue/ immune system, gland/ hormones or neurones/ nervous system) appears to be no longer appropriate or complete as well in lower vertebrates [28]. Clearly in this case, the head kidney in fish represents a tissue in which all three regulatory systems are integrally connected forming a centralized network to coordinate endocrine, neural, and immune, responses after stress.

## 6. Stress and adaptation in vertebrates

The adaptive value of responsiveness to stressors in animals in nature may provide invaluable information regarding the dynamics and flexibility of neuroendocrine responses. Absolute levels of transmitters or hormones may not matter in the induction of responses and survival. Relative elevation or inhibition related to previous experience may adjust specific neural centers to produce relevant output specifically related to the appropriate environmental context. The neural mechanisms for transduction of relevant information are necessarily very plastic, with many transmitters, neuromodulators and peripheral hormone systems interacting between them. These systems influence behavioral and physiological stress responses, but are also influenced by that output (Greenberg and Summers, 2002).

Taking the whole amount of data on the research in stress, it can be said that it generally focuses on the use of negative stimuli. However, positive stimuli such as immediate reaction or novel rewards can cause comparable physiological stress responses (Ulrich-Lai and Herman, 2009). It has been proposed that acute rises in corticosteroids following perturbations of the environment may actually avoid chronic stress as they may work primarily as “anti-stress” hormones. Free-living populations may have elevated circulating levels of corticosteroids under emergency stages. However, this situation may not always be advantageous and there is accumulating evidence from birds that the adrenocortical responses to perturbing factors are modulated both on seasonal and individual bases. These data suggest that corticosteroid secretions allow flexibility so that the response is integrated in relation to time of year, time of the day, as well as for individual differences owing to body condition, disease and social status (Wingfield and Kitaysky 2002).

Although GC responses are viewed as a major evolutionary mechanism to maximize fitness through stress management, phenotypic variability exists within animal populations, and it remains unclear whether inter-individual differences in stress physiology can explain variance in unequivocal components of fitness. For instance, it has been shown that the magnitude of the adrenocortical response to a standardized perturbation during development is negatively related to survival and recruitment in a wild population of birds, providing empirical evidence for a link between stress response, not exposure to stressors, and fitness in a vertebrate under natural conditions. Recent studies suggest that variability in the adrenocortical response to stress may be maintained if high and low responders represent alternative coping strategies, with differential adaptive value depending on environmental conditions (Blas et al., 2007). In fish it has been shown that such a coping strategy is an important determinant of the physiological response, either in behavior, physiology and even gene expression, and therefore much of the phenotypic expression

may be significantly conditioned by the coping strategy, not only in one species but specifically in groups of individuals (Mackenzie et al., 2009).

## 7. Conclusion

All living organisms have developed responses to face stress situations, and some at cellular level such as heat shock protein activation, have been well conserved along evolution. In vertebrates the physiological stress response is driven by the neuroendocrine axes which in turn affect many other physiological compartments until the homeostasis is regained. In this chapter, after revisiting the stress concept, we have reviewed the general character of the stress phenomenon and the cellular responses, and afterwards we summarize the physiological stress response in vertebrates: mammals, birds, reptiles, amphibians and fish. Finally, the consequences of the endocrine activation and the neuroimmunoendocrine integrated response to stressors have been reviewed in relation to the adaptive value of the stress reaction.

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## **Part 7**

# **Steroid Induced Osteoporosis**



# Genistein Aglycone Demonstrates a Protective and Reversible Effect on the Development of Steroid-Induced Secondary Osteoporosis and Increases Bone Breaking Strength in Rats

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## 1. Introduction

Glucocorticoids are used in the treatment of inflammatory and autoimmune diseases, cancers, and following organ transplantation. Glucocorticoid-induced osteoporosis is one of the primary side effects of glucocorticoid use resulting in increased risk of fractures. In a large meta-analysis study of glucocorticoid users, for example, van Staa et al (2002) found a relative risk increase of 1.91 for any fracture, 2.86 for vertebral fracture, 1.61 for hip fracture, and 1.13 for forearm fracture. Glucocorticoid-induced osteoporosis is characterized by low bone turnover and fractures, which occur in 30-50% of patients (van Staa et al., 2000). Glucocorticoids affect predominantly cancellous or trabecular bone, increasing the risk of vertebral fractures, which may be asymptomatic and occur early during the first months of glucocorticoid treatment (Laan et al., 1993; van Staa et al., 2000) and (Angeli et al., 2006). Trabecular bone accounts for approximately 20% of the total mass of bone (Bertazzo & Bertran, 2006) and also contributes to the ability of bone to tolerate stress to avoid fracture, especially in the spine. Long-term use of glucocorticoids also increases the risk of all osteoporotic fractures.

Published reports suggest no course of glucocorticoid therapy is safe for the skeleton. Regimens of daily prednisone at doses as low as 2.5 mg have been associated with an increased risk of hip and vertebral fractures. The risk increases by 5-fold, with prednisone doses above 7.5 mg daily. A dramatic 17-fold increase in vertebral fracture incidence was observed in subjects who used prednisone continuously more than 10 mg per day for longer than 3 months with increased fracture incidence in postmenopausal females and elderly males. The risk of osteoporotic fractures was also elevated in patients undergoing cyclic corticosteroid treatment at high doses (Van Staa et al., 2006) Although variable in onset,

fracture risk decreases after discontinuation of oral corticosteroids (Vestergaard et al., 2008). The risk of osteoporosis associated with inhaled glucocorticoids or with budesonide, a topical steroid used in inflammatory bowel disease, is small because their absorption is limited. Due to the high risk of fracture in glucocorticoid-administered patients, treatment guidelines exist.

Treatment guidelines for the use of glucocorticoids have been established which advise that if prednisolone is administered at  $\geq 5$  mg per day for three months or longer requires regular monitoring of bone mineral density (BMD) and treatment to prevent osteoporosis must be initiated (American college of Rheumatology, 2001; Adler & Hochberg, 2003; National Osteoporosis Society, 1998). Vitamin D and calcium are also recommended for the management of all patients treated with glucocorticoids. Bisphosphonates should be considered for the prevention and treatment of this disorder, because they can prevent the initial loss of bone mass from glucocorticoids. Alendronate, risedronate, and zoledronic acid were shown to prevent and reverse the loss of BMD in glucocorticoid-induced osteoporosis with greater effects than those observed with vitamin D and calcium (Doga et al., 2008) and (Amin et al., 2002). In fact, bisphosphonates induce improvement of BMD that is 2-fold greater than that observed during vitamin D treatment alone (4.6% vs. 2.0%, respectively) (Amin et al., 2002). Anabolic therapy is also utilized for treatment of glucocorticoid-induced osteoporosis. Teriparatide causes a greater increase in BMD than alendronate and greater reduction in the risk of vertebral fractures (Saag et al., 2007). Even with these evidentiary clinical trials and guidelines, patient bone loss is, in general, poorly managed (Eastell et al., 1998; Walsh et al., 1996; Gudbjornsson et al., 2002). In glucocorticoid-induced osteoporosis, fractures also occur at higher BMDs than in postmenopausal osteoporosis in untreated women (van Staa et al., 2003). Consequently, guidelines for the treatment of postmenopausal osteoporosis are not applicable to glucocorticoid-induced osteoporosis, and patients should be treated at BMD T-scores of  $\leq -1.0$  to  $-1.5$  (Compston, 2004). In addition, vertebral fractures may be asymptomatic and often require radiological diagnosis before treatment. Since the incidence of fractures is higher in the spine compared to other areas of the body, glucocorticoid therapy appears to affect rapid bone remodelling. During the initial phases of glucocorticoid exposure bone resorption is increased (van Staa et al., 2000). Glucocorticoids inhibit the formation of mature osteoblasts, but also activate apoptosis in these cell types (Jones & Sambrook, 1994; Canalis et al., 2007). Osteoprotegerin (OPG) expression, a key factor involved in modulating maturation of osteoclasts, is reduced also by glucocorticoids resulting in increased osteoclastogenesis (Orcel, 2005). Therefore, the combination of reduced osteoblast formation, increased osteoclast maturation leads to accelerated bone loss while on glucocorticoid therapy. Therapies are needed which modulate osteoclast as well as osteoblast activity to restore a more normal balance to the bone remodeling process in glucocorticoid treated patients.

## 2. Glucocorticoid-induced osteoporosis: Is there a role for genistein?

Among the anabolic compounds tested in recent years genistein aglycone seems a promising agent able to stimulate bone formation and to reduce bone resorption, acting *via* a genomic as well as a non-genomic pathways. Genistein is an isoflavone found in small quantities in certain legumes throughout the plant kingdom. Soybeans are a particularly rich source of genistin, the glucosidal precursor of genistein, although the concentration varies with the strain, location and environmental conditions of cultivation

of the plant. Another widely utilized source of genistin is *Sophora japonica* L (Tian et al., 2004). The glucosidal form and acetyl- and malonyl-glucosides are the major isoforms found in soybean derivatives. The conversion from the glucosides to the aglycone form occurs in the gut, where the sugar residue is removed with the generation of genistein (Larkin et al., 2008).

Genistein is freely absorbed from the intestine and a large fraction is converted to the 7 $\beta$ -O-glucuronide as it crosses the brush border and ultimately enters the portal vein (Sfakianos et al., 1997), a process that is influenced by intestinal bacteria (Setchell et al., 2002; Day et al., 1998). Recent *ex vivo* data using isolated human gastrointestinal tract tissue also suggest that genistein may also undergo sulfonation in the small intestine, though the extent to which these sulfonates are absorbed following dietary intake is unknown (Ronis et al., 2006). The exact percentages of glucuronidated and sulfonated metabolites after crossing the lumen are also unknown, although it is clear that only a small percentage of the parent molecule remains as unconjugated genistein once it reaches the liver. Once in the liver, genistein undergoes additional biotransformation *via* CYP450-mediated hydroxylation (Hu et al., 2003) followed by glucuronidation and sulfonation by UDP-glucuronosyl transferase and sulphotransferases, respectively (Sfakianos et al. 1997). Genistein 7 $\beta$ -O-glucuronide can be recovered from bile after infusion of genistein into the small bowel of rats (Prasain et al., 2006). As much as 70% of the recovered genistein from bile is in the form of glucuronidated conjugates with smaller amounts reappearing in the distal duodenum and jejunum (Sfakianos et al., 1997). The vast majority of circulating genistein in serum has been found to be in the form of glucuronidated and sulfonated conjugates which represent excretion forms of the molecule (Setchell et al., 2002). Little is known about the bioactivity of conjugated isoflavones. Genistein is composed of two benzene rings (A and B) linked through a heterocyclic pyrane C ring. The hydroxyl group on the A ring confers to genistein a greater activity both *in vitro* and *in vivo*, compared to other isoflavones (Choi et al., 2008).

In addition to competing with endogenous estrogens for binding to the estrogen receptors at high concentrations, genistein may exert anti-estrogenic effects by several potential mechanisms (Tham et al., 1998). Genistein has both ER agonist and antagonist activity in different cell types and works in a promoter specific manner in gene activation *via* ERs. The ER domain structure is typical of nuclear receptors. The amino-terminal region is involved in trans-activation of gene expression and has been termed the activation function domain or AF1 domain. The middle region contains a two-zinc finger structure, which plays an important role in binding to specific DNA response elements and in receptor dimerization. The carboxyl-terminal region contains an activation domain, termed AF2. A ligand binding domain, lying within AF2, is crucial for binding to receptor specific ligands and also co-repressors and co-activators. This binding interaction affects receptor dimerization, nuclear translocation and modulation of target gene expression by AF2. The ligand-binding domain is structurally conserved among the nuclear receptor family and consists of between 10 and 12 $\alpha$  helices folded in a globular domain. A central hydrophobic pocket accommodates the cognate ligand, which upon binding induces a conformational change in the ligand-binding domain, exposing a coactivator-docking site on ligand binding surface. However the binding of SERMs to ERs results in a different conformation change in which the coactivator-binding site is blocked from interaction with coactivator, thereby blocking AF2 mediated transcriptional activity (Liu et al., 2003). Several classes of nuclear receptor coactivators interact with ERs receptors and are specific for either ER $\alpha$  or ER $\beta$ . Using specifying coactivator recruitment and fluorescence resonance energy transfer, the genistein

interaction with ER receptors was investigated (Kuiper et al., 1998). Compounds with estrogen agonism induce agonist-mediated recruitment and allow fluorescence resonance energy transfer. Compounds with estrogen antagonism block the agonist-mediated recruitment of coactivators and prevents fluorescence resonance energy transfer. Genistein showed full agonism for ER  $\alpha$  and only partial agonism for ER  $\beta$ , but higher affinity for ER $\beta$  than ER $\alpha$  (Kuiper et al., 1998).

A second action of genistein on membrane associated protein (membrane receptors) has been proposed, and remains still not fully elucidated. In this mechanism, several possible second messengers, including kinase enzymes, are activated and in turn stimulate or inhibit downstream pathways of metabolism and protein products. The duration of this effect *via* a membrane protein is short lasting and of limited efficacy. The positive effects of genistein on primary osteoporosis, due to estrogen fall, have been extensively investigated by our group as well as by others and have been recently summarized in a review article (Bitto et al., 2010); however pre-clinical evidence point out a role for genistein also in secondary osteoporosis induced by corticosteroid use.

### 2.1 Evidence from *in vitro* studies

Genistein has been most investigated for its effect on the proliferation and differentiation of a number of cell types. Through its effect on tyrosine kinase, genistein is able to modulate cell cycle progression in the S phase, to induce G2/M arrest and to induce apoptosis (Matsukawa et al., 1993). Numerous *in vitro* studies with human or animal osteoclast- or osteoblast-like cell lines have been carried out with consistent observations of direct effects of genistein on both cell types. Effects of genistein on bone metabolism derived from direct and indirect actions on bone cells and can be summarized in stimulation of osteoblastic bone formation and inhibition of osteoclastic bone resorption (Gao & Yamaguchi, 1999a; Sugimoto & Yamaguchi, 2000; Chen et al., 2003; Heim et al., 2004). *In vitro* studies indicate that genistein is able to stimulate osteoblastic activity and inhibit osteoclast formation and action at a range of concentrations ( $10^{-5}$ – $10^{-7}$  M) consistent with the levels observed in human subjects after ingestion of genistein. With regard to effects on osteoclast genistein inhibits their formation and function (Amano et al., 1998; Gao & Yamaguchi, 2000; Yamagishi et al., 2001; Albertazzi, 2002; Blair et al., 1996; Gao & Yamaguchi, 1999a; Kajiya et al., 2000; Williams et al., 1998). Genistein suppress osteoclast activity by a number of possible mechanisms, including induction of apoptosis, activation of protein tyrosine phosphatase, inhibition of cytokines, changes in intracellular  $\text{Ca}^{2+}$ , and membrane depolarization. More in details, in mouse marrow cultures genistein has a potent inhibitory effect on osteoclast-like cell formation, and this effect is similar to others anti-bone-resorbing agents, such as calcitonin and  $17\beta$ -estradiol, and may involve cAMP signaling. Sliwiński and coworkers (2005) compared the effects of genistein, estradiol and raloxifene, compounds affecting in different ways estrogen receptors, on formation and viability of osteoclast from neonatal rat bone marrow; the results shown that all treatments decrease the number of osteoclasts formed from bone marrow, particularly genistein at the lower concentration decreases the number of osteoclasts by induction cell death, while at higher concentration genistein prevents the formation of osteoclasts. These observations are consistent with the results of another study (Rickard et al. 2003), showing that genistein, acting as agonist of estrogen receptors, mimicked estradiol in affecting gene expression of progesterone receptor, the proteoglycan versican, alkaline phosphatase and osteopontin, and production of

interleukin-6 protein. However, actions of genistein at the cellular level depend on the target tissue, receptor status of the tissue, and the level of endogenous estrogen. Both estrogen receptors ER $\alpha$  and ER $\beta$  are present in bone tissue, although the expression of these subtypes varies considerably during differentiation. The greatly increased expression of ER $\beta$  during bone mineralization is particularly pertinent to the potential hormonal effects of isoflavones because compounds such as genistein show a much higher affinity for ER $\beta$  than for ER $\alpha$  (Arts et al., 1997).

One of the mechanism by which genistein induces apoptosis of mature osteoclasts involves the Ca<sup>2+</sup> signaling, the inhibition of protein kinase and the activation of protein tyrosine phosphatase in osteoclasts. In this study the suppressive effect of genistein was completely abolished by the presence of inhibitors of Ca<sup>2+</sup>-dependent protein kinases (Gao & Yamaguchi, 1999b). The most important cytokine machinery, which is involved in bone metabolism, is osteoprotegerin (OPG)/receptor activator of nuclear factor- $\kappa$ B (RANK)/RANK ligand (RANKL) system. The molecular triad OPG/RANK/RANKL plays important roles in bone remodeling. RANKL is expressed by osteoblasts and is necessary and sufficient for osteoclastogenesis. RANKL binds to its receptor RANK, present at the surface of osteoclast precursors and mature osteoclasts, inducing osteoclast formation and activation. RANKL activity can be blocked by the soluble decoy receptor OPG, resulting in prevention of bone resorption. Studies, *in vitro*, have found that genistein induces apoptosis and inhibits RANKL signaling-related gene expression (Uchiyama & Yamaguchi, 2007).

Genistein was also reported to increase osteoblastic differentiation and affect osteoblast activity (Rickard et al., 2003; Pan et al., 2005; Morris et al., 2006; Okumura et al., 2006). In osteoblasts genistein stimulates a concentration-dependent increase in alkaline phosphatase activity. In osteoblastic MC3T3-E1 cells, genistein increases protein content, DNA content and alkaline phosphatase activity (Yamaguchi & Sugimoto, 2000; Sugimoto & Yamaguchi, 2000). This effect has also been demonstrated in tissue cultures. In femoral metaphyseal tissue from elderly female rats genistein increases Ca<sup>2+</sup> content and alkaline phosphatase activity. This effect is blocked by tamoxifen, indicating an ER-mediated pathway, and also by cycloheximide, suggesting that this isoflavone influence transcriptional or translational events (Yamaguchi & Gao, 1997, 1998). On the other hand, genistein also causes apoptosis of osteoblasts by activating caspase-3 and cleaving adhesion molecules such as cadherins and catenins (Hunter et al., 2001). In normal fetal osteoblast cells, genistein increases the progesterone receptor and alkaline phosphatase gene expression and inhibits osteopontin and interleukin-6 gene expression (Rickard et al. 2003).

## 2.2 Evidence from *in vivo* studies

The manner by which glucocorticoids induce bone loss is complex and incompletely understood (Patschan et al., 2001; Canalis et al., 2007), in part because there are no animal models absolutely comparable to humans. A major effect on the skeleton is a decrease in bone formation and unchanged or enhanced bone resorption (Lane et al., 2001). The main effect of glucocorticoids on bone is inhibition of osteoblastogenesis, augmented continued osteoclastogenesis and osteocyte apoptosis, leading to a decrease in bone formation, a rapid weakening of bone architecture and an increase in fracture risk (Manolagas & Weinstein, 1999). Chronic administration of steroids also causes avascular necrosis *via* an apoptotic mechanism of osteocytes and osteoblasts (Weinstein et al., 2000, Bekler et al., 2007). Once osteonecrosis occurs, glucocorticoids also cause inhibition of bone regeneration (Takano-

Murakami et al., 2009). In a rat model, we studied genistein preservative effects on methylprednisolone-induced bone loss and osteonecrosis of the femoral head (Bitto et al., 2009). In our study (Figure 1), genistein succeeded in preventing osteoporosis and osteonecrosis of the femoral head when co-administered with the glucocorticoid. The isoflavone statistically maintained bone mineral density (Figure 2) and content (Bitto et al., 2009) over the methylprednisolone-treated group and showed comparable efficacy with the vehicle group. Genistein co-administered with methylprednisolone also statistically maintained femoral bone's resistance to rupture compared with the methylprednisolone group (Figure 3C) and preserved the normal architecture of cartilage as well as both cortical and trabecular bones with a well-organized matrix in femoral head (Figure 3 A and B).

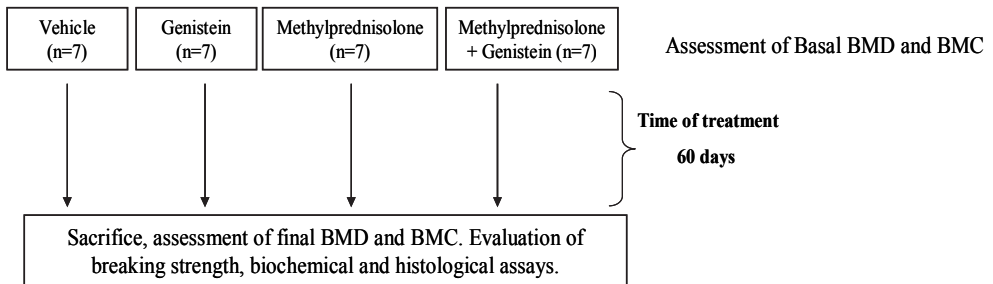


Fig. 1. Flow-chart of the experimental protocol.

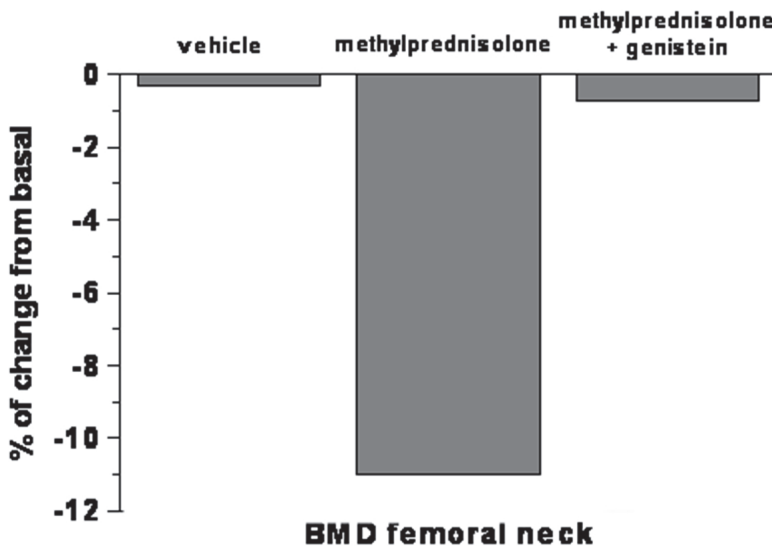


Fig. 2. Effects of aglycone genistein on femoral bone mineral density (BMD) in methylprednisolone-treated rats. Data are shown as % of variation from basal values in the same group of animals. \*  $p < 0.005$  vs. methylprednisolone.



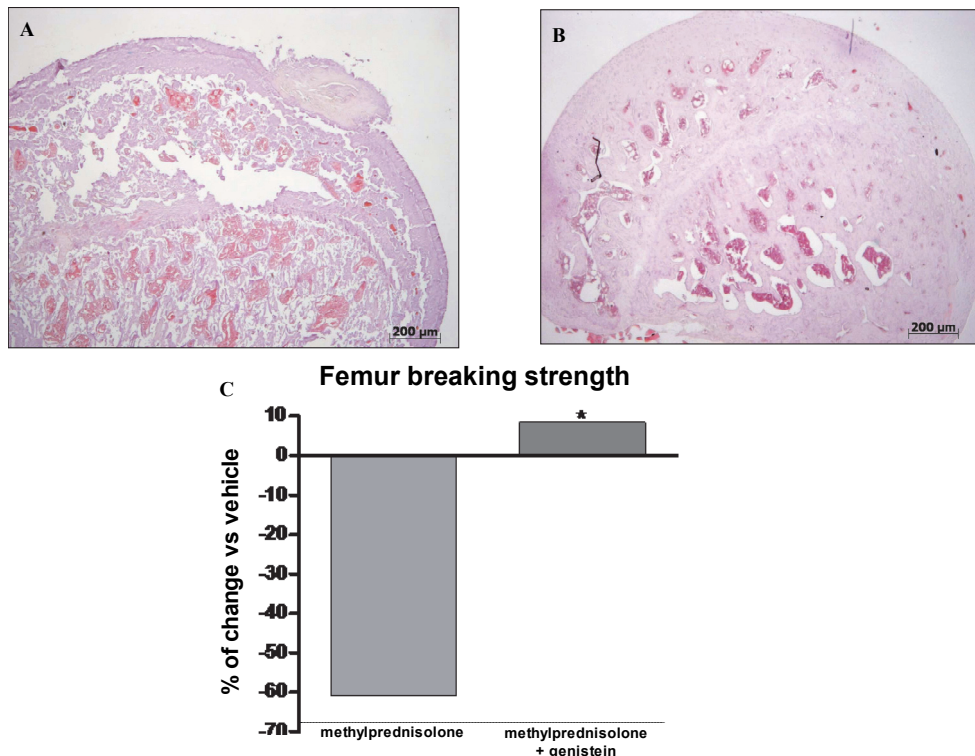


Fig. 3. A and B: Light microscopy of the bone structure of the femur head obtained from animals treated with methylprednisolone (A) or methylprednisolone plus genistein aglycone (B). (H&E original magnification X5). C: Femur breaking strength: \* $p < 0.001$  vs. methylprednisolone. Data are shown as % of variation from vehicle treated animals.

In addition, genistein caused a significant increase in b-ALP and OPG over methylprednisolone and vehicle confirming its role as an anabolic bone-forming agent (Figure 4A and B). Genistein administration also significantly reduced CTX circulating levels suggesting an anti-resorptive effect (Figure 4C).

Corticosteroid therapy has been shown to cause apoptosis in osteoblasts and inhibit the production of OPG required for bone formation, while RANKL accumulates resulting in further bone resorption (Bejar et al. 2005). Genistein has been shown to stimulate the production of osteoblasts *via* inhibition of a RANKL-resorptive mechanism by producing OPG (Viereck et al. 2002). This was also confirmed in our study where genistein increased OPG levels in glucocorticoid-treated animals. Osteoprotegerin is a glycoprotein secreted by osteoblasts in a differentiation-dependent manner and acts as a 'decoy receptor' (a soluble receptor that acts as antagonist) for RANKL regulating osteoclast functions and lifespan. In postmenopausal women with bone loss, genistein stimulated the production of OPG, down-regulated RANKL and decreased the RANKL/OPG ratio suggesting a direct effect on the RANK cytokine system (Marini et al., 2008). This work on genistein also suggests that increases in OPG in humans modulates the maturation process of osteoclast formation, thus

clarifying the putative antiresorptive effect being a non-apoptotic process, rather than an apoptotic process induced in antiresorptive therapy with bisphosphonates. Other mechanisms of genistein binding to glucocorticoid receptors have also been implicated.

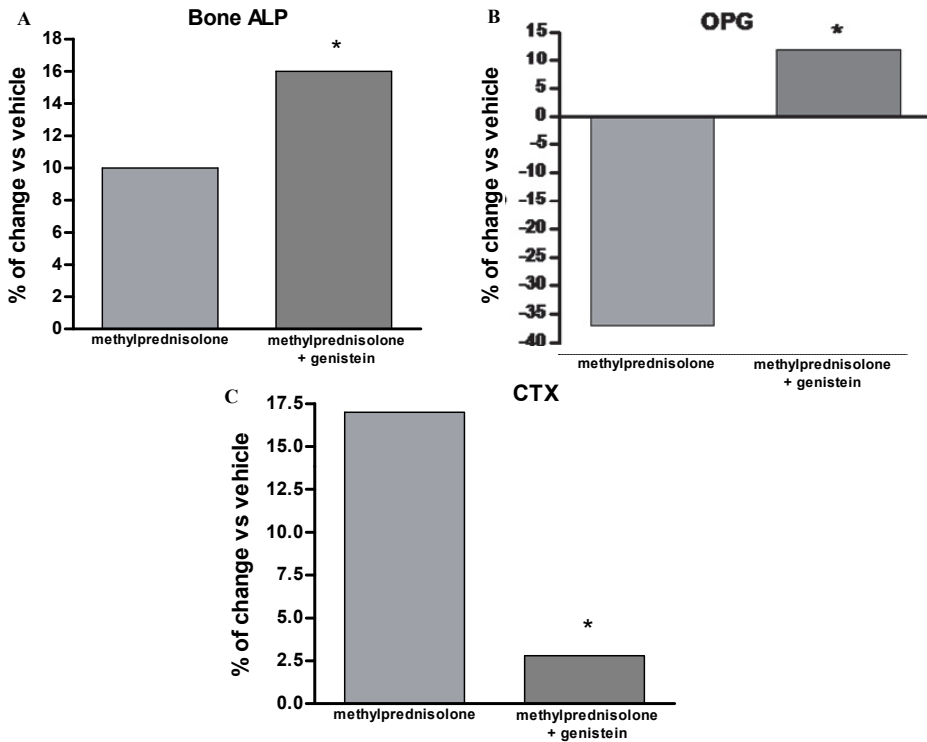


Fig. 4. A-C: Effects of aglycone genistein on serum bone-alkaline phosphatase (b-ALP), osteoprotegerin (OPG) and collagen C-telopeptides (CTX). Data are shown as % of variation from vehicle treated animals. b-ALP: \* $p < 0.005$  vs methylprednisolone. OPG: \* $p < 0.001$  vs. methylprednisolone. CTX: \* $p < 0.001$  vs. methylprednisolone.

It has been demonstrated that genistein inhibits glucocorticoid receptor transactivation and may also induce a proteasomal degradation of the glucocorticoid receptor complex *via* the p53 and ubiquitin pathways (Kinyamu & Archer 2003). Another mechanism might involve genistein activity as a tyrosine kinase inhibitor *via* the limitation of the subcellular nuclear transport and the recycling of the glucocorticoid receptors, blunting in turn the effects of glucocorticoids on bone (Yang et al., 1997).

### 3. Conclusions

Several studies and reports show a decrease in BMD and an increased risk of fractures during glucocorticoid use as well as an increase in osteonecrosis with chronic steroid use. Approximately 30% of all fractures of the hip and almost half of all fractures of the spine can

be attributed to chronic, high-dose glucocorticoid administration in humans (van Staa et al., 2001). Prior and current exposure to glucocorticoids increases the risk of fractures beyond that explained by values of BMD (Civitelli & Ziambaras, 2008). Pharmacological intervention for prevention of glucocorticoid-induced osteoporosis is needed depending on dose, expected duration of treatment, age and gender of the patient, and sometimes BMD at the start of the glucocorticoid therapy. At present, calcium and vitamin D<sub>3</sub> supplementation are considered as important support for the prevention of glucocorticoid-induced osteoporosis (Williams et al., 2004). Bisphosphonates are largely used to avoid bone loss and are cost effective in certain subgroups of patients depending on age, gender, glucocorticoid dose, and previous fracture history (Williams et al., 2004; Prinsloo & Hosking, 2006). Unfortunately, calcium and vitamin D<sub>3</sub> supplementation may not be enough to stave off bone deterioration and bisphosphonates have safety risks associated with long-term use, such as esophageal burns, bone and muscular pain (Ettinger et al., 1998; Wysowski & Chang, 2005). Osteonecrosis of the jaw, although rare and found primarily in cancer patients undergoing dental surgery, is a very serious and debilitating side effect of bisphosphonate use (Durie et al., 2005). Therefore, a safe and effective treatment for the prevention of bone loss and osteonecrosis of the femoral head in glucocorticoid-treated subjects is still needed. Collectively, our results strongly suggest that genistein might be a new potential therapy for the prevention of glucocorticoid-induced osteoporosis, the most important secondary cause of osteoporosis in humans. And in the minority of cases, genistein may prevent necrotic deterioration of the femoral head. Usually, drugs used in management of osteoporosis have been classified as predominantly 'antiresorptive agents' or as 'bone-forming agents', but, on the basis of the present results, genistein might represent the first therapy to overcome this classification combining a powerful bone-forming as well as an anti-resorptive activity.

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## **Part 8**

# **Chronic Renal Disease and Endocrinology**



# Hormonal Actions and Interactions in Proximal Tubule Cells Associated with the Development of Chronic Kidney Disease

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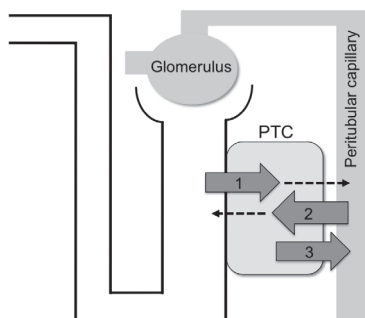
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## 1. Introduction

As the first tubular segment of the kidney between the Bowman's capsule and the loop of Henle, the proximal tubule is exposed to initial glomerular filtrates. Proximal tubule cells (PTCs) have a variety of functions including 1) reabsorption and intracellular processing of glomerular-filtered substances such as proteins, peptides, glucose, amino acids, uric acid, sodium, potassium, phosphate, bicarbonate and water via apical membrane receptors, transporters, and channels; 2) uptake of substances such as protein-bound compounds via basolateral membrane transporters, followed by metabolism or secretion to the urinary space; and 3) synthesis of bioactive substances that are released to peritubular capillaries (Fig. 1).



Normal functions of PTCs (indicated by arrows) include: 1) reabsorption and intracellular processing of glomerular-filtered substances via apical membrane receptors, transporters, and channels; 2) uptake of substances via basolateral membrane transporters followed by metabolism or secretion into the urinary space; and 3) synthesis of bioactive substances that are released to peritubular capillaries.

Fig. 1. Normal functions of proximal tubule cells (PTCs)

Impairment of these diverse functions is likely to affect systemic hemodynamic and metabolic homeostasis and can lead to the development of chronic kidney disease (CKD) and cardiovascular disease (CVD) (Saito, Kaseda et al. 2010). In particular, PTC injury occurs during the early stages of diabetic nephropathy, a leading cause of CKD.

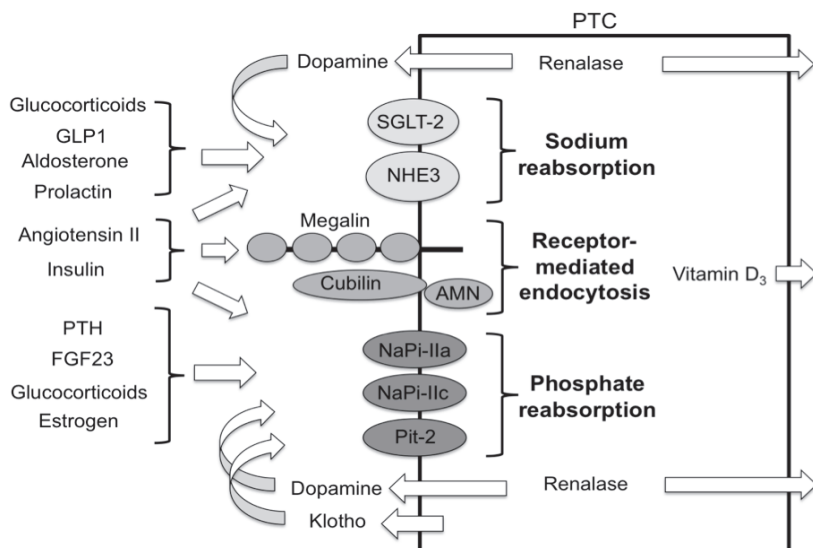
In this review, we focus on actions and interactions of various hormones and hormone-like substances on the regulation of apical membrane receptors and transporters that mediate three major functions of PTCs: 1) receptor-mediated protein endocytosis, 2) sodium reabsorption, and 3) phosphate reabsorption, which are particularly associated with the pathophysiology of CKD and the increased risk of CVD (Fig. 2).

## 2. Hormonal actions and interactions for regulating protein endocytosis in PTCs

Receptor-mediated endocytosis is a pivotal function of PTCs through which the cells reabsorb and metabolize proteins (and other substances) from glomerular filtrates (Saito, Sato et al. 2010). This reabsorption process is extremely efficient as urine is virtually protein-free in humans, and it accounts for the essential conservation of nutrients, carrier-bound vitamins, and trace elements filtered by glomeruli. Impairment of the process results in a loss of such substances and development of proteinuria.

### 2.1 Endocytic receptors involved in protein reabsorption in PTCs

The two major endocytic receptors expressed at the apical membranes of PTCs are megalin and cubilin (Saito, Sato et al. 2010) which act cooperatively in the uptake of glomerular-filtered proteins and mediate their metabolism in PTCs (Fig. 2).



Some hormones, such as dopamine, renalase, vitamin D<sub>3</sub> and klotho, are synthesized in PTCs and others come from other organs. Renalase acts to metabolize dopamine.

AMN, amnionless; FGF23, fibroblast growth factor 23; GLP1, glucagon-like peptide 1; NaPi-IIa, sodium-dependent phosphate cotransporter type IIa; NaPi-IIc, sodium-dependent phosphate cotransporter type IIc; NHE3, Na<sup>+</sup>/H<sup>+</sup> exchanger isoform 3; Pit-2, sodium-dependent phosphate symporter 2; PTC, proximal tubule cell; PTH, parathormone; SGLT2, sodium-glucose cotransporter 2

Fig. 2. Hormonal actions and interactions on the functions of receptors and transporters involved in receptor-mediated protein endocytosis and reabsorption of sodium and phosphate in the apical membrane of PTCs.

### 2.1.1 Megalin

Megalin is a large (~600 kDa) glycoprotein member of the low-density lipoprotein receptor family that is primarily expressed in clathrin-coated pits (Saito, Sato et al. 2010). Megalin-ligand complexes are internalized by invagination of clathrin-coated pits mediated by multiple adaptor proteins and motor molecules, forming endosomal vesicles. Acidification of the intravesicular lumen dissociates the ligands from megalin and they are transported to lysosomes for degradation or storage or are secreted into the cytosol for further processing or transport. Megalin is then recycled to the apical membranes through a recycling compartment. Megalin thus plays a critical role in reabsorption and metabolism of glomerular-filtered substances including albumin and low molecular weight proteins. Vitamin D-binding protein (DBP) is an endocytic ligand of megalin, and megalin knockout mice display decreased utilization of vitamin D for its activation in PTCs (Nykjaer et al. 1999; Leheste et al. 2003) (Fig. 3).

Decreased megalin expression in PTCs has been observed in the early diabetic stages of experimental animals (Tojo et al. 2001; Russo et al. 2007), and it has been suggested that the functions of megalin are also impaired in patients during the early stages of diabetic nephropathy, since low molecular weight proteinuria is frequently observed in patients at these stages (Pontuch et al. 1992; Hong et al. 2003). Therefore, the altered regulation of megalin expression and its functions must be primarily responsible for the early development of proteinuria/albuminuria in diabetic patients.

Megalin also interacts with various membrane receptors and transporters such as the cubilin/amnionless complex, sodium-hydrogen exchanger isoform 3 (NHE3) and sodium-dependent phosphate cotransporter type IIa (NaPi-IIa) as discussed in the following sections.

### 2.1.2 Cubilin

Cubilin is a 460-kDa peripheral glycoprotein that lacks transmembrane and intracellular segments but is anchored to apical PTC membranes. It was originally identified as the receptor for intrinsic factor-vitamin B<sub>12</sub> complex (Seetharam et al. 1988; Seetharam et al. 1997). Cubilin requires interaction with megalin to regulate its endocytic functions (Yammani, Seetharam, and Seetharam 2001; Kozyraki et al. 2001); however, it is bound more firmly by a protein called amnionless forming the complex CUBAM (Fyfe et al. 2004; Coudroy et al. 2005). Cubilin gene defects are the cause of hereditary megaloblastic anaemia 1 and Imerslund-Gräsbeck syndrome, also known as selective vitamin B<sub>12</sub> malabsorption with proteinuria (Aminoff et al. 1999). Cubilin is also involved in the absorption of various protein ligands present in glomerular filtrates, including albumin, transferrin and DBP (Christensen, Verroust, and Nielsen 2009). A recent meta-analysis of genome-wide association studies identified a missense cubilin gene variant associated with levels of albuminuria in both the general population and in diabetic individuals (Boger et al. 2011).

### 2.1.3 Amnionless

Amnionless is a 38–50 kDa membrane protein with a single transmembrane domain that was initially identified as a component required for normal development of the trunk mesoderm derived from the middle streak (Kalantry et al. 2001). In addition, defects of the amnionless gene cause hereditary megaloblastic anaemia (Tanner et al. 2003).

## 2.2 Hormones regulating the expression and function of endocytic receptors

Angiotensin II and insulin are two major hormones involved in regulating the expression and function of megalin (Hosojima et al. 2009) (Fig. 2), but no hormones have been identified that regulate cubilin and amnionless expression.

### 2.2.1 Angiotensin II

Angiotensin II downregulates the expression and function of megalin via the extracellular-signal-regulated kinase (ERK)1/2 signaling pathway in cultured PTCs (Hosojima et al. 2009). Intrarenal levels of angiotensin II are augmented in CKD (Kobori et al. 2007), which is involved in the development of hypertension as discussed below. Angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers are thus likely to target the increased action of angiotensin II in the kidney. These agents may be effective in suppressing the reduced expression of megalin in CKD and in preventing proteinuria/albuminuria (Tojo et al. 2003).

Megalín also appears to regulate the intrarenal actions of angiotensin II by internalizing angiotensin II (Gonzalez-Villalobos et al. 2005) and angiotensin-(1-7) (Gonzalez-Villalobos et al. 2006), as well as mediating the endocytosis of angiotensinogen that is filtered by glomeruli (Pohl et al. 2010).

### 2.2.2 Insulin

Insulin is involved in the upregulation of megalin, which in turn may be associated with increased endocytosis and metabolism of glomerular-filtered proteins, leading to intracellular metabolic overload. Cross-talk between insulin-insulin receptor substrate/phosphoinositide 3-kinase and angiotensin II-ERK1/2 signaling pathways was previously observed in cultured PTCs (Hosojima et al. 2009), and the balance between the actions and interactions of angiotensin II and insulin may also be important in regulating the expression of megalin *in vivo*. Hyperinsulinemia associated with metabolic syndrome is thought to stimulate insulin signaling in PTCs, while impaired insulin signaling (so-called insulin resistance) is suggested to occur in kidney cells in type 2 diabetic models; moreover, the intrarenal activation of angiotensin II appears to be associated with the development of insulin resistance (Tiwari et al. 2007). Recently, megalin was shown to be downregulated by the lipopolysaccharide-tumor necrosis factor- $\alpha$ -ERK1/2 signaling pathway that may also impair insulin signaling in diabetes (Takeyama et al. 2011). In addition, positive control of megalin expression has been demonstrated by peroxisome proliferator-activated receptor isoforms peroxisome proliferator-activated receptor (PPAR) $\alpha$  and PPAR $\gamma$  and their insulin-sensitizing agonists (Cabezas et al. 2011).

## 3. Hormonal actions and interactions for regulating sodium reabsorption in PTCs

Sodium reabsorption from glomerular filtrates is an important function of PTCs in the maintenance of systemic hemodynamics. Impairment of this function is associated with the abnormal regulation of blood pressure, especially in patients with CKD. Moreover, increased PTC sodium reabsorption causes glomerular hyperfiltration via the tubuloglomerular feedback mechanism, particularly in diabetes (Thomson, Vallon, and Blantz 2004).

### **3.1 Major transporters involved in sodium reabsorption and regulated by hormones in PTCs**

Various transporters and channels are involved in sodium reabsorption in the apical and basolateral membranes of PTCs. Here, we focus on three types of apical transporters (Fig. 2).

#### **3.1.1 Sodium-hydrogen exchanger isoform 3**

Sodium-hydrogen exchanger isoform 3 (NHE3), the main NHE isoform of PTCs, mediates isotonic reabsorption of approximately two-thirds of filtered NaCl and water, the reabsorption of bicarbonate, and the secretion of ammonium (Bobulescu and Moe 2009). It also contributes to the reabsorption of filtered citrate, amino acids, and oligopeptides by providing H<sup>+</sup> used by H<sup>+</sup>-coupled cotransporters. Indeed, enhanced NHE3 activity is assumed to be a factor for the increased sodium reabsorption and the development of hypertension in diabetes. NHE3 was reported to interact with megalin in PTC intermicrovillar clefts (Biemesderfer et al. 1999; Biemesderfer, DeGray, and Aronson 2001) and, following endocytosis with megalin, it is postulated to utilize the outward transvesicular sodium gradient of endocytic vesicles and early endosomes to drive the inward movement of H<sup>+</sup> and endosomal acidification. This is important for dissociating reabsorbed ligand proteins from megalin both for further processing and megalin recycling to the cell surface. Hormonal regulation of NHE3 has been intensively investigated and reviewed elsewhere (Bobulescu and Moe 2009).

#### **3.1.2 Sodium-glucose cotransporter-2**

Glucose is freely filtered by glomeruli before being almost entirely reabsorbed into the circulation from PTCs. Reabsorption of the majority of glucose is achieved by sodium-glucose cotransporter-2 (SGLT2), which is present in the S1 segment of proximal tubules. Expression of SGLT2 is enhanced in the kidney of diabetic Zucker rats (Tabatabai et al. 2009). SGLT2 inhibitors currently in development are expected not only to treat hyperglycemia but also to have an effect on hypertension and glomerular hyperfiltration in diabetic patients (Bailey et al. 2010; List and Whaley 2011).

#### **3.1.3 Sodium-dependent phosphate cotransporters**

Sodium-dependent phosphate cotransporters reabsorb inorganic phosphate in a sodium-dependent manner in PTCs. These will be discussed in detail in section 4.1.

### **3.2 Hormones regulating the expression and function of the transporters involved in sodium reabsorption in PTCs**

Many hormones are involved in the regulation of sodium reabsorption in PTCs, particularly through the function of NHE3 (Fig. 2).

#### **3.2.1 Angiotensin II**

Angiotensin II is important for increasing sodium uptake by PTCs. Intrarenal levels of angiotensin II are augmented in CKD and are also involved in the development of hypertension (Kobori et al. 2007; Navar et al. 2011). The action of angiotensin II on NHE3 has been intensively investigated, indicating biphasic dosage-dependent acute effects and chronic stimulatory effects on NHE3 (Bobulescu and Moe 2009). Classically, angiotensin II has been thought to act on its specific receptors in PTC plasma membranes by endocrine

and local tissue paracrine mechanisms. However, more recently it was also shown to function in an autocrine or intracrine manner (Li et al. 2011).

### 3.2.2 Insulin

Insulin is an important hormone that regulates sodium uptake as well as glucose metabolism in PTCs. In patients with metabolic syndrome, serum levels of insulin are elevated by a mechanism of insulin resistance in the liver and skeletal muscles. Increased levels of insulin are likely to augment sodium uptake in PTCs (Baum 1987) as well as other nephron segments such as thick ascending limbs (Mandon et al. 1993) and collecting ducts (Takahashi, Ito, and Abe 1996), causing extracellular fluid expansion and hypertension. Insulin has been shown to acutely and chronically stimulate the function of NHE3 in cultured PTCs (Fuster et al. 2007), and its infusion is associated with increased blood pressure in rats (Brands et al. 1991) and humans (Gans et al. 1991).

Diabetic kidneys, however, have been demonstrated to have impaired expression of renal insulin receptors (Tiwari et al. 2007). In kidney-specific insulin receptor KO mice, basal systolic blood pressure was increased and natriuresis was reduced in response to a volume load (Tiwari et al. 2008). The greatest relative reduction of the insulin receptor in these mice was found in the thick ascending limb and collecting duct, and renal production of NO was likely to be reduced. A previously uncharacterized role of the insulin receptor in these nephron segments may thus be associated with sodium and water reabsorption via NO production.

### 3.2.3 Glucocorticoids

Glucocorticoids, whether endogenous, as in Cushing syndrome, or exogenous, via pharmacologic provision, induce hypertension (Mangos et al. 2003). They directly stimulate NHE3 through both acute and chronic mechanisms (Bobulescu and Moe 2009). The acute effect of glucocorticoids on cell surface NHE3 is mediated by the activation of NHE3 exocytosis (Bobulescu et al. 2005) and a glucocorticoid receptor-dependent mechanism that activates serum and glucocorticoid-inducible kinase1 (SGK1) in a nongenomic manner (Wang et al. 2007). Glucocorticoids have also been found to enhance the function of insulin in stimulating NHE3 activity (Klasic et al. 2002).

### 3.2.4 Glucagon-like peptide 1

Glucagon-like peptide 1 (GLP1) receptors are expressed in pancreatic cells and other cells such as PTCs (Schlatter et al. 2007). Administration of recombinant GLP1 to Dahl salt-sensitive rats resulted in an antihypertensive effect, due mainly to its diuretic and natriuretic actions (Yu et al. 2003). GLP1 was also found to induce natriuresis in healthy subjects and insulin-resistant obese men through its action on PTCs (Gutzwiller et al. 2004). Hyperglomerular filtration in obese men was improved following this treatment, which was likely to be mediated by tubuloglomerular feedback mechanisms.

GLP1 receptor agonistic agents have been used in the treatment of hyperglycemia by acting on GLP1 receptors in pancreatic  $\beta$ -cells to stimulate insulin synthesis. The GLP1 receptor agonist exendin-4 also decreases sodium reabsorption in PTCs by affecting NHE3 activity (Carraro-Lacroix, Malnic, and Girardi 2009) and reduces blood pressure in a salt-sensitive hypertension mice model (Hirata et al. 2009). Such agents are also suggested to be clinically relevant in reducing blood pressure (Vilsboll et al. 2007; Klonoff et al. 2008).



### **3.2.5 Aldosterone**

Aldosterone stimulates the activity and surface expression of NHE3 in human primary PTCs, which is inhibited by the mineralocorticoid receptor blocker spironolactone and by the epidermal growth factor receptor (EGFR)-kinase inhibitor (Drumm et al. 2006). Aldosterone was found to regulate NHE3 by a nongenomic mechanism (Good, George, and Watts 2006). Aldosterone/mineralocorticoid receptor stimulation is also known to affect PTCs by inducing cellular senescence (Fan et al. 2011), suggesting that it is a potential therapeutic target in CKD.

### **3.2.6 Prolactin, dopamine and renalase**

Prolactin is also a natriuretic hormone that inhibits Na<sup>+</sup>, K<sup>+</sup>, ATPase activity in PTCs, depending on the renal dopamine system (Ibarra et al. 2005). Dopamine is known to both acutely and chronically inhibit the function of NHE3 by reducing its cellular expression (Bobulescu and Moe 2009). Renalase, a secreted amine oxidase that is synthesized in the kidney, metabolizes circulating catecholamines (Xu et al. 2005). Urinary renalase metabolizes urinary catecholamines, and perhaps regulates dopamine concentration in luminal fluid, and modulate the function of NHE3 for proximal tubular sodium transport (Desir 2011).

## **4. Hormonal actions and interactions for regulating phosphate reabsorption in PTCs**

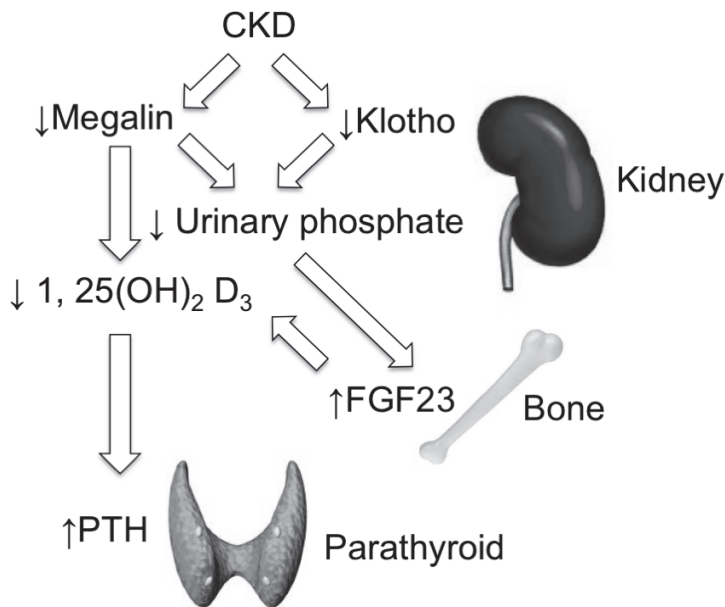
Phosphate uptake is an important function of PTCs which is regulated by the actions and interactions of various hormones and hormone-like substances, such as parathormone (PTH), fibroblast growth factor 23 (FGF23), klotho, glucocorticoids, vitamin D<sub>3</sub>, dopamine and so on (Figs. 2 and 3). Phosphate regulation is particularly associated with the development of cardiovascular disease and high mortality in patients with CKD, independently of the glomerular filtration rate (Kestenbaum et al. 2005). Therefore, the regulation of phosphate uptake in PTCs is clinically important.

### **4.1 Transporters involved in PTC phosphate reabsorption that is hormonally regulated**

At least three types of sodium-dependent phosphate transporters have been identified in the brush border membrane of PTCs: NaPi-IIa, NaPi-IIc, and sodium-dependent phosphate symporter 2 (Pit-2) (Fig. 2). Renal reabsorption of inorganic phosphate is mainly (about 70%) mediated by NaPi-IIa in the adult kidney (Ohkido et al. 2003).

#### **4.1.1 NaPi-IIa**

NaPi-IIa is a major phosphate transporter of PTCs, and changes in renal phosphate handling are primarily attributable to altered NaPi-IIa brush border membrane expression (Hernando et al. 2005). NaPi-IIa is expressed in the S1-S3 segment of superficial and juxtamedullary nephrons (Picard et al. 2010) and mediates the electrogenic transport of inorganic phosphate coupled to three sodium ions (Murer, Forster, and Biber 2004). NaPi-IIa is regulated by several hormones and other factors, as described in section 4.2. Adequate steady-state expression of NaPi-IIa and its inactivation by endocytosis and intracellular translocation require the presence of megalin (Bachmann et al. 2004), such that megalin dysfunction is likely to impair NaPi-IIa inactivation, inducing the increased reabsorption of phosphate (Fig. 3).



In CKD, renal expression of megalin and klotho is decreased, resulting in increased urinary phosphate excretion. Decreased megalin expression also causes decreased renal synthesis of  $1,25(\text{OH})_2 \text{D}_3$ . Synthesis of FGF23 in the bone is then stimulated, which also suppresses renal  $1,25(\text{OH})_2 \text{D}_3$  synthesis, increasing the synthesis of PTH in the parathyroid.

Fig. 3. Presumed hormonal network involved in phosphate regulation in CKD

#### 4.1.2 NaPi-IIc

NaPi-IIc is a growth-related transporter that is more active in the young and has a reduced role in adults (Segawa et al. 2002). NaPi-IIc transports inorganic phosphate in an electroneutral fashion and is highly pH dependent, exhibiting more transport at a more alkaline pH (Segawa et al. 2002). It is expressed in the S1 segment of PTCs (Picard et al. 2010) and mutations of the NaPi-IIc gene were found to cause hereditary hypophosphatemic rickets with hypercalciuria (HHRH), a Mendelian disorder of renal inorganic phosphate reabsorption (Bergwitz et al. 2006; Lorenz-Depiereux et al. 2006).

#### 4.1.3 Pit-2

Pit-2, expressed in S1 and weakly in S2 segments of superficial and juxtamedullary nephrons (Picard et al. 2010), mediates the electrogenic transport of inorganic phosphate (Ravera et al. 2007).

### 4.2 Hormones regulating the expression and function of PTC transporters involved in phosphate reabsorption

Several hormones and hormone-like substances are involved in the regulation of phosphate cotransporters. In particular, the actions and interactions of PTH, fibroblast growth factor 23 (FGF23), klotho and vitamin  $\text{D}_3$  are important in maintaining phosphate homeostasis and the development of CKD-mineral bone disease (MBD) (Fig. 2 and 3).

#### 4.2.1 PTH

PTH was first identified as an inactivator of NaPi-IIa through endocytic retrieval and degradation (Bacic et al. 2006). It was then found to be also associated with the endocytic regulation of NaPi-IIc (Segawa et al. 2007), although its acute effects differentially regulate these cotransporters including Pit-2 (Picard et al. 2010). The synthesis of PTH is increased in the progression of secondary hyperparathyroidism in CKD.

#### 4.2.2 FGF23

FGF23 is a 32-kDa protein that is secreted mainly by bone osteocytes (Liu et al. 2006). Administration of FGF23 to mice decreases the renal expression of both NaPi-IIa and NaPi-IIc and induces hypophosphatemia predominantly via FGF receptor 1 (Gattineni et al. 2009). FGF23 also suppresses 1,25(OH)<sub>2</sub>D<sub>3</sub> by inhibiting 1 $\alpha$ -hydroxylase (CYP27B1), which converts 25-hydroxyvitamin D<sub>3</sub> [25(OH)D<sub>3</sub>] to 1,25(OH)<sub>2</sub>D<sub>3</sub>, and by stimulating 24-hydroxylase (CYP24), which converts 1,25(OH)<sub>2</sub>D<sub>3</sub> to inactive metabolites in PTCs (Shimada et al. 2004). Increased FGF23 appears to play a causative role in the abnormal renal phosphate handling and vitamin D<sub>3</sub> metabolism of rats with CKD, leading to the development of secondary hyperparathyroidism and increased production of PTH (Hasegawa et al. 2010). The ternary complex of klotho-FGF23-FGF receptor in distal convoluted tubules (DCTs) has been thought to be a prerequisite for the exertion of FGF23 effects (Kurosu et al. 2006), and, indeed, initial FGF23-mediated signaling occurs in DCTs (Farrow et al. 2009). However, it remains undetermined how FGF23-mediated signaling in DCTs leads to the regulation of NaPi-IIa and NaPi-IIc in PTCs. It may be possible that FGF23 has a direct action on PTCs (Huang and Moe 2011).

#### 4.2.3 Klotho

Klotho was first identified as an aging suppressor gene product (Kuro-o et al. 1997). It is a single-pass transmembrane protein expressed predominantly in the kidney, intensely in DCTs and to a lesser extent in PTCs and parathyroid gland (Kuro-o 2011). *Klotho*<sup>-/-</sup> and *Fgf23*<sup>-/-</sup> mice show very similar phenotypes such as premature aging and abnormal mineral metabolism (Kuro-o 2011). As mentioned above, klotho acts with FGF receptor as a coreceptor in the binding of FGF23 in DCTs for phosphate and vitamin D<sub>3</sub> regulation (Kurosu et al. 2006). The Klotho-FGF23-FGF receptor complex in DCTs may produce putative paracrine factors on PTCs where phosphate transporters and enzymes for vitamin D<sub>3</sub> regulation are located (Huang and Moe 2011). The extracellular domain of Klotho is also known to be cleaved from plasma membranes, secreted to the circulation and present in urine (Hu et al. 2011); this soluble klotho form may directly act on PTCs. Klotho was also found to act as a PTC-synthesized autocrine factor in the FGF23-independent inhibition of NaPi-IIa function by  $\beta$ -glucuronidase-like enzymatic activity (Hu et al. 2011). Decreased Klotho expression in the kidney may be one of initial factors triggering dysregulation of various hormones involved in phosphate homeostasis and the development of secondary hyperparathyroidism in CKD (Kuro-o 2011).

#### 4.2.4 Vitamin D<sub>3</sub>

As mentioned above, megalin and cubilin take up the 25(OH)D<sub>3</sub>/DBP complex from glomerular filtrates (Nykjaer et al. 1999; Nykjaer et al. 2001; Leheste et al. 2003). In PTCs, 25(OH)D<sub>3</sub> is dissociated from DBP and converted by 1 $\alpha$ -hydroxylase to the biologically active form 1,25(OH)<sub>2</sub>D<sub>3</sub>, which is released to peritubular capillaries. Therefore, dysfunction of these endocytic receptors is an important cause of deficiency of both 25(OH)D<sub>3</sub> and

1,25(OH)<sub>2</sub>D<sub>3</sub> in CKD. The activation of vitamin D<sub>3</sub> is also upregulated by PTH and downregulated by FGF23 (Shimada et al. 2004). Vitamin D<sub>3</sub> deficiency develops very early in the course of CKD, especially in diabetic nephropathy, and is associated with the development of CVD or mortality in patients at the predialysis stage (Mehrotra et al. 2009).

#### 4.2.5 Other hormones

Dopamine mediates renal phosphate excretion, which is affected by the function of renalase (Weinman et al. 2011). Chronic infusion of angiotensin II into rats was demonstrated to increase the expression of NaPi-IIa in PTC brush border membranes by posttranscriptional mechanisms (Xu et al. 2004). This increase of NaPi-IIa may be associated with an angiotensin II-mediated decrease in the expression of megalin that plays a role in endocytosis and the degradation of NaPi-IIa (Bachmann et al. 2004). Insulin may also be involved in regulating sodium-dependent phosphate transporters (Kunkler et al. 1991; Li et al. 1996). Glucocorticoids inhibit phosphate uptake in primary cultured rabbit PTCs via both nongenomic and genomic mechanisms (Park, Taub, and Han 2001). In addition, estrogen causes the PTH-independent downregulation of NaPi-IIa in PTCs (Faroqui et al. 2008).

### 5. Conclusion

There are a variety of hormonal actions and interactions involved in the regulation of important PTC functions such as receptor-mediated endocytosis and the reabsorption of sodium and phosphate. The impaired counterbalance of these hormones is likely to be associated with the development and/or progression of CKD. Elucidation of the mechanisms of such hormonal actions and interactions would therefore be of great benefit in the future development of novel therapeutic strategies for CKD.

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## **Part 9**

# **Estrogens and Endothelium**



# Estradiol Regulation of Prostanoids Production in Endothelium

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## 1. Introduction

The endothelium is a continuous cellular monolayer lining the interior of the blood vessels and heart. Classically considered to exert its actions only as a mechanical barrier, also plays important roles in vascular physiology. Endothelium participates in numerous metabolic and regulatory functions such as the control of primary hemostasis, blood coagulation and fibrinolysis, interaction with lipoprotein metabolism, platelet and leukocyte interactions with the vessel wall, presentation of histocompatibility antigens, regulation of vascular tone and growth, and of blood pressure. The endothelium exerts these actions through the release of such vasoactive compounds as prostacyclin, thromboxane A<sub>2</sub>, nitric oxide (NO), bradykinin, endothelin, angiotensin, and free radicals that control the functions of vascular smooth muscle cells and of circulating blood cells (Ross, 1999; Vapaatalo & Mervaala, 2001).

The integrity and functionality of the arterial endothelium play a crucial role in the physiology of circulation (Filipe *et al.*, 2008) and, as a consequence, in preventing the development of cardiovascular diseases, whose genesis is currently considered a consequence of the anatomical and functional disruption of the endothelium (Spyridopoulos *et al.*, 1997). When the ability of the endothelial cells to release relaxing is reduced, and in particular if the propensity to produce contractile factors is enhanced, endothelial dysfunction appears as a first step in the sequence of events that leads to atherosclerosis and coronary disease. Thus, no single mechanism is responsible for all endothelium dependent responses and their modulation by pathophysiological states leads to endothelial dysfunction characterized by an imbalance in endothelial regulators (Vanhoutte *et al.*, 2009).

Clinical and experimental data support the consideration of endothelium as a target for estradiol and other sexual hormones. A number of studies have demonstrated a favourable profile for estrogens in both experimental animal, as well as in *in vitro* models (Turgeon *et al.*, 2006). However, the protective effect detected in a considerable number of observational clinical studies (Barrett-Connor & Grady, 1998) has not been confirmed by randomized placebo-controlled trials (Hulley *et al.*, 1998; Grady *et al.*, 2002), which described clinical complications, such as thrombosis in veins and coronary arteries, developed in postmenopausal women during the administration of exogenous hormones (Cano *et al.*, 2007).

A number of studies have demonstrated that estrogens exert significant benefits on the cardiovascular system, and at least a part of these benefits are attributed to the direct effect of estradiol on vascular endothelial cells (Mendelsohn & Karas, 1999; Rubanyi *et al.*, 2002; Sader & Celermajer, 2002). Estradiol is able to stimulate endothelial NO production through several mechanisms, including increased expression of NO synthases (mainly endothelial NO synthase), increased L-arginine availability, non-genomic activation of second messengers (Simoncini, 2009), translocation to intracellular sites, modulation of NO degrading sites (Tostes *et al.*, 2003), and modulation of endogenous antagonist levels (Monsalve *et al.*, 2007).

Additionally, estradiol is able to exert antioxidant actions on endothelium (Shwaery *et al.*, 1998; Hermenegildo *et al.*, 2002), to modulate the renin-angiotensin system (Farhat *et al.*, 1996; Alvarez *et al.*, 2002), and to decrease endothelin-1 production (Mikkola *et al.*, 1995; Akishita *et al.*, 1998). Furthermore, estradiol regulates endothelial cell expression of adhesion molecules (Caulin-Glaser *et al.*, 1996; Abu-Taha *et al.*, 2009).

Estradiol has also been implicated on the regulation of prostanoids production in endothelial cells. Two main vascular prostanoids, prostacyclin and thromboxane A<sub>2</sub>, play an essential role in the maintenance of vascular homeostasis. Prostacyclin is a vasodilator and an inhibitor of platelet aggregation; in contrast, thromboxane A<sub>2</sub> is a vasoconstrictor and a promoter of platelet aggregation. As a consequence of their opposing roles, an imbalance in prostacyclin or thromboxane production has been implicated in the physiopathology of many thrombotic and cardiovascular disorders. In this chapter, we will discuss clinical and experimental data that document the endothelial effects of estradiol on prostanoid production and regulation, and their vascular consequences.

## 2. Prostanoids in endothelium

Prostanoids are unstable lipid mediators that, after intracellular biosynthesis, are released outside the endothelial cell. It is believed that prostanoids work mostly locally in an autocrine or paracrine way. Prostanoids are ubiquitous compounds that coordinate a wide variety of physiological and pathological processes via membrane receptors on the surface of target cells (FitzGerald, 2002). Prostanoids include prostaglandins (such as prostaglandins D<sub>2</sub>, E<sub>2</sub>, F<sub>2</sub>, F<sub>2α</sub>, PGI<sub>2</sub> -or prostacyclin-) and thromboxanes (such as TXA<sub>2</sub>) (Smith *et al.*, 2000; Linton & Fazio, 2002).

Prostanoids production is mainly regulated by the availability of free arachidonic acid and the activity of cyclooxygenases (COX). Release of arachidonate from cell membrane phospholipids is mediated through phospholipases, mainly phospholipase A<sub>2</sub>. Once arachidonate is released, it is sequentially converted to prostaglandins G<sub>2</sub> and H<sub>2</sub> by two COX isoenzymes (COX-1 and COX-2). The unstable prostaglandin H<sub>2</sub> is then transformed into biologically active prostanoids by specific synthases in different cells. Prostaglandins interact with G-protein-coupled receptors, classified in function of their preferential affinity towards the five primary prostaglandins (Tsuboi *et al.*, 2002) (Figure 1).

COX isoenzymes (also known as prostaglandin endoperoxide synthases or prostaglandin H synthases) are the rate-limiting step in the formation of prostanoids from arachidonic acid and represent the main control mechanism for prostaglandin production (Smith *et al.*, 2000). Both enzymes have similar biochemical activity in converting arachidonic acid to PGH<sub>2</sub> in tissue or in recombinant enzyme systems (Vane *et al.*, 1998).

COX-1 and COX-2 are encoded by two different genes derived from human chromosomes 9 and 1, respectively. In spite of there is a 60 - 65% homology between the isoforms within

species, the activity and the pattern of gene expression of these enzymes are differentially regulated (Davidge, 2001).

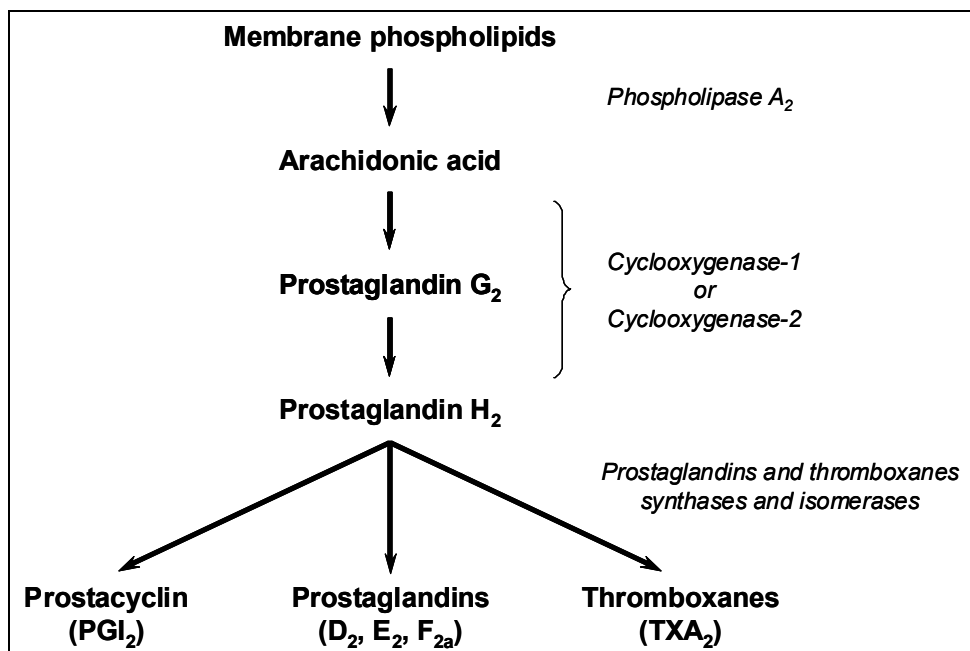


Fig. 1. Biosynthesis of prostaglandins and thromboxanes.

COX-1 has been considered to be the constitutively expressed protein, while COX-2 is induced at sites of inflammation. Following that hypothesis, COX-1 would generate prostaglandins for physiological, housekeeping functions like gastrointestinal mucosal integrity and regulation of renal blood flow, while COX-2 would form the prostaglandins responsible for inflammatory symptoms (Smith *et al.*, 2000; Parente & Perretti, 2003).

But this separation of functions is so not clear. For instance, COX-2 is constitutively expressed in some regions of the central nervous system, and in renal and uterus tissues, suggesting it may play a role under physiologic conditions (Kim *et al.*, 1999; FitzGerald, 2002; Cheng & Harris, 2004). In fact, both COX-1 and COX-2 are involved not only in physiological, but also in pathological processes. The importance of this topic has impelled many outstanding reviews (Vane *et al.*, 1998; Parente & Perretti, 2003; Cipollone *et al.*, 2008; Vanhoutte, 2009).

Regarding the vascular system, both isoenzymes are expressed in endothelium and smooth muscle cells. However, endothelial cells contain up to 20 times more COX than smooth muscle cells (DeWitt *et al.*, 1983). As mentioned before for other organs, COX-1 has usually been considered in endothelium as the constitutive isoform, while COX-2 is induced by a number of cardiovascular risk factors, such as cytokines, cholesterol, lipoproteins, and hypoxia. Actually, both COX isoenzymes share characteristics of constitutive and inducible enzymes in endothelium. Shear stress induces COX-1 gene expression in human umbilical vein endothelial cells (HUVEC) (Doroudi *et al.*, 2000), while clinical studies with a selective

inhibitor of COX-2 (celecoxib) have shown that this enzyme exerts control of most systemic prostacyclin production in healthy humans (McAdam *et al.*, 1999).

Although prostaglandins E<sub>2</sub> and F<sub>2 $\alpha$</sub>  can contribute to vascular phenotype, two main prostanoids play an essential role in vascular physiology: thromboxane A<sub>2</sub> and prostacyclin. On the one hand, in the cardiovascular system, thromboxane A<sub>2</sub> is predominantly derived from platelet COX-1, but can also be produced by other cell types including the endothelial cells. The stimulation of thromboxane receptors elicits not only platelet aggregation and smooth muscle contraction, but also the expression of adhesion molecules and the adhesion and infiltration of monocytes/macrophages (Nakahata, 2008). As platelets are anucleate, there can be no induction of COX-2 enzyme synthesis (Patrignani *et al.*, 1999). Thromboxane A<sub>2</sub> can also be synthesized from endothelial cells (Sellers & Stallone, 2008). The third possibility is a transcellular formation of thromboxane A<sub>2</sub> by platelets from prostaglandin H<sub>2</sub> released by endothelial cells (Camacho & Vila, 2000). Thromboxane A<sub>2</sub> promotes irreversible platelet aggregation, vasoconstriction, and smooth muscle proliferation and, consequently, plays an important role as a mediator of not only haemostasis, but also of acute vascular occlusive events and atherogenesis (FitzGerald, 2002; Weir *et al.*, 2003).

On the other hand, in most blood vessels prostacyclin is the main prostanoid produced by endothelial cells, and the endothelium is its major source (Moncada & Vane, 1978). Both COX-1 and COX-2 isoenzymes contribute to the production of endothelial prostacyclin. By stimulating its preferential receptor, prostacyclin is a potent inhibitor of platelet adhesion to the endothelial cell surface and of platelet aggregation, and generally acts as an endothelium-derived vasodilator and an inhibitor of vascular smooth muscle migration and proliferation (Moncada & Vane, 1978; Fetalvero *et al.*, 2007).

Under physiological conditions, endothelial prostacyclin is a counterregulatory influence to platelet-derived thromboxane, and eicosanoids produced by the COX pathways generally induce vasorelaxation. Nevertheless, in some pathologic circumstances, such as oxidative stress and dyslipidemia, there may be an imbalance where COX-dependent vasoconstrictors and platelet aggregation become more predominant. Reactive oxygen species, such as superoxide anion and hydrogen peroxide are derived from multiple sources within inflammatory leukocytes and vascular tissues including NADPH oxidase, uncoupled endothelial and inducible endothelial NO synthase, xanthine oxidase, cyclooxygenases, lipoxygenases, cytochrome P450 monooxygenases and excess substrate utilization by mitochondria. Additionally, NO reacts with superoxide anion to form the extremely potent oxidant, peroxynitrite. Low concentrations of peroxynitrite selectively nitrate and inactivate prostacyclin synthase, which not only eliminates the vasodilatory, growth-inhibiting, antithrombotic, and antiadhesive effects of prostacyclin, but also increases release of the potent vasoconstrictor, prothrombotic, growth- and adhesion-promoting agents, prostaglandin H<sub>2</sub> and thromboxane A<sub>2</sub> being in general deleterious to vascular function (Zou *et al.*, 2004; Schildknecht & Ullrich, 2009; Feletou *et al.*, 2010).

### 3. Estrogens actions on endothelium

Estradiol is the most potent estrogen in humans, and exerts its actions mainly through binding and activation of estrogen receptors (ER). Two major subtypes of ER (ER $\alpha$  and ER $\beta$ ) have been identified. These receptors vary not only in their tissue distributions, but also in their agonist/antagonist profile of several compounds (Cano & Hermenegildo, 2000). Both



types of ER belong to the class of intracellular receptors classically defined as nuclear ligand-activated transcription factors. Activation of these receptors by the corresponding hormones affects gene expression by acting on specific sequences in the target genes, known as estrogen-response elements, and by modulating transcriptional events.

The contribution of both receptors to the regulation of vascular effects is still under study. There is controversy about whether estrogenic effects are mediated through ER $\alpha$ , ER $\beta$  or both. In the cardiovascular system, ER $\alpha$  and ER $\beta$  have been identified by different techniques in the endothelium, smooth muscle cells and adventitia and on adrenergic nerve endings of arteries from various territories and several species, including humans (Karas *et al.*, 1994; Kim-Schulze *et al.*, 1996; Venkov *et al.*, 1996; Register & Adams, 1998). Although it has been reported cultured endothelial cells do not express ER $\alpha$  (Toth *et al.*, 2008), other investigators have demonstrated the presence of both ER $\alpha$  and ER $\beta$  mRNA in endothelium (Wagner *et al.*, 2001) and data from our group demonstrate the protein expression of both ER $\alpha$  and ER $\beta$  in HUVEC (Sobrinho *et al.*, 2009; Sobrinho *et al.*, 2010).

Although the relative significance of both ER subtypes in the vascular actions of estrogens is still under study, data from mice lacking either ER $\alpha$  (Pare *et al.*, 2002; Arnal *et al.*, 2010) or ER $\beta$  (Zhu *et al.*, 2002), and also from a non-functional mutation of ER $\alpha$  in a male patient (Sudhir *et al.*, 1997), reveal an impaired vascular function. Therefore, ER $\alpha$  is probably critical for the production of estrogen vascular protective actions (Favre *et al.*, 2010), but ER $\beta$  may have relevant functions at the vascular level too (Simoncini *et al.*, 2004). The changes in vascular cell gene and protein expression mediated by those ER $\alpha$  and/or ER $\beta$  produce the classic, and better known, longer-term effects of estrogen (Cano & Hermenegildo, 2000).

ER act in the nucleus as ligand-activated transcription factors that regulate gene expression in response to hormone binding. ER can also regulate vascular cell gene expression in the absence of estrogen following activation by growth factor signalling pathways, a process referred to as ligand-independent transcriptional activation. In addition, ER are able to signal rapidly (from seconds to minutes), in an apparently non-nuclear manner, resulting in downstream activation of specific kinases and their effectors molecules (Mendelsohn & Karas, 2010).

The rapid effects of estrogens have been also explained by ER-independent mechanisms of action. For instance, the G protein-coupled receptor GPR30 has been proposed to be a third form of ER, although it is controversial whether GPR30 is a biologically-relevant ER or a collaborator in non-nuclear functions of the classical ER in certain contexts (Levin, 2009). Moreover, even though GPR30 is expressed in certain endothelial cells, there is currently a lack of clear evidence of a role for the receptor in estrogen action in endothelium (Wu *et al.*, 2011).

Rapid actions of estradiol include several endothelial pathways, being the activity of endothelial nitric oxide synthase the most studied (Chow *et al.*, 2010). Other signalling pathways acutely activated by estradiol in endothelium are several kinases, such as phosphatidylinositol 3-kinase (PI3K) (Razandi *et al.*, 2000), protein kinase C (PKC) (Akarasreenont *et al.*, 2000) and Rho-associated kinase (ROCK) (Simoncini *et al.*, 2006; Oviedo *et al.*, 2011). Rapid actions are responsible for the acute and potent vasorelaxation induced by estradiol both at physiological (Teoh *et al.*, 2000; Tep-areenan *et al.*, 2003) or supraphysiological (Naderali *et al.*, 2001; Salom *et al.*, 2002) concentrations, and it has been demonstrated in isolated vessels from animals such as rats (Tep-areenan *et al.*, 2003), pigs (Teoh *et al.*, 2000), rabbits (Salom *et al.*, 2002), guinea pigs (Naderali *et al.*, 2001), and even from human beings (Chester *et al.*, 1995).

In addition, steroid hormone genomic and non-genomic effects may occur simultaneously and may act at different levels, revealing the complexity of estrogen regulation of vascular function (Tostes *et al.*, 2003).

#### 4. Estrogens actions on prostanoids vascular regulation

The actions of estrogens on prostanoids vascular production will be presented in two sections: first, those experimental data that are rapid in onset and short in duration (within minutes; “non-genomic” effects); second, those experimental data that are delayed in onset and prolonged in duration (require from hours to days to occur; “genomic” effects).

##### 4.1 Rapid effects of estrogens on prostanoids vascular production

Physiological concentrations of estradiol cause a rapid stimulation of prostacyclin synthesis (within 15 min) in two types of cultured endothelial cells, ovine fetal pulmonary artery endothelial cells (PAEC) (Sherman *et al.*, 2002) and HUVEC (Alvarez *et al.*, 2002). In both cases, the use of the ER-antagonist ICI182780 reveals that the increased prostacyclin production is mediated through ER activation. In ovine PAEC, it seems mediated through ER $\beta$ , since prostacyclin stimulation with estradiol was fully blocked by the ER $\beta$ - antagonist RR-tetrahydrochrysen. Moreover, in ovine PAEC, the increased prostacyclin production is not related to changes in COX-1 or COX-2 protein abundance, thus reinforcing the implication of non-genomic mechanisms (Table 1) (Sherman *et al.*, 2002).

COX-pathway activity is involved in the acute relaxation afforded by estradiol. In rat mesenteric vessels and aorta, COX inhibition with indomethacin enhances the vasodilator potency of estradiol, suggesting an endogenous release of vasoconstrictor prostanoids (Tepareenan *et al.*, 2003). But estradiol also indirectly affects vasoactivity to contracting and relaxing substances. In epicardial porcine coronary arteries activation of ER $\alpha$ , but not of ER $\beta$ , reduces thromboxane A<sub>2</sub> vasoconstriction, indicating another indirect and vasodilator function of ER $\alpha$  (Traupe *et al.*, 2007). Other data suggest that the estrogen-dependent constrictor prostanoid mechanism in the vascular wall may play an important role in the regulation of vascular tone in the female, in both normal and pathophysiological states. In that way, endogenous estrogen acts as an important regulator of constrictor prostanoid function in female rat aorta, involving the potentiation of COX-2 and thromboxane synthase expression in vascular wall (Li *et al.*, 2008).

Data from studies performed on postmenopausal women also supports the importance of COX-pathway in acute estrogen-induced vasodilation. Estradiol induces a rapid increase of cholinergic cutaneous vasodilation, which is eliminated in women treated for 6 weeks with celecoxib, suggesting that the COX-2 pathway plays a specific role in the rapid estradiol-induced vasodilation in postmenopausal women (Calkin *et al.*, 2002).

Some studies have been performed at times that do not clarify whether the observed effects are genomic or non-genomic actions. Estrogen stimulates rat aortic endothelial cell release of prostacyclin after 1 hour of incubation without affecting COX-1 levels (Myers *et al.*, 1996). That stimulation could be mediated by NO, since endogenous NO increased production of prostacyclin and thromboxane A<sub>2</sub> in bovine coronary microvascular endothelial cells within 1 hour, without affecting COX-1 or COX-2 expressions (Davidge *et al.*, 1995). At molecular level, exposure of HUVEC for 40 min to estradiol significantly increases the expression of roughly 250 genes, measured by DNA microarray analysis. Among them, the COX-2 gene was the strongest up-regulated by estradiol. In fact, estradiol induced COX-2 gene

expression and new COX-2 protein synthesis by 40 and 60 min, respectively, and quickly stimulated the secretion of prostacyclin and prostaglandin E<sub>2</sub> in an ER-dependent manner (Pedram *et al.*, 2002). A similar, significant induction of COX-2 mRNA levels is obtained in human placental villous endothelial cells exposed to estradiol up to 1 hour, without an increased protein production (Su *et al.*, 2009).

Cell type	Time	E <sub>2</sub> (nM)	Prostacyclin production	COX-1	COX-2	Reference
<b><u>Rapid effects</u></b>						
Ovine fetal PAEC	15 min	10	↑ 52 %	↔	↔	(Sherman <i>et al.</i> , 2002)
HUVEC	15 min	1	↑ 57 %	NA	NA	(Alvarez <i>et al.</i> , 2002)
HUVEC	30 min	1	↑ 134 %	NA	NA	(Alvarez <i>et al.</i> , 2002)
<b><u>Delayed effects</u></b>						
HUVEC	24 h	10	↑ 26 %	NA	NA	(Mikkola <i>et al.</i> , 1995)
HUVEC	24 h	0.1	↑ 16 %	NA	NA	(Mikkola <i>et al.</i> , 1996)
HUVEC	24 h	1	↑ 78 %	↔	↑	(Akarasereenont <i>et al.</i> , 2000)
HUVEC	24 h	1	↑ 30 %	↑	↔	(Sobrinho <i>et al.</i> , 2009)
HUVEC	24 h	10	↑ 33 %	↑	↔	(Sobrinho <i>et al.</i> , 2010)
Human coronary EC	24 h	100	↑ 45 %	NA	NA	(Mueck <i>et al.</i> , 2002)
Bovine coronary EC	24 h	1	↓ 83 %	↔	↔	(Stewart <i>et al.</i> , 1999)
Ovine fetal PAEC	48 h	10	↑ 64 %	↑	NA	(Jun <i>et al.</i> , 1998)

Table 1. Summary of estradiol effects on cultured cell production of prostacyclin. E<sub>2</sub>: concentration of estradiol in nM. Prostacyclin production is expressed as increased (↑) or decreased (↓) percentage of control values. COX-1 and COX-2 expression: increased (↑), decreased (↓), unchanged (↔) or not available (NA). PAEC: pulmonary artery endothelial cells. HUVEC: human umbilical vein endothelial cells. EC: endothelial cells.

#### 4.2 Delayed (genomic) effects of estrogens on prostanoids vascular production

Estrogen also exerts vascular delayed effects on the metabolism of prostaglandins and the activity of COX, as has been demonstrated in studies performed both in cultured cells, as well as in isolated vascular preparations.

A time-course analysis performed in HUVEC, demonstrates that estradiol effects on prostacyclin production were evident only after 8 or 24 hours (10 and 1 nM estradiol, respectively), suggesting an ER-mediated genomic effect (Sobrinho *et al.*, 2010). Moreover, physiological concentrations of estradiol stimulate the production of prostaglandins, mainly

prostacyclin, in a variety of cultured endothelial cells, such as ovine fetal PAEC (Jun *et al.*, 1998), HUVEC (Mikkola *et al.*, 1995; Mikkola *et al.*, 1996; Akarasereenont *et al.*, 2000; Sobrino *et al.*, 2010), and human coronary endothelial cell (Mueck *et al.*, 2002). In the vast majority of experiments, cells were exposed to estradiol during 24-48 hours, and the increase of prostacyclin above control values ranged from 16 % to 78 % (Table 1).

In addition, it has been shown that estradiol stimulates production of prostaglandins in a variety of preparations of arteries, including ovine uterine arteries (Vagnoni & Magness, 1998; Janowiak *et al.*, 1998; Habermehl *et al.*, 2000), mesenteric arteries from ovariectomized rats (Davidge & Zhang, 1998; Armstrong *et al.*, 2002), rat cerebral blood vessels (Ospina *et al.*, 2002; Ospina *et al.*, 2003), and aorta from ovariectomized monkeys (O'Sullivan *et al.*, 2001). The increase of prostacyclin production is of a similar magnitude to that obtained with cultured endothelial cells.

Concerning endothelial production of thromboxane A<sub>2</sub> under estradiol, the literature indirectly suggests that estrogen would have a beneficial effect by decreasing the production of thrombogenic compounds. In men, there is a decrease in the formation of thromboxane A<sub>2</sub> after the use of high dosage intra-muscular estrogen therapy (Henriksson *et al.*, 1996). Moreover, the ratio of *in vivo* prostacyclin to thromboxane A<sub>2</sub> formation increases 2-fold during estrogen replacement therapy (Mueck *et al.*, 2001). Nevertheless, it has also been documented that estrogen increases platelet activation with the liberation of thromboxane A<sub>2</sub> in women treated with hormone replacement therapy (Oliveira *et al.*, 2005) and that estrogen enhances the constrictor prostanoid function in female rat aorta (Li *et al.*, 2008). However, in cultured endothelial cells, thromboxane A<sub>2</sub> production remains unchanged when measured after exposure to estradiol (Sobrino *et al.*, 2010).

The regulatory role of estradiol on the biosynthesis pathway of prostanoids in endothelium (Figure 1) has been scarcely studied, with the only exception of COX-1 and COX-2 expressions. Concerning the first enzyme, soluble phospholipase A<sub>2</sub> is highly expressed in HUVEC and umbilical smooth muscle cells (Ost *et al.*, 1998), but it does not appear to be regulated by estradiol. Phospholipase A<sub>2</sub> expression remains unchanged after exposure to estradiol in HUVEC (Sobrino *et al.*, 2010) or in human myometrial cells prepared from second trimester pregnant women (Korita *et al.*, 2004).

The roles of COX-1 and/or COX-2 isoenzymes in endothelial cells exposed to estradiol have been studied in some cases, and both enzymes have been implicated in estradiol effects. In ovine fetal PAEC after exposure to estradiol, both COX-1 mRNA and protein expressions were up-regulated (Jun *et al.*, 1998), whereas in HUVEC estradiol only induces COX-2 expression protein (Akarasereenont *et al.*, 2000). Genome-wide analysis performed in HUVEC exposed to estradiol for 24 hours demonstrated that COX-1 was among the 5 % of proteins which expression was changed more than 1.5 fold-times compared to controls, data that was verified by western blot analysis (Sobrino *et al.*, 2009).

Prostacyclin increased levels after vessel stimulation with estradiol have been mainly associated with enhanced expression of COX-1. In this way, COX-1 protein content is increased in rat (Ospina *et al.*, 2002) and mice (Geary *et al.*, 2001) cerebral blood vessels, and in ovine uterine arteries in response to treatment with estrogen (Rupnow *et al.*, 2002). Indeed, COX-1 (but not COX-2) expression is increased in the endothelium of the ovine uterine artery during the follicular phase of the ovarian cycle and during pregnancy, where estrogen levels are highest (Janowiak *et al.*, 1998; Habermehl *et al.*, 2000). In spite of the classic consideration of COX-2 as an inducible enzyme, it seems that COX-2 does not contribute to the estradiol-increased prostacyclin production. Estrogen has even been found

to decrease COX-2 expression in mesenteric arteries from ovariectomized aged rats (Armstrong *et al.*, 2002).

However, similar experiments have occasionally produced contradictory results. Exposure of culture bovine coronary microvascular endothelial cells to physiological concentrations of estradiol for 24 hours reduced both prostacyclin and thromboxane A<sub>2</sub> production (Stewart *et al.*, 1999). In the case of whole tissue experiments, arteries respond in a different way to estradiol. This is the case of artery segments from different vascular beds (thoracic aorta, pulmonary artery, ear artery and coronary arteries) from adult male rabbits in which estradiol may cause both vasorelaxation and vasoconstriction (Saetrum *et al.*, 2002). Moreover, in thoracic aorta the contractile effects of estrogen are mediated through the release of vasoconstrictor prostaglandins (Saetrum *et al.*, 2002).

These discrepancies could indicate a difference in the effects of estrogen depending on its previous serum concentration, the reproductive condition of the animal and/or the vascular bed being studied (Armstrong *et al.*, 2002; Saetrum *et al.*, 2002; Tostes *et al.*, 2003).

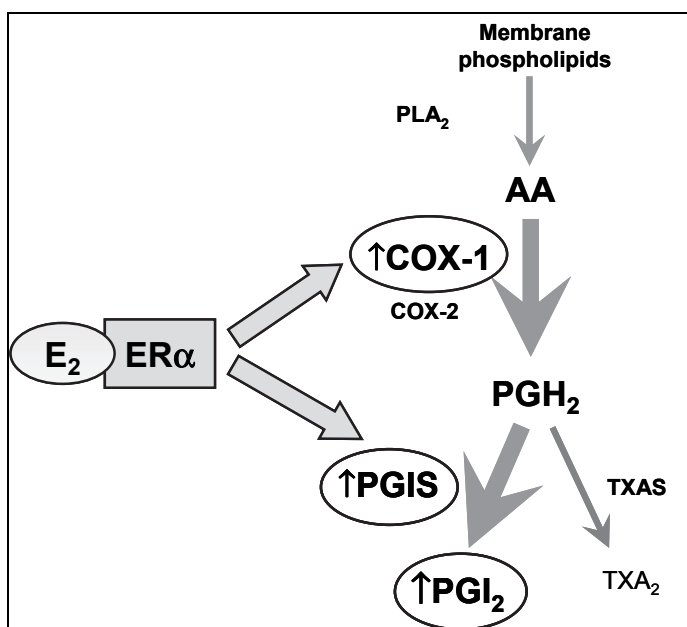


Fig. 2. Estradiol-induced modifications on prostanoid pathway through ER $\alpha$ . Estradiol (E<sub>2</sub>), acting on ER $\alpha$ , increased cyclooxygenase-1 (COX-1) and prostacyclin synthase (PGIS) expression, resulting in an increased prostacyclin (PGI<sub>2</sub>) production. Phospholipase A<sub>2</sub>, cyclooxygenase-2 (COX-2) and thromboxane synthase (TXAS) expressions, along with thromboxane A<sub>2</sub> production (TXA<sub>2</sub>), remained unchanged after exposure to estradiol.

The regulatory actions of estradiol on the prostanoids biosynthesis pathway are mediated through ER activation. The use of ICI182780, a non-selective ER-antagonists, completely blocks estradiol-stimulated prostacyclin production in ovine fetal PAEC (Jun *et al.*, 1998), HUVEC (Sobrino *et al.*, 2009), and tamoxifen blocked it in HUVEC (Mikkola *et al.*, 1995; Mikkola *et al.*, 1996).

Moreover, data support a specific role for ER $\alpha$  on estradiol-stimulated prostacyclin production. Recently, selective ER $\alpha$  agonist 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1h-pyrazole (PPT), selective ER $\beta$  agonist (diarylpropionitril; DPN), and selective ER $\alpha$  antagonist, methyl-piperidino-pyrazole (MPP), have become available (Krom *et al.*, 2007). With their use, it has been demonstrated that estradiol increased prostacyclin production, and COX-1 and prostacyclin synthase protein and gene expressions through ER $\alpha$ , whereas COX-2, phospholipases and thromboxane synthase expression remained unaltered (Sobrinho *et al.*, 2010). The regulatory role of estradiol through ER $\alpha$  is supported by other studies. Estradiol increases levels of COX-1 in cerebral blood vessels from wild-type mice but was ineffective in ER $\alpha$  knockout mice (Geary *et al.*, 2001) and also increases COX-1 expression through ER $\alpha$  in ovine endothelial cells transfected with the human COX-1 promoter (Gibson *et al.*, 2005).

## 5. Conclusion

Remarkable progress has been made in elucidating the effects of estrogens on the vascular system, and the cellular and molecular mechanisms by which those effects are achieved. The analysis of published data supports estradiol increases prostacyclin vascular production in accordance with experimental and clinical research. This effect seems to be mediated mainly through ER $\alpha$ -dependent pathways which involve an increase in COX-1 and, in some cases, COX-2 activation and/or expression, and an augment in prostacyclin synthase expression, without affecting thromboxane A2 production. As a consequence, estradiol may reduce vascular tone and improve endothelium-dependent vasodilation (Figure 2).

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## **Part 10**

### **Factors Affecting Farrowing**



# Factors Affecting Physiology and Endocrinology of Farrowing

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## 1. Introduction

The highly prolific sow, defined as one giving birth to 16 or more liveborn piglets, presents a challenge to reproductive management of a sow herd (Boulot et al., 2008). From the point of view of reproductive management of the sow, feeding plays a key role. Therefore, recent findings related to feeding and how it affects farrowing, lactation and oestrus management are given the highest priority in the present paper. We report on recent findings related to feeding sows a high fiber diet during the period preceding parturition and its beneficial effect on gut function and duration of farrowing (Oliviero et al., 2009). In addition to feeding, arrangement of the farrowing pen (crate vs. pen; barren vs. enriched) appears as a critical factor determining the course of parturition. Our latest findings suggest that prohibiting the sow to exhibit nest building behaviour (Algers and Uvnäs - Moberg, 2007) prolongs parturition by an average of 90 minutes (Oliviero et al., 2008; 2010).

In addition, feeding sows with a high fiber diet during pregnancy, apart from being a beneficial feeding strategy from the welfare point of view, appears to increase the ad libitum feed intake during lactation. This effect seems to be carried over to the average daily gain of piglets, especially during the neonatal period (Quesnel et al., 2009, Peltoniemi et al., 2009). Amount of feed eaten by sows during lactation, on the other hand, appears as a key in enhancing gonadotrophin secretion and follicle development throughout lactation, however these effects of feeding become more evident towards the end of lactation (Kauffold et al., 2008). Follicles grow after weaning, as triggered by gonadotrophins FSH and LH, occurs the faster the better the stimulation by gonadotrophins has been prior to weaning (Prunier and Quesnel, 2000; Kauffold et al., 2008).

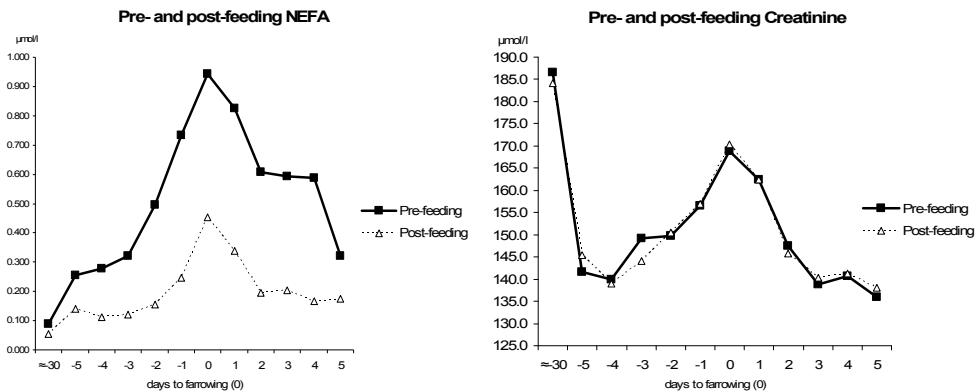
## 2. Successful farrowing

Successful farrowing can be defined as (1) sows given a chance to exhibit species specific nest building behaviour, (2) duration of farrowing not exceeding 5 hours, (3) all piglets in the litter born alive and (4) the first sucklings resulting in all newborn piglets receiving colostrum.

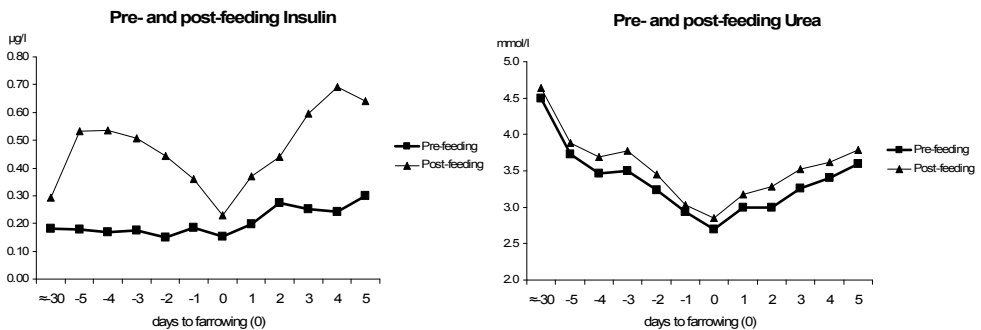
As fetuses grow fast at the end of pregnancy, there is a need to increase the energy intake by the sow. A common feeding strategy has been to put sows on lactation diet during the period before farrowing, in anticipation of the great energy requirement during lactation.

While being understandable as a strategy from the energy intake point of view during lactation, this strategy by large ignores the intestinal function and dietary metabolism during and shortly after farrowing.

In one of our studies (Oliviero et al., 2009) we investigated the energy balance related parameters of the sow around farrowing. The parameters indicating catabolism (NEFA and creatinine) increased significantly a few days before farrowing, showing a positive peak almost on the day of parturition (Figure 1a), concerning NEFA this is in agreement with the results of Le Cozler et al. (1999). On the other hand, the metabolic markers of dietary energy (urea, insulin and glucose), decreased significantly with the approach of farrowing, yielding a negative peak on the day of parturition (Figure 1b). This may be the result of an internal regulation of the metabolism as the sow approaches farrowing. At this time, metabolism of



(a)



(b)

Fig. 1. Energy balance related parameters in sows (n = 41). At the top (a) there are the parameters indicating catabolism (NEFA and Creatinine) which have high levels at farrowing; at the bottom (b) there are the metabolic markers of dietary energy which on the contrary have low levels at farrowing.



the external source of energy decreases and the main source of energy becomes the internal body reserves (fat and muscles) as the peaks of NEFA and creatinine demonstrate. In this sensitive phase, diet and intestinal activity seems of secondary importance. The parturition process clearly takes priority over digestive and intestinal activity, but the demand for energy remains high or even higher, and it's supplied mobilising internal reserves. Therefore, around farrowing it appears important, to promote good intestinal activity with an adequate amount of fibre rather than just increase dietary energy intake.

We observed that after having been on a traditional lactation diet with 3,8 % of crude fiber prior to farrowing, sows showed an increased constipation incidence of up to 22 % for several days after farrowing (Figure 2; Oliviero et al., 2009). Gut function slows down during parturition anyway and overgrowth of bacteria in a gut filled with an energy-rich diet may lead into activation of the GALT (gut associated lymphoid tissue) system (Oliviero et al, 2009; Reiner et al., 2009). The activation of the GALT system would stimulate PGE<sub>2</sub> release, which may further suppress intestinal function until a leak of endotoxins through the gut wall occurs, bringing about a systemic response and clinical symptoms found in post partum dysgalactia syndrome, PDS (Reiner et al., 2009). Therefore, avoiding constipation by any means should be of interest to managers of any well functioning piglet producing unit.

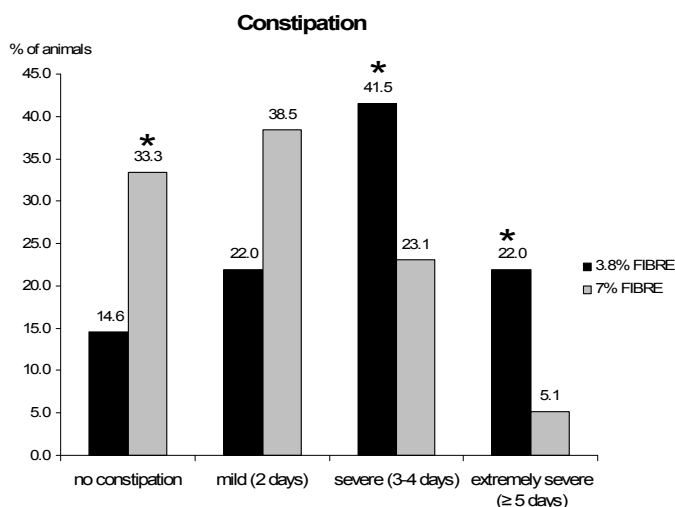


Fig. 2. Incidence of different grades of constipation in the 3.8% FIBRE group (n = 40) and in the 7% FIBRE group (n = 41), during the observational period (from 5 days before to 5 days after farrowing). Consecutive days of constipation are recorded, and for constipation is meant absence produced of feces. Asterisks represent significant differences between the two groups with  $P < 0.05$ .

While feeding sows laxatives like Glauber salt, cooked linseed mixture and commercial laxatives are among traditional measures to avoid constipation, our experiences support adding more fiber into sow diets before farrowing (Oliviero et al., 2009). A 7-11 % crude fiber content prior to farrowing appears as a reasonable measure to prevent constipation and an important part of management of successful farrowing (Oliviero et al., 2009; Quesnel

et al., 2009; Peltoniemi et al., 2009). The first beneficial function of a high fiber diet is the improved intestinal activity (Oliviero et al., 2009). However, other beneficial effects relating to the use of high fiber diets have also been reported. These include improved intestinal immunity due to an increased mucin production as well as improved energy utilization of the feed consumed.

Water intake of the sow before farrowing is an important parameter to monitor, since it is elementary to milk production. On average, sows drink 10-30 liters of water around farrowing (Oliviero et al., 2009). However, variation between individual sows was considerably large and sows on the high fiber diet drank significantly more than did sows on the traditional diet (Figure 3; Oliviero et al., 2009). This was attributed to either stimulating effect of fiber on water intake as such or the increased volume related to the higher fiber diet possibly explaining the difference compared to the traditional pre-farrowing diet (Oliviero et al., 2009). Whatever the cause, these findings encourage use of diets containing more fiber prior to farrowing. The usual flow recommendation of drinking water from nipples is 3-4 liters / minute and this is one of the easiest parameters to be measured on a health check call in a farrowing unit.

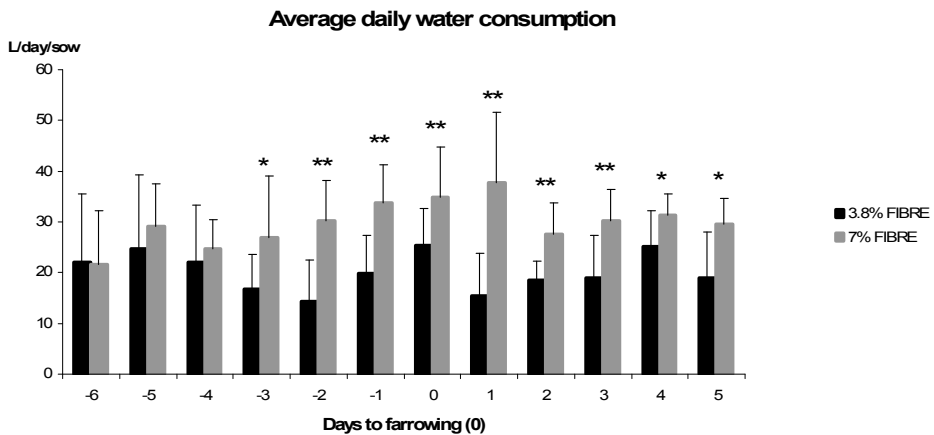


Fig. 3. Individual average daily water consumption and respective SD bars in the FIBRE group (n = 12) and in the LACT group (n = 12). The asterisk indicate the degree of significance (\* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ).

Data from our group show that sows not given a chance to express nest building behaviour have higher circulating cortisol concentrations before farrowing and lower oxytocin concentrations during the expulsion phase of farrowing (Figure 4; Oliviero et al., 2008a). From the practical viewpoint, however, maybe the most important observation was the one related to the duration of farrowing. If placed in a crate and not given a chance to build up a nest, it took an average of 1,5 hours longer from our experimental sows to deliver the litter than sows placed in a free pen with nesting material (Figure 4; Oliviero et al., 2008a; 2009). Furthermore, the average interval between piglets was considerably longer in crated sows with no nest building material (25 minutes CRATE vs. 16 minutes in PEN; Oliviero et al., 2008a).

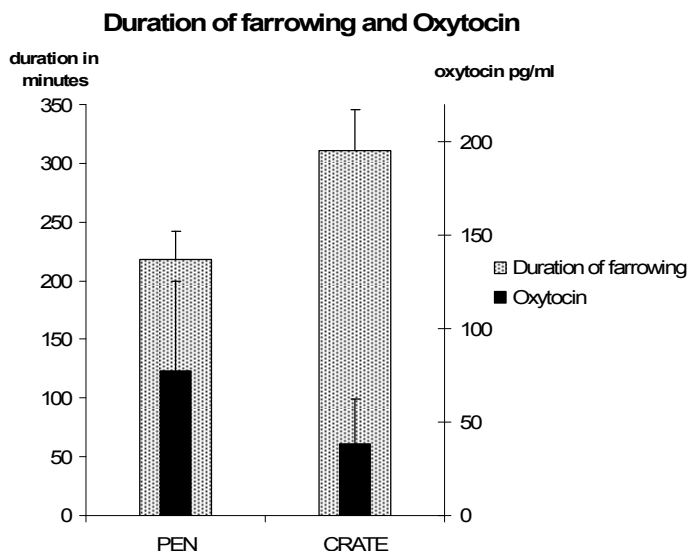


Fig. 4. Average duration of farrowing and average Oxytocin post-expulsion pulses in the PEN ( $n = 9$ ) and CRATE ( $n = 9$ ) groups of sows (mean  $\pm$  SD). Data from Oliviero et al., 2008.

Another explanation could be hormonal impairment due to the higher level of fat circulating in overfed sows. A higher level of fat can affect lipid-soluble steroids, and especially the progesterone:oestrogen ratio, which is known to affect oxytocin receptor activation (McCracken et al., 1999; Russell et al., 2003). Abnormalities due to oxytocin receptor activation may weaken the expulsive phase of parturition. A decline in progesterone and a concomitant increase in the oestrogen profile should occur 36 to 24 hours prior to parturition (Cowart, 2007). We observed a delayed decline in progesterone beyond day 1 after parturition in a number of our trial sows (Figure 5; Oliviero et al., 2008a), a delay that may be linked to obesity and the prolongation of parturition. If so, progesterone bound to fat may be too stable to promptly react to CL regression.

We have recorded duration of farrowing in several studies by now (Oliviero et al., 2008; 2010). From these studies it is apparent that a farrowing lasting longer than 5 hours is deemed unsuccessful and easily leads to complications for both the dam and the newborn. Therefore, we suggest that with the modern sow lines, a 5 hours threshold may be applied when making a difference between successful and unsuccessful farrowing. Several factors, as constipation, body condition of the sow, parity, breed and number of stillborn piglets affect duration of farrowing (Oliviero et al., 2010). Constipation, as discussed above, may be alleviated by increasing fiber content of the diet. Backfat thickness of higher than 17 mm, however, also appeared as a risk factor for prolonged farrowing in the genetically lean Finnish sow population (Oliviero et al., 2010).

Number of piglets stillborn prolonged duration of farrowing (Oliviero et al., 2010). It is known from previous studies that fetuses are usually alive shortly before farrowing. The great majority (>80%) of piglets that are deemed to born still, die during the course of parturition. It is therefore reasonable to assume that dystocia, whether due to the dam or the

fetus, is the major cause for piglets born still. According to Jackson (1972), in the sow, inertia uteri (37%) used to be considered as the most common cause for dystocia followed by the breech presentation (14,5%), obstruction of the birth canal (13%), simultaneous presentation (10%), downward torsion of the uterus (9%), downward deviation of the head (4%) and fetal oversize (3,5%). However, these robust classifications do not account for the more precise causes for intrapartum deaths of piglets, such as strangulation or early rupture of the umbilical cord or considerations relating to uterine contractions.

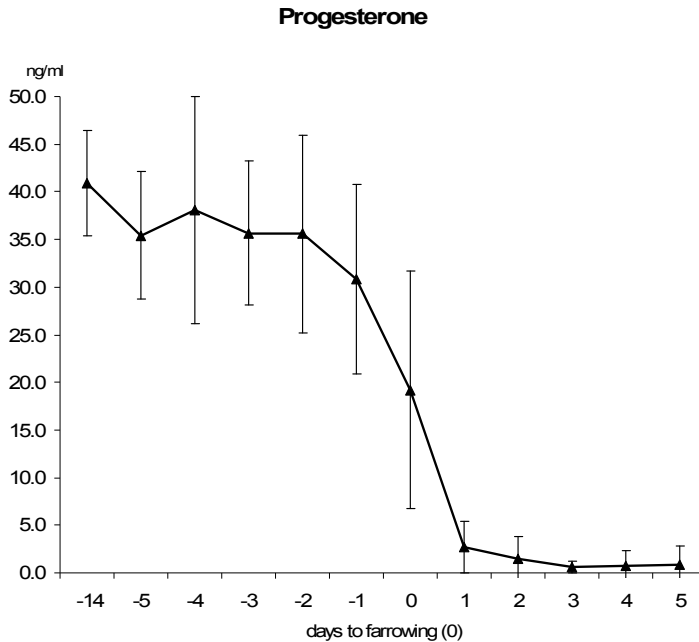


Fig. 5. Some sows showed a delayed decline in circulating progesterone before farrowing. The sows in this study ( $n = 38$ ) showed still high levels of progesterone on the day of farrowing.

We found that also the level of back fat affected the duration of farrowing even though the sows used in this study were not particularly fat (Figure 6). Sows with a back fat average higher than 17 mm had an average duration of farrowing of  $385 \pm 197$  min ( $n = 48$ ), whereas those with a back fat average of less than 17 mm had an average duration of farrowing of  $230 \pm 103$  min ( $n = 124$ ;  $p < 0.001$ ). One explanation could be a progesterone impairment due to the higher level of fat in those sows as described in the paragraph above. Another hypothesis could be that fat sows have more adipose layers around the birth canal (Coward, 2007), thereby reducing the diameter of the birth canal, which can create a physical obstacle to birth during the expulsive phase with delayed farrowing.

It is very well known, that piglets need their passive immunity acquired through colostrum, otherwise they will not be able to cope with the environmental infective pressure. As every piglet is in need of colostrum and one that is missing out is clearly the one at risk of loosing

life already in the early days. Colostrum transmission through the gut wall of the piglet can only occur during the first day of life and the amount of colostrum is not increasing according to the number of piglets born. Therefore, in a large litter, supervision is required, not only during the process of parturition, but also attending that even the last piglets born will receive adequate amount of colostrum. We found that, after stillborn, the most common primary cause of perinatal mortality is starvation (Table 1). Piglets which are unable to adequately feed themselves within the first days of life have less chance to survive and more chances to get crushed by the sow because of their state of lethargy which reduce their ability to move and react fast.

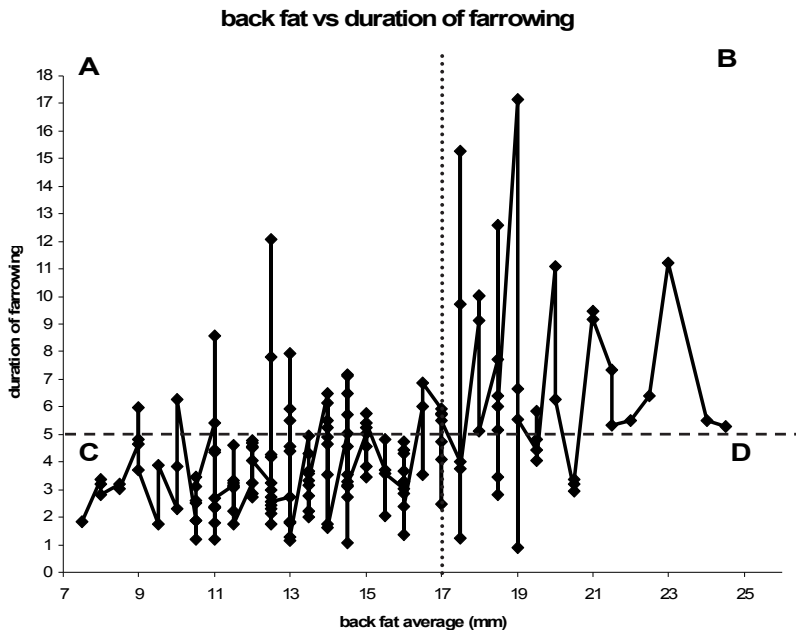


Fig. 6. Individual sows plotted by back fat average (mm) and the duration of farrowing (hours). The horizontal dashed line discriminates prolonged farrowing (> 5 h; A, B); the vertical dotted line discriminates the fatter sows (B, D). Most of the fatter sows had duration of farrowing longer than 5 hours (area B), while most of the thinner sows had farrowings duration shorter than 5 hours (area C).

### 3. Lactation performance of the sow and piglets

To care for a large litter, adequate feed intake of the sow is of great significance. A drop in feed intake around farrowing can be considered as physiological. Stepwise rise in feed intake by 0,5 kg of feed / day until day 10 or so will be enough to reach the target daily intake of about 8 kg, equal to about 100 MJ ME for a sow nursing 12 piglets (Peltoniemi and Kemp., 2009). A too steep rise in feed intake after farrowing will jeopardize the success of the whole lactation, since there will be a decline in intake, followed by fluctuation of it for most of the duration of lactation.

Cause of mortality	<i>n</i>	%
Stillborn	50	34.5
Starvation	30	20.7
Trauma	27	18.6
Infections	17	11.7
Other*	21	14.5
Total	145	100

\*Anaemia, malformed, autolysis.

Table 1. Primary causes of piglet mortality (from birth to day 5 of life) determined after post-mortem examination (41 litters).

A balanced nutritional program designed to avoid excessive weight loss during lactation is of great importance for successful lactation. It has been shown that feeding the sow with higher amounts of fiber during pregnancy, intake of feed during lactation can be increased with a corresponding increase in performance of piglets (Quesnel et al., 2009). It has also been shown, that increasing fiber intake through the reproductive life of the sow appears as a feasible approach, contributing to improved welfare of the sow as well as good reproductive performance of the sow and improved growth performance of piglets (Peltoniemi et al., 2009).

Piglet mortality and mean birth weight appear to be closely associated with litter size. When litter size increased from 10 to 15, number of stillborn piglets went up from 0,3 to 1,0 and the proportion of piglets weighing less than 1 kg went up from 3 to 15 %, respectively (Boulot et al., 2008). In large litters, supervision of farrowing is therefore necessary. The first issue there is an accurate prediction of farrowing. We have developed techniques, whereby prediction of farrowing becomes possible and feasible. Movement sensors monitoring impending farrowing can be used to predict the expulsion phase of farrowing 24 hours prior to the first birth of the first piglet (Oliviero et al., 2008b). Other modern technology, such as use of thermocameras may be applied to detect hypothermic newborn piglets that require immediate attention by the caretakers.

We argue that the present development regarding piglets born in the pig industry is not on a sustainable basis. Instead of the number of piglets born alive, more research effort should be aimed at increasing the birth weight of piglets born and decreasing the still birth rate. Moreover, more attention should be paid to the quality of newborn piglets, the quality of piglets weaned and the quality of fattening pigs. The quality of piglets and fattening pigs in the pig production may be achieved by long term studies, starting during the fetal period, focusing on early development of the piglet and finally exploring the fattening phase of the pig.

#### 4. Gonadotrophins and follicle development

Intake of feed by sows during lactation appears as a key in enhancing gonadotrophin secretion and follicle development throughout lactation, however these effects of feeding become more evident towards the end of lactation (Kauffold et al., 2008). Follicle growth after weaning, as triggered by gonadotrophins FSH and LH, then occurs the faster the better the stimulation by gonadotrophins has been prior to weaning (Kauffold et al., 2008).

Decreasing lactation length is a commonly taken strategy in Europe to hasten the reproductive cycle of the high producing sow and avoid excessive weight loss. However, the sustainability and ethical grounds for these strategies need thorough attention in the near future.

After weaning follicles grow approximately 1 mm / day. They reach the ovulatory size at about 7-10 mm within a week after weaning. The most recent findings in our group suggest that follicles ovulating at the size of 7 or 8 mm result in improved fertilization rates and larger litters than follicles ovulating at < 7 mm or > 8 mm (Vehmas et al., unpublished). Ultrasound technology (US) can nowadays be effectively applied in mating units to monitor insemination accuracy of insemination operators. In problem farms, inseminations occurring too early or too late in relation to ovulation can be picked up by US and relevant recommendations to change the AI strategy can be given. The optimal insemination time is estimated to be 0-16 hours prior to ovulation, which occurs at approximately when two thirds of the standing oestrus has passed.

Follicle development can also be monitored with regard to possible not-expected physiological or pathological findings at the ovary. After weaning, detection of corpora lutea may indicate lactational oestrus, while cystic follicles, for instance, are among classical examples of application of ultrasound technology in sow herds.

## 5. Conclusions

Successful farrowing includes components of maternal behavior, duration of farrowing, piglet mortality and colostrum intake. Feeding is considered as the major factor in the reproductive management of the hyperprolific sow. New insights such as adding more fiber to sow diets during pregnancy and especially in the period prior to farrowing prevent constipation, increase water intake of the sow around parturition and increase milk intake and performance of piglets. Use of modern technology in supervision of farrowing may improve losses related to large litters. Use of ultrasound technology after weaning to monitor follicular growth may further improve litter size. In breeding programs, new components of maternal characteristics such as maternal behavior, ease of parturition, colostrum production, and piglet quality parameters may be taken to further improve success rate of reproductive management.

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