BIOMARKERS OF KIDNEY DISEASE

EDITED BY

CHARLES L. EDELSTEIN

Biomarkers in
KIDNEY DISEASE

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To My Wife Freda and Children
Craig, Jeremy and Joy

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FOREWORD

The prevention or attenuation of the severity of disease necessitates early detection. In recent years this has been a focus relative to kidney disease. **Biomarkers in Kidney Disease** edited by Charles Edelstein summarizes advances in early detection and assessment of severity in an array of important kidney diseases. State of the art techniques, including metabolomics and proteomics, are discussed in areas of acute kidney injury, kidney transplantation, renal cancer, diabetic nephropathy and other glomerular diseases, as well as in preeclampsia.

Biomarkers in Kidney Disease is a seminal book, because nephrology has lagged behind other subspecialties in performing interventional trials which can improve the lives of their patients. A major reason is because the tools to detect kidney disease at the early stage have heretofore not been available. As in all diseases, prevention and attenuation of severity necessitates early intervention. The emergence of sensitive biomarkers of early kidney disease now has the potential to allow early detection and intervention. With this book there is now a source which provides up to date and important information by distinguished authors about biomarkers available to detect early kidney disease.

Louis Pasteur stated, "Science knows no country, because knowledge belongs to humanity and is the torch which illuminates the world." Charles Edelstein and colleagues have illuminated the emerging field of early detection in **Biomarkers of Kidney Disease**.

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PREFACE

The importance of developing and defining biomarkers of kidney diseases that can be used for early diagnosis, assessment of severity, and long term prognosis has been emphasized by the American Society of Nephrology and the National Institutes of Diabetes, Digestive and Kidney Diseases (NIDDK). Over the last ten years, there has been exponential growth in research on biomarkers of kidney diseases. Preclinical studies have been taken to the bedside and it is now possible to use biomarkers to diagnose certain kidney diseases at an earlier stage than has been possible with conventional tests. This prospect of early diagnosis and treatment of kidney diseases has made biomarker research one of the most exciting areas of kidney research.

Biomarkers of Kidney Disease offers a thorough examination of the latest findings in the field for both the practicing physician as well as the biomedical researcher. Coverage includes biomarkers of acute kidney injury, chronic kidney disease, kidney transplant rejection, delayed kidney allograft function, renal cell cancer, glomerular disease, diabetic nephrop athy, and preeclampsia. This book is the most comprehensive reference yet published on the topic of biomarkers of kidney diseases.

Dr. Prasad Devarajan, a pioneer in taking biomarkers from the bench to the bedside, makes the case that biomarkers are the essential tools for the implementation of personalized medicine. He reviews how novel biomarkers were discovered and validated, and he systematically lays out the general characteristics of an ideal biomarker.

For the physician interpreting or planning biomarker studies, the chapter by Drs. Chirag R. Parikh and Heather Thiessen Philbrook discusses both traditional and emerging statistical methods for evaluating the classi fication performance of biomarkers.

Proteomic and metabolomic profiling of body fluids and tissues can provide a landscape of simultaneous changes in thousands of proteins and metabolites during the body's responses to diseases and drug treatments. Dr. Uwe Christians, who has state of the art laboratories at the University of Colorado for biomarker discovery using mass spectrometry, proteomics, and metabolomics, has written two comprehensive chapters on the use of metabolomics and proteomics in kidney diseases.

BUN and serum creatinine are not very sensitive and specific markers of kidney function in AKI as they are influenced by many renal and non renal factors independent of kidney function. Drs. Charles Edelstein and Sarah Faubel review the numerous biomarkers of AKI that are released by the "injured" kidney, many of which increase before serum creatinine. Dr. Alkesh Jani, a transplant nephrologist, has written the chapter on biomarkers for the early diagnosis of delayed kidney graft function or rejection.

Cystatin C was found in the urine in 1961. Twenty years later at the University of Lund in Sweden, Drs. Anders Grubb and Helga Lofberg isolated and sequenced this "mysterious" protein as part of the cystatin family of proteins. We are fortunate to have Dr. Grubb write the chapter on cystatin C as a biomarker in kidney diseases.

Novel biological therapies for renal cell cancer are being used and there is a need to identify markers that predict response to a particular agent. The current field of renal cancer biomarkers is comprehensively reviewed by Dr. Roz Banks and colleagues.

Diabetic nephropathy and glomerulonephritis are the commonest causes of ESRD in the USA. Dr. Jon Klein and colleagues review the role that proteomics has played in answering the "how, when and why" of diabetic nephropathy. Biomarkers for the early diagnosis, early prediction of flares and prediction of outcome in patients with glomerulonephritis are reviewed by Dr. John M. Arthur and colleagues.

Preeclampsia is the most common renal complication of pregnancy and is a leading cause of maternal and perinatal morbidity and mortality. Dr. Ananth Karumanchi and colleagues review their exciting work that circulating angiogenic factors like soluble Fms like tyrosine kinase 1 (sFlt 1), in addition to heralding the onset of preeclampsia, may also cause the disease.

The advances in our knowledge of biomarkers has never been greater. It is my privilege to edit a book written by distinguished authors who have contributed to the exciting advances in our knowledge of biomarkers.

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CHAPTER 1

Characteristics of an Ideal Biomarker of Kidney Diseases

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Contents

1. THE DISCOVERY OF BIOMARKERS

The quest for biomarkers is as old as medicine itself. From the earliest days of diagnostic medicine in ancient Egypt, to the misguided science of phrenology (the belief that skull measurements could predict personality traits), to the powerful discoveries of modern science, we have been search ing for measurable biological cues that will allow us insight into the physiological workings of the human organism. In its simplest definition, a biomarker is anything that can be measured to extract information about a biological state or process. The NIH Biomarkers Definitions Working Group has defined a biological marker (biomarker) as "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.[1]"

Biomarkers appear in every form. Body temperature, in the form of a fever, can signal infection. Blood pressure and cholesterol levels can predict cardiovascular risk. Tracking biomarkers such as height and weight can give clues to normal human growth and development. Such general biomarkers have been used for decades or even centuries and have remained powerful tools for tracking general biological activity. However, the era of person alized medicine is well upon us. Ushered in by the remarkable genomic and proteomic advances in our understanding of health and disease, personalized

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medicine promises a more precise determination of disease predisposition, diagnosis and prognosis, earlier preventive and therapeutic interventions, a more efficient drug development process, and a safer and more fiscally responsive approach to medicine. Biomarkers are the essential tools for the implementation of personalized medicine. The quest for the advancement of personalized medicine pushes us further and further into the realm of molecular medicine to discover biomarkers with increasing sensitivity and specificity. For most of our history, biomarker discovery has relied on intimate knowledge of the pathophysiology of the diseases being studied. Biological substances that we knew were related to a disease state were investigated to see if they could serve as diagnostic markers, provide a target for therapy or lend further insight into the etiology of the disease. While this can be tedious, and relies heavily on prior knowledge of the disease mechanism, this hypothesis driven method of research almost always provides useful scientific results, whether positive or negative.

The biomarker development process has typically been divided into five phases, as shown in Table 1.1. The preclinical discovery phase requires high quality, well characterized tissue or body fluid samples from care fully chosen animal or human models of the disease under investigation. In recent years, the ready availability of powerful tools to scan both the genome and the proteome of an organism have revolutionized and greatly accel erated biomarker discovery. Microarrays that can measure the entire complement of messenger RNA in a given sample type have yielded

Table 1.1 Phases of biomarker discovery, translation and validation

Phase	Terminology	Action steps
Phase 1	Preclinical discovery	• Discover biomarkers in tissues or body fluids • Confirm and prioritize promising candidates
Phase 2	Assay development	• Develop and optimize clinically useful assay • Test on existing samples of established disease
Phase 3	Retrospective study	• Test biomarker in completed clinical trial • Test if biomarker detects the disease early • Evaluate sensitivity, specificity, ROC
Phase 4	Prospective screening	• Use biomarker to screen population • Identify extent and characteristics of disease • Identify false referral rate
Phase 5	Disease control	• Determine impact of screening on reducing disease burden

Adapted from Devarajan 2007.[15]

a number of promising biomarkers of kidney disease, such as neutrophil gelatinase associated lipocalin (NGAL), an early predictor of acute kidney injury (AKI) and a powerful risk marker of chronic kidney disease (CKD) progression which we will discuss later, and have also led to the discovery of novel disease mechanisms in many fields.[2–4] This approach can be combined with other techniques, such as laser capture microdissection, to target specific areas of diseased tissue to give mechanistic clues not possible just a decade ago. Even with this level of specificity, these techniques can yield a daunting array of data that must be sifted through for relevance. One example of this in human kidney disease was a study performed by Bennett et al[5] in which the authors looked at gene expression profiles of laser captured glomeruli from kidney biopsies in patients with focal segmental glomerulosclerosis. The investigators were able to examine gene expression exclusively in the histo logical center of this disease, and still found well over 100 genes differentially expressed compared to glomeruli from control tissues. A shortcoming of such transcriptomic profiling approaches is that you cannot directly measure biological fluids. Another problem with this approach is that ultimately messenger RNA does not always reflect protein levels or activity and must be further confirmed at the protein level prior to larger validation studies.

Proteomic approaches move a step beyond genomic studies and screen the actual proteins and peptides present in a sample. This approach allows one to go beyond simple translation of mRNA into protein, and allows a look into protein regulation, post translational modifications such as glycosylation and methylation, and even disease specific fragmentation. There are a number of proteomic approaches including gel electrophoresis and modern mass spectometry techniques such as matrix assisted laser desorption ionization time of flight (MALDI TOF) mass spectrometry and surface enhanced laser desorption ionization time of flight (SELDI TOF) mass spectrometry. These techniques are capable of identifying and quantifying proteins and peptides in exceedingly large numbers.[6] The urinary proteome itself is quite large, with laboratories having identified over 1500 proteins to date.[7,8] The blood proteome is even larger, with over 3000 non redundant proteins identified in the plasma alone.[9–11] Adding the proteome of the cellular component of blood will yield thousands more.[12,13] To this end we have entered what has been termed an "open loop"[14] or unbiased approach to biomarker discovery. This is in stark contrast to the hypothesis driven approach of our past. With such a vast pool of potential biomarkers from readily available, non invasive sources

one must take care to plan and design the proper experimental approach to ensure parsimony.

2. CHARACTERISTICS OF AN IDEAL BIOMARKER

Prior to beginning the search for biomarkers of renal disease, one has to ask — What are the ideal characteristics of a renal biomarker? To be certain what constitutes an ideal biomarker is highly dependent upon the disease you are investigating. However, certain universal characteristics are important for any biomarker: (1) they should be non invasive, easily measured, inexpensive, and produce rapid results; (2) they should be from readily available sources, such as blood or urine; (3) they should have a high sensitivity, allowing early detection, and no overlap in values between diseased patients and healthy controls; (4) they should have a high specificity, being greatly upregulated (or downregulated) specifically in the diseased samples and unaffected by comorbid conditions; (5) biomarker levels should vary rapidly in response to treatment; (6) biomarker levels should aid in risk stratification and possess prognostic value in terms of real outcomes; and (7) biomarkers should be biologically plausible and provide insight into the underlying disease mechanism.[1,15]

Of course, very few biomarkers will meet all of the characteristics of an ideal marker, but let us discuss these characteristics in a little more detail. First, a biomarker should be non invasive. For example, many chronic kidney diseases present with a range of proteinuria. Currently the preferred method for differentiating nephrotic syndrome producing chronic kidney diseases such as focal segmental glomerulosclerosis, membranous nephropathy or minimal change disease is an invasive biopsy. In addition to the health risks, these procedures cause undue anxiety, especially in pediatric populations. While typically a safe procedure, there are associated risks, especially for those patients with contraindications to percutaneous renal biopsies who must elect for an "open", or operative renal biopsy. A recent study found major (cardiac arrest, stroke, sepsis) and minor (wound infection, pneumonia, arrhythmia, postoperative retroperitoneal bleed, deep vein thrombosis) complication rates of 6.1% and 27% in a group of 115 open biopsy patients from 1991 to 2006.[16] While these are relatively rare occurrences, they illustrate the need for less invasive diagnostic procedures.

Regarding the source of biomarkers, the most readily available ones are urine and blood. These are substances obtained in the normal care of a patient, easily collected at the bedside, and associated with little to no

health risks to the patient. Each source has desirable and negative charac teristics. Urine is an excellent source of biomarkers produced in the kidney[17] and thus may give better mechanistic insight into specific renal pathologies. Urine is less complex than serum and thus is easier to screen for potential biomarkers. Collection of urine is easy enough, and it can be readily employed in home testing kits. The handling of urine, however, greatly influences the stability of its proteins and measurements should be made immediately after collection or the urine should be promptly frozen at 80°C to avoid degradation. Finally, urinary biomarker studies typically adjust for urine creatinine to account for differences in urine concentration due to hydration status and medications such as diuretics. However, the utility of urine creatinine in biomarker correction has been questioned due to its variable excretion throughout the day and its dependence on normal renal function. Serum or plasma can also be a good source of biomarkers and is even available in anuric patients. Serum is less prone to bacterial contamination than urine and is considered more stable. Serum biomarkers, however, are more likely to represent a systemic response to disease, rather than an organ response. There are exceptions, such as the troponins in cardiac disease. The real problem with serum as a source of biomarkers lies in the discovery phase. Serum has a wide range of protein concentrations across several orders of magnitude, with a small number of proteins (such as albumin) accounting for a large percentage of the volume. This can be akin to trying to spot a single strand of cotton in a large tapestry. The more abundant proteins simply overwhelm the signal of those in less abundance. While there exist assays to remove these high abundance proteins from serum, many potential biomarkers have for example been shown to bind to albumin.[18] Thus, when you deplete the albumin, the rest of the tapestry unravels with it and you may lose proteins relevant to your disease.

The sensitivity and specificity of a biomarker go hand in hand. The receiver operating characteristic (ROC) curve is a binary classification test, based on the sensitivity and specificity of a biomarker at certain cutoff points. ROC curves are often used to determine the clinical diagnostic value of a marker.[15,19] The area under the ROC curve (AUC) is a common statistic derived from ROC curves. An AUC of 1.0 represents a perfect biomarker, while an AUC of 0.5 is a result that is no better than expected by chance. An AUC of 0.75 or greater is generally considered a good biomarker, while an AUC of 0.90 is considered an excellent biomarker.[15] However, even a sensitive biomarker with what experimentally would be considered an excellent specificity of 90%, would still yield a false positive rate of 10%,

which may be unacceptably high for clinical use as a stand alone marker.[14] As a result, the best approach clinically may be to find multiple biomarkers that can be combined as part of a panel to achieve even higher specificity.

Lack of specificity and slow response to alterations in disease severity or treatment are primary reasons why serum creatinine is an unsatisfactory biomarker for renal disease, especially in cases of acute kidney injury (AKI). Firstly, serum creatinine levels change with factors unrelated to renal disease, such as age, gender, diet, muscle mass, muscle metabolism, race, strenuous exercise and hydration status. Creatinine levels are also influenced by certain drugs.[20,21] Furthermore, in AKI, serum creatinine is not a real time indi cator of kidney function, because the patients are not in steady state; so rises in serum creatinine occur long after the renal injury is sustained. In fact, serum creatinine concentrations may not change until approximately 50% of kidney function has been lost. This makes serum creatinine a poor diag nostic marker for AKI, since treatments need to be administered soon after injury to be effective. Animal studies have shown that treatments that can prevent or alleviate AKI need to begin well before the serum creatinine level begins to rise.[17,22,23] Because so many variables affect creatinine levels, it also lacks precision in assessing disease progression or risk stratification. Finally, it is well known that significant renal disease such as fibrosis can exist with little or no change in creatinine because of renal reserve or enhanced tubular secretion of creatinine.[24,25] Nephrology remains in the 1950s in its use of serum creatinine. Despite having few of the outlined characteristics of an ideal biomarker, serum creatinine remains in widespread use as an indicator of renal function and is the sole FDA approved diagnostic marker of AKI. The problems with creatinine have been evident for over thirty years,[25] yet until recently little progress had been made in the search for replacement markers that will aid in earlier, more accurate and specific diagnosis of renal disease.

3. BIOMARKERS IN ACUTE KIDNEY INJURY

AKI is a serious clinical problem and is increasing in incidence, lacks satisfactory therapeutic options and presents an enormous financial burden to society. Conservative estimates have placed the annual health care expenditures attributable to hospital acquired AKI at greater than 10 billion dollars in the United States alone.[26,27] AKI is a major side effect of other medical procedures and can result from insults ranging from ischemia reperfusion injury (IRI) following cardiopulmonary bypass surgery or renal

transplant to damage from nephrotoxic agents such as contrast used in CT or cysplatin used in chemotherapy. Although many new insights into the mechanisms of AKI have been advanced in recent years and novel interventions in animal models have shown promise, translational efforts in humans have been disappointing. There are many plausible reasons for this lack of success, among them is a paucity of early diagnostic markers of AKI leading to delayed initiation of therapy and incomplete pathophysiological understanding of the disease process.[15]

Another major hindrance to the successful implementation of new therapies is the lack of a consensus definition of AKI (previously known as acute renal failure, or ARF). In fact, the Acute Dialysis Quality Initiative (ADQI) workgroup found that over 30 definitions for ARF were used in the literature. The definitions varied from a 25% increase over baseline serum creatinine to the need for dialysis.[28] The term AKI is of relatively recent origin and was proposed to better account for the diverse spectrum of molecular, biochemical and structural processes that characterize the AKI syndrome.[29] In order to better classify AKI, the RIFLE classification system (Table 1.2) was developed (Risk—Injury—Failure—Loss—End stage renal

Table 1.2 RIFLE criteria (acute dialysis quality initiative)

Stage	Serum creatinine criteria	GFR criteria	Urine output criteria
R = Risk for renal dysfunction	Increase in serum creatinine $\geq$ 1.5× baseline	Decrease in GFR $\geq$ 25%	$<$ 0.5 mL/kg/h for 6 h
I = Injury to the kidney	Increase in serum creatinine $\geq$ 2.0× baseline	Decrease in GFR $\geq$ 50%	$<$ 0.5 mL/kg/h for 12 h
F = Failure of kidney function	Increase in serum creatinine $\geq$ 3.0× baseline OR serum creatinine $\geq$ 4.0 mg/dL in the setting of an acute rise $\geq$ 0.5 mg/dL	Decrease in GFR $\geq$ 75%	$<$ 0.3 mL/kg/h for 24 h or anuria for 12 h
L = Loss of kidney function	Persistent failure $>$ 4 weeks		
E = End stage renal disease (ESRD)	Persistent failure $>$ 3 months		

Adapted from Bellomo et al.[30]

disease).[30] The first three classes represent degrees of injury and the last two are outcome measures. This system has shown to correlate well with mortality rates.[31] In order to further refine the definition of AKI, the Acute Kidney Injury Network (AKIN) was created, which proposed a modified version of the RIFLE classification, known as the AKIN criteria. The AKIN criteria define AKI as an abrupt (within 48 h) reduction in kidney function as measured by an absolute increase in serum creatinine ≥ 0.3 mg/dL, a percentage increase in serum creatinine $\geq 50\%$, or documented oliguria (< 0.5 mL/kg/h) for more than 6 h.[32] Minor modifications of the RIFLE criteria (Table 1.3) include broadening the "risk" category of RIFLE to include an increase in serum creatinine of at least 0.3 mg/dL in order to increase the sensitivity of RIFLE for detecting AKI at an earlier time point. In addition, the AKIN criteria sets a window on first documentation of any criteria to 48 h and categorizes patients in the "failure" category of RIFLE if they are treated with renal replacement therapy, regardless of either changes in creatinine or urine output. Finally, AKIN replaces the three levels of severity R, I and F with stages 1, 2 and 3.[33]

Many conventional markers of kidney function have suffered from a lack of specificity and poor standardized assays. The insensitivity of these measurements, such as casts and fractional secretion of sodium, make them poor candidates for the early detection of AKI. As mentioned, creatinine is an unreliable marker of acute changes in kidney function due to its slow response time and the fact that many variables can alter creatinine levels.[34] The failure of two clinical trials on promising new interventions in AKI,

Table 1.3 Comparison of the RIFLE criteria with the AKIN staging criteria

RIFLE stage	RIFLE criteria	AKIN stage	AKIN criteria
R	$\geq$ 150% increase in serum creatinine, or $>$ 25% GFR decrease	I	$\geq$ 150% or $\geq$ 0.3 mg/dL increase in serum creatinine
I	$\geq$ 200% increase in serum creatinine, or $>$ 50% GFR decrease	II	$>$ 200% increase in serum creatinine
F	$\geq$ 300% increase in serum creatinine, or serum creatinine of $\geq$ 4.0 mg/dL in setting of increase $\geq$ 0.5 mg/dL, or $>$ 75% GFR decrease	III	$>$ 300% increase in serum creatinine, or serum creatinine of $\geq$ 4.0 mg/dL in setting of increase $\geq$ 0.5 mg/dL

Note: The urine output criteria are the same for both RIFLE and AKIN.

human insulin like growth factor 1 and anaritide, is at least partly attrib utable to the lack of early biomarkers for AKI.[35,36] Despite these and other potential advances in clinical care and groundbreaking research into the mechanisms of AKI, it remains a devastating clinical condition and studies suggest its incidence may be increasing.[37–39] AKI has been reported to complicate up to 7% of all hospital admissions[40,41] and as high as 25% of intensive care unit (ICU) admissions.[42] The prognosis of AKI has remained quite poor over the past 50 years with a mortality rate of 40–80% in the intensive care setting.[23,43] Identification of novel AKI biomarkers has been designated a top priority by the American Society of Nephrology and the concept of developing a new collection of tools for earlier diagnosis of disease states is a prominent feature in the National Institutes of Health road map or biomedical research.[29,44]

Besides establishing the early diagnosis, biomarkers are needed for several other purposes in AKI (summarized in Table 1.4). Thus, biomarkers are needed for: (1) pinpointing the location of primary injury (proximal tubule, distal tubule, interstitium or vasculature); (2) determining the duration of kidney failure (AKI, chronic kidney disease or "acute on chronic" kidney disease); (3) discerning AKI subtypes (prerenal, intrinsic renal or postrenal); (4) identifying AKI etiologies (ischemia, toxins, sepsis or a combination); (5) differentiating AKI from other forms of acute kidney disease (urinary tract infection, glomerulonephritis or interstitial nephritis); (6) risk stratification and prognostication (duration and severity of AKI, need for renal replacement therapy, length of hospital stay and mortality); (7) defining the course of AKI; and (8) monitoring the response to AKI interventions.[15] Biomarkers are also desperately needed for use as surrogate endpoints in clinical trials evaluating potential therapeutics for AKI.

Table 1.4 Areas of need for biomarkers in AKI

Biomarkers are needed to determine:
1. Location of injury
2. Duration of AKI
3. AKI subtypes
4. AKI etiologies
5. Differentiate from other forms of acute kidney disease
6. Risk stratification and prognostication
7. Defining course of AKI
8. Monitoring interventions

Surrogate markers are precise measurements that can accurately correlate with a clinical endpoint.[1] Surrogate endpoints can expedite clinical trials evaluating the safety and efficacy of new drug applications. If the intervention has the desired effect on the surrogate endpoint, then further evaluations are warranted to directly address the effect of the intervention on the appropriate clinical endpoint. This linking of the surrogate endpoint to the clinical endpoint is referred to as validation and is an essential step in the biomarker discovery process.

With respect to the desirable characteristics of AKI biomarkers, the most important remain those that are clinically applicable and can lead to early diagnosis and treatment of AKI. Other important properties of clinically relevant biomarkers of AKI are similar in concept to the properties of ideal biomarkers in general. Specific characteristics should include: (1) measurements from non invasive sources, such as blood or urine; (2) easy to perform either at bedside or in a standard clinical laboratory; (3) measurements should be reliable and have a rapid turnaround time; (4) they should be sensitive for early detection and have a wide dynamic range of values with cutoffs to allow for risk stratification; (5) they should be highly specific, and ideally allow for AKI subtype classification; and (6) they should be inexpensive to allow for broad global use.

Several promising candidates for clinical use as biomarkers in AKI are under intense contemporary study and some have already been approved for clinical use in much of the world. Many of these biomarkers will be discussed in more detail in other chapters, but we will offer a brief description of the major candidates. Perhaps the most widely applicable marker found to date for the early diagnosis of AKI is neutrophil gelatinase associated lipocalin (NGAL). NGAL was discovered by cDNA microarray analysis to be induced very early following ischemic or nephrotoxic injury and the protein is easily detectable in urine and plasma soon after AKI,[4,45–49] but is also elevated in patients with systemic or urinary tract infections, as well as those with pre existing renal conditions. The current status of NGAL as an AKI biomarker is further discussed below. Another emerging candidate for inclusion in an AKI panel of biomarkers is the pro inflammatory cytokine interleukin 18 (IL 18), which is induced in the proximal tubule after AKI. IL 18 is specific to ischemic AKI and other forms of acute tubular necrosis (ATN), and it doesn't appear to be affected by chronic kidney disease or urinary tract infections (UTIs).[50–55] Serum cystatin C is another candidate for inclusion in an AKI panel. Cystatin C is produced in the blood and is filtered by the glomerulus, then completely reabsorbed by the proximal

tubules and not normally excreted in the urine.[56] Serum cystatin C is primarily a sensitive marker of glomerular filtration rate reduction and not kidney injury, but has been shown to predict AKI earlier than serum creatinine in the intensive care setting.[22] Kidney injury molecule 1 (KIM 1) is a transmembrane protein upregulated in de differentiated proximal tubule cells after ischemic or nephrotoxic injury, but not expressed in normal kidney.[57] KIM 1 is later detecting AKI than NGAL or IL 18 (e.g. 12–24 h vs 2–6 h post CPB (cardiopulmonary bypass), respectively),[56] but shows promise differentiating between subtypes of AKI.

4. BIOMARKERS IN CHRONIC KIDNEY DISEASE

Chronic kidney disease (CKD) is a devastating illness that has reached epidemic proportions and continues to increase in incidence at an alarming rate. Estimates place the prevalence of CKD in the general population at 10–13%.[58] Medicare costs for patients in the United States with CKD reached $57.5 billion in 2007. This is an increase of 5.1 times the associated costs in 1993. For those patients progressing to end stage renal disease (ESRD), the mortality levels even exceed those of most malignancies.[59] Even those with mild CKD have greatly increased risk of premature death when compared to the general population, mainly due to associated cardiovascular disease.[60] CKD is a complex disease that often affects multiple organ systems and often coexists with numerous associated conditions, such as cardiovascular disease, diabetes mellitus, lupus, chronic inflammation. In many cases these conditions are independently associated with cardiovas cular disease, implying a vicious circle in which cardiovascular disease can lead to CKD, which worsens cardiovascular disease and down the line.

The 'gold standard' measurement for CKD is the 'true' glomerular filtration rate (GFR) as tracked by 24 h urine isotope clearance. This method is quite expensive and not always practical in the clinical setting. A commonly used clinical surrogate for nuclear GFR is serum creatinine clearance. However, as noted previously, the accuracy of serum creatinine is greatly affected by a number of patient dependent and independent variables. Additionally, serum creatinine may fall to one third of its normal level in advanced kidney disease, unrelated to its renal clearance.[56] Even serial 24 h creatinine measurements fail to determine risk progression in approximately 20% of CKD patients.[61] Even when accurate, 24 h creatinine clearance fails to offer reliable prognosis of CKD progression. CKD is defined by the presence of kidney damage or a glomerular filtration rate less than

60 mL/min/1.73 m^2 for 3 months or greater, regardless of cause. However, significant increases in cardiovascular disease risk occur at more subtle loss of kidney function (a GFR of approximately 75 mL/min/1.73 m^2),[60] so it is inherently important that CKD be caught in its earliest stages when possible.

Proteinuria is another useful marker of progressive functional decline in renal function. Proteinuria has been shown to directly represent kidney damage and higher levels of proteinuria correlate well with a more rapid progression of kidney disease.[62] Proteinuria is the earliest known marker of kidney damage in glomerular diseases, diabetes and hypertension, and is the most common marker of kidney damage in the adult population. However, proteinuria has limitations. Proteinuria may occur long after the renal injury has occurred and it is not always present in many types of renal disease.[56] Treatments, such as lowering urinary protein excretion using renin angiotensin system blockade and controlling hypertension, can reduce CKD progression rates.[63] CKD is often not caught until shortly before the onset of symptomatic kidney failure, so it is typically too late to prevent many adverse outcomes.[64] At this point, early diagnosis would entail routine testing of asymptomatic individuals in at risk categories for development of CKD and allow for determining staging (Table 1.5) and appropriate treat ment options for those individuals identified as having renal disease.

The need for biomarkers that can aid in diagnosing, distinguishing subtypes and prognosticating the severity of CKD and associated conditions are greatly needed, as the risk factors of this population are different from that of the general population. The search for biomarkers of CKD, especially those for the early diagnosis, is more difficult than that of AKI because the timing and nature of the insult is harder to pin down. With AKI, it is easy to

Table 1.5 Stages of chronic kidney disease

Stage	Description	GFR level
Normal	Healthy kidneys	$\geq$ 90 mL/min/1.73 m^2
1	Kidney damage with normal or high GFR	$\geq$ 90 mL/min/1.73 m^2
2	Kidney damage and mild decrease in GFR	60 89 mL/min/1.73 m^2
3	Moderate decrease in GFR	30 59 mL/min/1.73 m^2
4	Severe decrease in GFR	15 29 mL/min/1.73 m^2
5	Kidney failure	< 15 mL/min/1.73 m^2 or dialysis

Adapted from KDOQI Clinical Practice Guidelines for Chronic Kidney Disease: Evaluation, Classification, and Stratification, 2002.[101]

pick a population undergoing a procedure such as contrast administration or cardiopulmonary bypass, where the timing and nature of the insult can be tightly controlled and measurements can be made in scheduled periods before and after the potential injury has occurred. With pediatric pop ulations, it is often the case that you can control for many comorbid conditions, such as prolonged cardiovascular disease, effects of obesity and lifestyle that can affect renal function, and isolate the AKI incident from other variables that might influence potential biomarker levels. Such is not the case with CKD. Individuals with acquired or even hereditary forms of CKD can go years without knowing of their condition until it becomes severe enough to adversely affect the general health of the individual. Since many comorbid conditions are likely to exist, the results of biomarker studies on these individuals may be subject to high individual variability and be difficult to interpret and subsequently reproduce.

As is the case with AKI, biomarkers are needed in many areas of CKD, including the following: (1) determining the site of predominant kidney damage (e.g. glomerular, tubular); (2) providing insight into disease mechanism; (3) prognostication of disease progression (e.g. if it is deter mined that an individual is more likely to progress to ESRD, more aggressive treatments may be employed); (4) subtype classification and ability to direct course of treatment (e.g. distinguishing progressive focal segmental glomerular sclerosis, which is normally resistant to steroid treatment, from minimal change disease, which is non progressive and typically sensitive to steroid treatment); (5) determining risk of complica tions from comorbid conditions such as cardiovascular disease; and (6) more sensitive and reliable surrogate measurements for the estimation of GFR. In terms of characteristics of clinically applicable biomarkers for CKD, they should be nearly identical to those discussed for AKI (Table 1.4).

For CKD, due to its complexity and co existence with other conditions, it is even more unlikely that any one marker can be found to possess all of the ideal characteristics of a biomarker. Ongoing research has produced some promising candidates for possible inclusion in a panel of biomarkers for CKD, some of which are performing double duty as markers of AKI. In addition to its place in the upper echelon of promising AKI biomarkers, NGAL has been shown to be a potential marker for CKD severity and progression.[65] Likewise, cystatin C is a promising marker of GFR in both AKI and CKD.[66] It should be noted that more studies are needed to determine if cystatin C is truly a better marker of GFR than serum creat inine. Asymmetric dimethylarginine (ADMA) is a nitric oxide synthase

inhibitor and a marker of endothelial function. Increases in ADMA levels are predictive of CKD progression rates and are a risk factor for mortality in ESRD patients.[67] Liver type fatty acid binding protein (L FABP) is expressed in the proximal tubule of the kidney and its elevation has been shown to predict progression in CKD.[68] Larger longitudinal studies are needed to determine the utility of L FABP and the other biomarkers mentioned in predicting CKD progression in multiple etiologies.

5. THE EXAMPLE OF NGAL AS A BIOMARKER OF ACUTE KIDNEY INJURY

Preclinical transcriptome profiling studies identified *Ngal* (also known as lipocalin 2 or *lcn2*) to be one of the most upregulated genes in the kidney very early after acute injury in animal models.[2,3] Downstream proteomic analyses also revealed NGAL to be one of the most highly induced proteins in the kidney after ischemic or nephrotoxic AKI in animal models.[4,23,48] The serendipitous finding that NGAL protein was easily detected in the urine soon after AKI in animal studies has initiated a number of translational studies to evaluate NGAL as a non invasive biomarker in human AKI. In a cross sectional study, adults with established AKI (doubling of serum creatinine) displayed a marked increase in urine and serum NGAL by Western blotting when compared to normal controls.[49] Urine and serum NGAL levels correlated with serum creatinine, and kidney biopsies in subjects with AKI showed intense accumulation of immunoreactive NGAL in cortical tubules, confirming NGAL as a sensitive index of established AKI in humans.

A number of studies have now implicated NGAL as an early diagnostic biomarker for AKI in common clinical situations. In several prospective studies of children who underwent elective cardiac surgery, AKI (defined as a 50% increase in serum creatinine) occurred 1–3 days after surgery.[45,54,69] In contrast, NGAL measurements by ELISA revealed a 10 fold or more increase in the urine and plasma, within 2–6 h of the surgery in those who subsequently developed AKI. Both urine and plasma NGAL were excellent independent predictors of AKI, with an area under the receiver operating characteristic curve (AUC ROC) of > 0.9 for the 2–6 h urine and plasma NGAL measurements. These findings have now been confirmed in prospective studies of adults who developed AKI after cardiac surgery, in whom urinary and/or plasma NGAL was significantly elevated by 1–3 h after the operation.[70–77] However, the AUC ROCs for the prediction of

AKI have ranged widely from 0.61 to 0.96. The somewhat inferior performance in some publications appear to be the result of several factors, including assay performances and sample storage conditions, but are also perhaps reflective of confounding variables such as older age groups, pre existing kidney disease, prolonged bypass times, chronic illness, and dia betes. The predictive performance of NGAL also depends on the definition of AKI employed, as well as on the severity of AKI.[77] For example, the predictive value of plasma NGAL post cardiac surgery was higher for more severe AKI (increase in serum creatinine $>50\%$; mean AUC ROC 0.79) compared to less severe AKI (increase in serum creatinine $>25\%$; mean AUC ROC 0.65). Similarly, the discriminatory ability of NGAL for AKI increased with increasing severity as classified by RIFLE criteria. Thus, the AUC ROC improved progressively for discrimination of R (0.72), I (0.79) and F (0.80) category of AKI.[77] Despite these numerous potential variables, a recent meta analysis of published studies in patients after cardiac surgery revealed an overall AUC ROC of 0.76 for prediction of AKI, when NGAL was measured within 6 h of initiation of cardiopulmonary bypass and AKI was defined as a $>50\%$ increase in serum creatinine.[78] This performance compares favorably with that of troponin for the prediction of myocardial infarction during its clinical implementation period.

NGAL has also been evaluated as a biomarker of AKI in kidney trans plantation. In this setting, AKI due to ischemia reperfusion injury can result in delayed graft function, most commonly defined as dialysis requirement within the first postoperative week. Protocol biopsies of kidneys obtained 1 h after vascular anastomosis revealed a significant correlation between NGAL staining intensity in the allograft and the subsequent development of delayed graft function.[79] In a prospective multicenter study of children and adults, urine NGAL levels in samples collected on the day of transplant identified those who subsequently developed delayed graft function (which typically occurred 2–4 days later), with an AUC ROC of 0.9.[53] This has now been confirmed in a larger multicenter cohort, in which urine NGAL measured within 6 h of kidney transplantation predicted subsequent delayed graft function with an AUC ROC of 0.81.[80]

Several investigators have examined the role of NGAL as a predictive biomarker of nephrotoxicity following contrast administration.[81,82] In a prospective study of children undergoing elective cardiac catheterization with contrast administration, both urine and plasma NGAL predicted contrast induced nephropathy (defined as a 50% increase in serum creatinine from baseline) within 2 h after contrast administration, with an

AUC ROC of 0.91–0.92.[82] In several studies of adults administered contrast, an early rise in both urine (4 h) and plasma (2 h) NGAL were documented, in comparison with a much later increase in plasma cystatin C levels (8–24 h after contrast administration), providing further support for NGAL as an early biomarker of contrast nephropathy. A recent meta analysis revealed an overall AUC ROC of 0.894 for prediction of AKI, when NGAL was measured within 6 h after contrast administration and AKI was defined as a $>25\%$ increase in serum creatinine.[78]

Urine and plasma NGAL measurements also represent early biomarkers of AKI in a very heterogeneous pediatric intensive care setting, being able to predict this complication about 2 days prior to the rise in serum creatinine, with high sensitivity and AUC ROCs of 0.68–0.78.[83,84] Several studies have now examined plasma and urine NGAL levels in critically ill adult populations.[85–88] Urine NGAL obtained on admission predicted subsequent AKI in multi trauma patients with an outstanding AUC ROC of 0.98.[85] However, in a more mixed population of all critical care admissions, the urine NGAL on admission was only moderately predictive of AKI with an AUC ROC of 0.71.[86] In studies of adult intensive care patients, plasma NGAL concentrations on admission constituted an excellent to outstanding biomarker for development of AKI within the next 2 days, with AUC ROC ranges of 0.78–0.92.[87,89] In subjects undergoing liver transplantation, a single plasma NGAL level obtained within 2 h of reperfusion was highly predictive of subsequent AKI, with an AUC ROC of 0.79.[90] Finally, in a study of adults in the emergency department setting, a single measurement of urine NGAL at the time of initial presentation predicted AKI with an outstanding AUC ROC of 0.95, and reliably distinguished prerenal azotemia from intrinsic AKI and from chronic kidney disease.[91] Thus, NGAL is a useful early AKI marker that predicts development of AKI even in heterogeneous groups of patients with multiple co morbidities and with unknown timing of kidney injury. However, it should be noted that patients with septic AKI display the highest concentrations of both plasma and urine NGAL when compared to those with non septic AKI,[88] a confounding factor that may add to the heterogeneity of the results in the critical care setting. A recent meta analysis revealed an overall AUC ROC of 0.73 for prediction of AKI, when NGAL was measured within 6 h of clinical contact with critically ill subjects and AKI was defined as a $>50\%$ increase in serum creatinine.[78]

Because of its high predictive properties for AKI, NGAL is also emerging as an early biomarker in interventional trials. For example,

a reduction in urine NGAL has been employed as an outcome variable in clinical trials demonstrating the improved efficacy of a modern hydroxyethylstarch preparation over albumin or gelatin in maintaining renal function in cardiac surgery patients.[92] Similarly, the response of urine NGAL was attenuated in adult cardiac surgery patients who experienced a lower incidence of AKI after sodium bicarbonate therapy when compared to sodium chloride.[93] Furthermore, adults who developed AKI after aprotinin use during cardiac surgery displayed a dramatic rise in urine NGAL in the immediate postoperative period, attesting to the potential use of NGAL for the prediction of nephrotoxic AKI.[94] Not surprisingly, NGAL measurements as an outcome variable are currently included in several ongoing clinical trials formally listed in ClinicalTrails.gov. The approach of using NGAL as a trigger to initiate and monitor novel therapies, and as a safety biomarker when using potentially nephrotoxic agents, is expected to increase.

A number of studies have demonstrated the utility of early NGAL measurements for predicting the severity and clinical outcomes of AKI. In children undergoing cardiac surgery, early postoperative plasma NGAL levels strongly correlated with duration and severity of AKI, length of hospital stay and mortality.[95] In a similar cohort, early urine NGAL levels highly correlated with duration and severity of AKI, length of hospital stay, dialysis requirement and death.[96] In a multicenter study of children with diarrhea associated hemolytic uremic syndrome, urine NGAL obtained early during the hospitalization predicted the severity of AKI and dialysis requirement with high sensitivity.[97] In adults undergoing cardiopulmonary bypass, those who subsequently required renal replacement therapy were found to have the highest urine NGAL values soon after surgery.[71 78] Similar results were documented in the adult critical care setting.[85 91] Collectively, the published studies revealed an excellent overall AUC ROC of 0.78 for prediction of subsequent dialysis requirement, when NGAL was measured within 6 h of clinical contact.[78] Furthermore, a number of studies conducted in the cardiac surgery and critical care populations have identi fied early NGAL measurements as a very good mortality marker, with an overall AUC ROC of 0.71 in these heterogeneous populations.[78]

The majority of NGAL results described in the literature have been obtained using research based assays, which are not practical in the clinical setting. In these regards, a major advance has been the development of a standardized point of care kit for the clinical measurement of plasma NGAL (Triage® NGAL Device, Biosite Incorporated). In children

undergoing cardiac surgery, the increase in plasma NGAL levels measured by the Triage® Device at various time points after cardiopulmonary bypass was proportional to the severity of AKI as classified by RIFLE criteria. In terms of diagnostic accuracy, the 2 h plasma NGAL measurement showed an AUC of 0.96, sensitivity of 0.84 and specificity of 0.94 for prediction of AKI using a cutoff value of 150 ng/mL.[95] Several additional publications have now confirmed the utility and accuracy of the Triage® NGAL Device in critically ill adults. The assay is facile with quantitative results available in 15 min, and requires only microliter quantities of whole blood or plasma. In addition, a urine NGAL immunoassay has been developed for a standard ized clinical platform (ARCHITECT® analyzer, Abbott Diagnostics). In children undergoing cardiac surgery, the increase in urine NGAL levels determined by ARCHITECT® analyzer at various time points after cardiopulmonary bypass was also proportional to the severity of AKI as classified by RIFLE critieria. The 2 h urine NGAL showed an AUC of 0.95, sensitivity of 0.79 and specificity of 0.92 for prediction of AKI using a cutoff value of 150 mg/mL.[96] This assay is also easy to perform with no manual pretreatment steps, a first result available within 35 min, and requires only 150 μL of urine. Both clinical assays are currently undergoing multi center validation in several clinical populations. Analysis of the published literature thus far indicates that the diagnostic accuracy of these clinical platforms for the prediction of AKI (AUC ROC 0.83) is superior to that of research based NGAL assays (AUC ROC 0.73).[78]

The genesis and sources of plasma and urinary NGAL following AKI require further clarification. Although plasma NGAL is freely filtered by the glomerulus, it is largely reabsorbed in the proximal tubules by efficient megalin dependent endocytosis. Direct evidence for this notion is derived from systemic injection of labeled NGAL, which becomes enriched in the proximal tubule but does not appear in the urine in animals.[49] Thus, any urinary excretion of NGAL is likely only when there is concomitant proximal renal tubular injury that precludes NGAL reabsorption and/or increases *de novo* NGAL synthesis. However, gene expression studies in AKI have demonstrated a rapid and massive upregulation of NGAL mRNA in the distal nephron segments – specifically in the thick ascending limb of Henle's loop and the collecting ducts. The resultant synthesis of NGAL protein in the distal nephron and secretion into the urine appears to comprise the major fraction of urinary NGAL. Supporting clinical evidence is provided by the consistent finding of a high fractional excretion of NGAL reported in human AKI studies.[49] The overexpression of NGAL in the distal

tubule and rapid secretion into the lower urinary tract is in accord with its teleological function as an antimicrobial strategy. It is also consistent with the proposed role for NGAL in promoting cell survival and proliferation, given the recent documentation of abundant apoptotic cell death in distal nephron segments in several animal and human models of AKI.[98]

With respect to plasma NGAL, the kidney itself does not appear to be a major source. In animal studies, direct ipsilateral renal vein sampling after unilateral ischemia indicates that the NGAL synthesized in the kidney is not introduced efficiently into the circulation, but is abundantly present in the ipsilateral ureter. However, it is now well known that AKI results in a dramatically increased NGAL mRNA expression in distant organs, especially the liver and lungs, and the overexpressed NGAL protein released into the circulation may constitute a distinct systemic pool.[99,100] Additional contributions to the systemic pool in AKI may derive from the fact that NGAL is an acute phase reactant and may be released from neutrophils, macrophages and other immune cells. Furthermore, any decrease in glomerular filtration rate resulting from AKI would be expected to decrease the renal clearance of NGAL, with subsequent accumulation in the systemic circulation. The relative contribution of these mechanisms to the rise in plasma NGAL after AKI remains to be determined.

Clearly, NGAL represents a novel predictive biomarker for AKI and its outcomes. However, NGAL appears to be most sensitive and specific in homogeneous patient populations with temporally predictable forms of AKI. Plasma NGAL measurements may be influenced by a number of coexisting variables such as CKD, chronic hypertension, systemic infections, inflammatory conditions, anemia, hypoxia and malignancies.[99,100] However, it should be noted that the increase in plasma NGAL in these situations is generally much less than those typically encountered in AKI. There is an emerging literature suggesting that urine NGAL is also a marker of CKD and its severity. In this population, urine NGAL levels are elevated and significantly correlated with serum creatinine, GFR and proteinuria. Urine NGAL has also been shown to represent an early biomarker for the degree of chronic injury in patients with IgA nephropathy and lupus nephritis, and may be increased in UTIs.[99,100] However, the levels of urine NGAL in these situations are significantly blunted compared to that typically measured in AKI.

Thus, NGAL as an AKI biomarker has successfully passed through the preclinical, assay development and initial clinical testing stages of the biomarker development process (Table 1.1). It has now entered the prospective screening stage, facilitated by the development of commercial

tools for the measurement of NGAL on large populations across different laboratories. But will any single biomarker such as NGAL suffice in AKI? In order to obtain all of the desired information that would characterize an ideal biomarker, a panel of validated biomarkers may be needed. Other AKI biomarker candidates may include interleukin 18 (IL 18), kidney injury molecule 1 (KIM 1), cystatin C and liver type fatty acid binding protein (L FABP), to name a few. The availability of a panel of AKI biomarkers could further revolutionize renal and critical care in the not too distant future.

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CHAPTER 2

Statistical Considerations in Analysis and Interpretation of Biomarker Studies

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Contents

1. INTRODUCTION

Biomarkers can be broadly defined as biological parameters, which objec tively can be measured and evaluated as indicators of normal biological processes, pathogenic processes or pharmacological responses to therapeutic interventions. The development of biomarkers into diagnostic tests can be categorized into three broad phases: biomarker discovery, the evaluation of biomarker classification performance and the impact of using biomarkers in

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Table 2.1 Research phases of biomarker development

Phase I: Biomarker discovery
Identify candidate biomarker for disease of interest
Phase II: Biomarker classification performance
Establish that the biomarker can discriminate between diseased and non diseased Determine if biomarker precedes current methods of diagnosis
Phase III: Biomarker use in clinical care
Assess the impact and additive benefit of the integration of the biomarker into clinical care Determine cost effectiveness and improvement in outcomes

clinical care (Table 2.1).[1] Each phase requires unique statistical considerations and tailored study design to accurately evaluate research objectives. There are several resources available for each phase of biomarker development, per taining to study design, statistical analysis and sample size calculations.[2–4]

In this chapter, we will focus on human studies evaluating the classifi cation performance of diagnostic biomarkers. We will use examples of biomarkers in acute kidney injury (AKI), in order to highlight concepts of the classification performance of biomarkers. The methodology and framework described herein can easily be extended for research and the development of biomarkers in other clinical settings. The statistical meth odology required for the assessment of classification performance of biomarker differs from the classical methods used in epidemiology or thera peutic research[5,6] (see the example in section 3.1.1 below). In biomarker development, we are focused on classification or discrimination (e.g. true positive and false positive rates), rather than measures of association (e.g. odds ratio, relative risks).

At the end of the biomarker discovery phase, we assume that a candidate biomarker has been identified for the disease of interest. During the second phase of biomarker development, we want to establish that the biomarker can: (1) discriminate between diseased and non diseased patients earlier than the current clinical standard; (2) explore covariates associated with the biomarker; and (3) validate the biomarker screening criteria and the combination of biomarkers, if applicable. In some cases, identifying the screen positive criteria and the combination of biomarkers also may be completed in this second phase. Several studies are usually required to complete the second stage of biomarker development. The final phase will examine the impact of biomarker usage in clinical care.[1,7–9]

2. PLANNING A STUDY

2.1. Research objectives for assessing biomarker performance

In planning a research study assessing biomarker performance, a well defined research question relevant to the phase of biomarker development is required and ample consideration should be given to defining the target population and ensuring that the data elements (both clinical information and sample processing details) will be collected in sufficient detail. The minimum biomarker performance level must be specified in advance and this will drive sample size requirements. The determination of a minimal required performance level should be based on the current clinical standards and consequences of potential misclassification by the biomarker.

The first research aim is to determine if the biomarker can discriminate between diseased and non diseased patients.[1,9] Generally, this can be completed in a retrospective study where the biomarker is collected at approximately the same time that the disease is diagnosed. The time dependent discriminatory ability of the biomarker will not be assessed in this study.

The next research aim is to evaluate whether the biomarker can discriminate between diseased and non diseased patients earlier than the current clinical standard.[1,9] For this, a prospective study is required, where specimens are taken at several time points prior to the clinical diagnosis. The biomarker then can be measured in all patients or in a subgroup of patients (nested case control study). To reduce bias, a patient's clinical information should be blinded when the specimen is assayed. To assess whether the biomarker can discriminate between diseased and non diseased patients earlier than current clinical care, time dependent receiver operating char acteristic (ROC) curves or ROC regression should be used.[2,10]

If the biomarker only will be measured in a subgroup of patients, consideration should be given to how the sample should be selected and if matching should be implemented.[1] The use of matching will introduce additional complexities in the analytical methods, such as accounting for the matching factors. Since the non diseased patients are no longer a represen tative sample of the target population, it will not be possible to evaluate the influence of matching factors with the biomarker, and the interpretation of false positives will change.[1]

2.2. Explore covariates that may affect biomarker values

An important step, often overlooked when evaluating the classification performance of a biomarker, is to determine the existence of factors that

influence a biomarker's prediction performance that are unrelated to the outcome of interest.[11] It is important to explore such factors by examining the distribution of the biomarker in the non diseased patients. Factors to consider may be related to patient demographics (e.g. age, race, gender), clinical parameters (e.g. protein in urine, oliguria; chronic kidney disease) or sample processing details (e.g. collection time, freezing time, length of storage). If there are factors identified relating to the biomarker in the non diseased patients, then diagnostic accuracy can be assessed separately (e.g. look at biomarker in adults and children separately), or an adjusted ROC curve analysis can be completed.[11] An adjusted ROC curve analysis is analogous to covariate adjustment in studies of association.

2.3. Avoid overfitting

A frequent criticism of biomarker discovery and validation is irreproducible results.[4,8] Often, this is because identification and validation of the biomarker was completed in the same data. If the identification of the screen positive criteria or the combination of biomarkers is to be completed in the same study as the evaluation of the biomarker's performance, methods need to be implemented to avoid overfitting. The most straightforward method is to split the data into two sets: a derivation and a validation dataset.[1,5,8] Alternatively, bootstrapping or cross validation methods could be applied[1,5,8] but they require more advanced statistical techniques.

3. STATISTICAL METHODS TO QUANTIFY CLASSIFICATION PERFORMANCE

The analytical methods required for a study depend on the research question and the study design. In this second phase of biomarker development, we are focused on evaluating the classification performance of the biomarker. In general, for both the retrospective and the prospective studies described above, we recommend quantifying the classification performance with true positive rates (TPR), false positive rates (FPR) and ROC curves. In medical literature, these rates are also referred to as sensitivity (TPR) and specificity (1 FPR).

3.1. True positive rate (TPR) and false positive rate (FPR)

If we compare the classification of the biomarker to the true disease status, the results can be categorized as a true positive, a false positive, a true negative or a false negative (Table 2.2). A true positive result occurs when the

Table 2.2 Biomarker classification by disease status

		True disease state	
		Diseased	**Non-diseased**
Biomarker test	Positive (diseased)	True positive (TP)	False positive (FP)
	Negative (non diseased)	False negative (FN)	True negative (TN)

True positive rate (TPR) Sensitivity TP/(TP + FN).
False positive rate (FPR) 1 − Specifity FP/(FP + TN).

biomarker correctly classifies the patient as a diseased patient, and similarly, a true negative result occurs when the biomarker correctly classifies the patient as a non diseased patient. A false positive or a false negative occurs when the biomarker incorrectly classifies a non diseased patient as a diseased patient, or a diseased patient as a non diseased patient, respectively. The true positive rate is the proportion of diseased patients that the biomarker correctly classified as diseased patients, and the false positive rate is the proportion of non diseased patients that the biomarker incorrectly classified as diseased patients. The range of possible values for both the TPR and FPR is between 0 and 1. A good biomarker has high TPR and low FPR.

3.1.1. Example − urine biomarker predicts AKI after cardiac surgery

A prospective cohort study followed 750 patients undergoing non emergent cardiac surgery. Within the first 5 days after surgery, 20% of patients developed acute kidney injury (defined by a 50% increase in serum creatinine from preoperative level). A urine biomarker was measured in all patients within the first 6 h after surgery. The optimal classification threshold of 30 ng/mL was identified and the classification table below was created to calculate the classification performance of the biomarker. At the optimal threshold, the TPR is 0.87 and the FPR is 0.12.

		AKI		
		Present	**Absent**	**Total**
Urine biomarker	Positive ($\geq$ 30 ng/mL)	130	70	200
	Negative ($<$ 30 ng/mL)	20	530	550
	Total	150	600	750

TPR 130/150 0.87.
FPR 70/600 0.12.
Odds ratio (130)(530)/(20)(70) 49.

For studies of classification, TPR and FPR should be used instead of an odds ratio. Classification performance can differ even if the odds ratio remains the same.[6] For example, the urine biomarker above had an odds

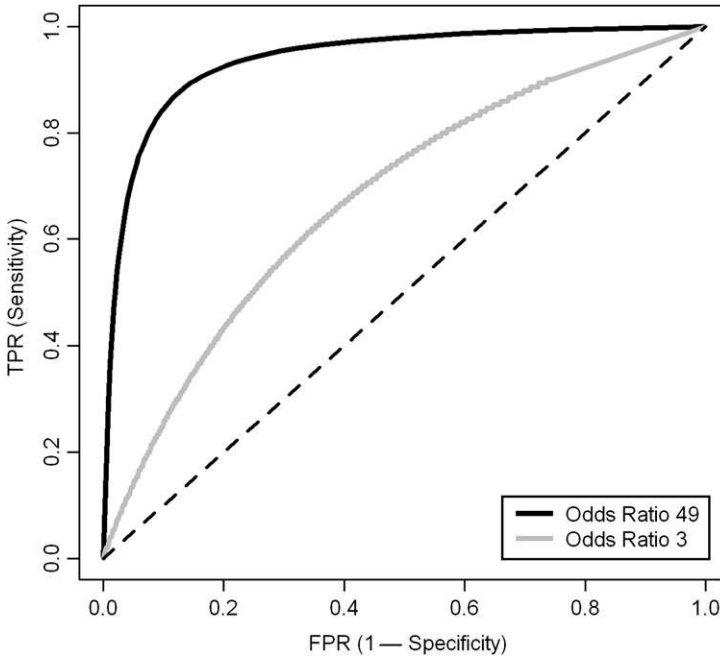

Figure 2.1 ***Relationship between odds ratio, false positive rates (FPR) and true positive rates (TPR).*** For a given odds ratio, there can be multiple combinations of FPR and TPR levels.

ratio of 49 (95%CI 30, 81) with a TPR of 0.87 and FPR of 0.12. In the table below, we have shown it is possible to have the same large odds ratio of 49 with different classification results (TPR 0.54 and FPR 0.02).

		AKI		
		Present	**Absent**	**Total**
Urine biomarker	Positive ($\geq$ 45 ng/mL)	81	14	95
	Negative ($<$ 45 ng/mL)	69	586	655
	Total	150	600	750

TPR 81/150 0.54.
FPR 14/600 0.02.
Odds ratio (81)(586)/(14)(69) 49.

The graph in Figure 2.1 demonstrates that for any odds ratio, there can be multiple combinations of TPR and FPR levels.[6]

3.2. ROC curve

The ROC curve provides a complete description of the biomarkers' classification performance. It is a single curve plotted on a graph with the

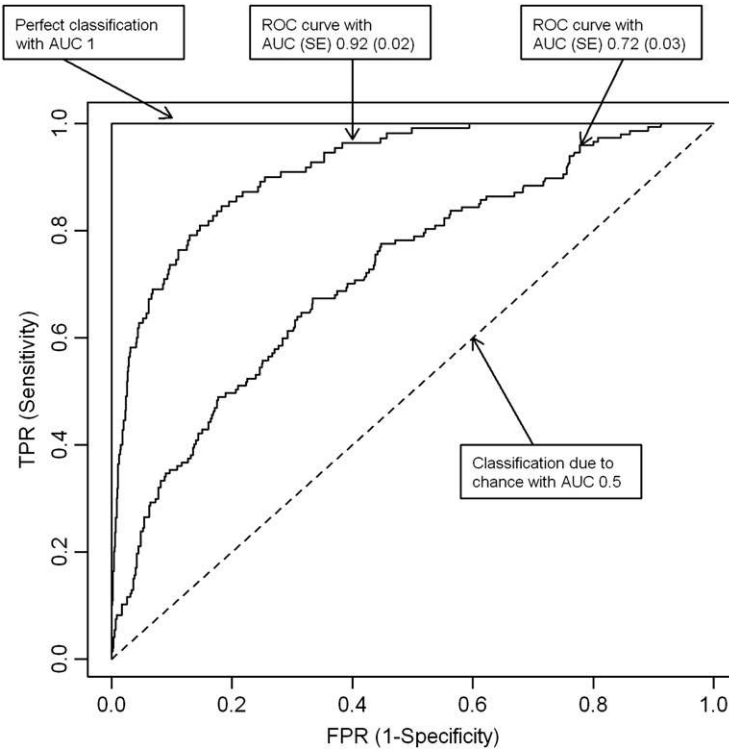

Figure 2.2 ***Example ROC curve.*** AUC, area under curve; FPR, false positive rate; TPR, true positive rate.

FPR on the horizontal axis and the TPR on the vertical axis (Figure 2.2). The curve is a plot of the classification performance (FPR, TPR) of the biomarker as the screen positive criteria changes. ROC curves can guide the selection of screen positive criteria.[2,12] Biomarkers with ROC curves closer to the top left hand corner have better classification performance. A perfect biomarker that accurately discriminates all diseased and non diseased patients would have an ROC curve along the left side of the graph and along the top of the graph. A diagonal line is included on ROC curves to demonstrate the performance of a biomarker purely due to chance. If the entire ROC curve lies below the diagonal line, this indicates that the distribution of the biomarker is opposite to that of usual convention (e.g. lower values of the biomarker are associated with diseased patients).[2,13] In such situations, transform the biomarker data so that it follows usual convention (e.g. biomarker values multiplied by negative one) and recreate

the ROC curve. The top left hand corner of the ROC curve corresponds to the biomarker level where specificity and sensitivity are optimized.

3.3. Area under the curve (AUC)

If it is difficult to produce an ROC curve or if there are too many biomarkers to compare with ROC curves, summary indices of ROC curves are frequently used. The area under the ROC curve (AUC) is probably the most widely used summary index. The AUC ranges from 0.5 (the area under the diagonal line representing discrimination based on random chance) to 1 (the area of the entire square representing perfect discrimination). The AUC can be interpreted as the probability of the biomarker value being higher in a diseased patient compared with a non diseased patient, if the diseased and non diseased patients are randomly chosen.[2,13]

ROC curves and AUC can be calculated using most statistical software packages. The area under the curve can be estimated by the c index (usually calculated by the trapezoidal rule) or by the Mann−Whitney U statistic.[14] The trapezoidal rule and U statistic are nearly identical when the biomarker is continuous,[15] but if the biomarker only has a few distinct values (5 or 6), the trapezoidal rule systematically underestimates the true area.[16]

3.4. Optimal classification threshold

Another summary index frequently reported is the set of FPR and TPR that corresponds to a particular screening threshold. Often, the optimal classification threshold is defined as the cut point with the maximum difference between the TPR and FPR (e.g. the Youden Index calculated as max(TPR − FPR) or equivalently max(sensitivity + specificity − 1)). This definition may not be the optimal threshold, depending on the clinical context. For example, for a biomarker to be accepted in clinical practice, it must have a better classification performance than the existing test, which has a FPR of 10%. Thus, the optimal threshold in this scenario would be defined as the maximum TPR for an FPR of at the most 10%.

3.5. Partial area under the curve

In some contexts, it might be of interest to summarize the classification performance of the biomarker based on more than one screening threshold, but less than the full range of FPR values. The partial area under the curve can be used to describe the classification performance within a range of FPR values. For example, certain settings may require very low FPR values (e.g. ≤ 0.05); therefore, only the AUC between FPR values of 0 and 0.05

would be of interest. There are other summary indices that have been proposed for this measure but are not discussed here.[2,13]

4. SAMPLE SIZE CALCULATIONS

Sample size calculations should be linked to the statistical methods used in the analysis. Margaret Pepe has developed rigorous methodology for sample size calculations.[2] Here, we will provide examples of sample sizes for a continuous biomarker based on TPR and FPR using Pepe's methodology.

For the sample size calculation, we will determine if the TPR is above some minimally acceptable value for a given minimally acceptable FPR. The following assumptions are required for the calculations: significance level, power level, disease event rate, and the ratio of the variability of the biomarker in diseased and non diseased patients. We assume the variances of the biomarker in diseased and non diseased patients are equal, in order to provide us with the largest sample sizes. In addition, we assume a signifi cance level of 5% and 80% power.

For example, suppose we are evaluating a new continuous biomarker for a disease with an event rate of 7%. The largest acceptable false positive rate is 5% (corresponds to specificity of 95%) and at that rate, the biomarker must have a true positive rate of at least 5% (TPR null) in order to be considered a useful biomarker. It is expected that the biomarker will have a TPR of at least 10% (TPR alternative). Given these assumptions, 3300 patients are required (231 diseased patients and 3069 non diseased patients). A smaller sample size is required with a higher event rate and larger effect sizes (Figure 2.3). Thus, 190 patients will be required for a biomarker with expected TPR rate of 40%, where the event rate of disease is 20%.

5. EMERGING METHODS

New statistical methodologies are being developed to improve the analysis and interpretation of biomarkers. Currently these methods are not widely used in presentation of biomarker results. However, some of these techniques may become popular and we wanted the readers to become familiar with these terms. Here, we briefly describe a few of these new methodologies. We also provide references for readers who are interested in further details.

5.1. Standardized placement values

To aid in the comparison of several biomarkers, it has been proposed to standardize results to the distribution of the biomarker in non diseased

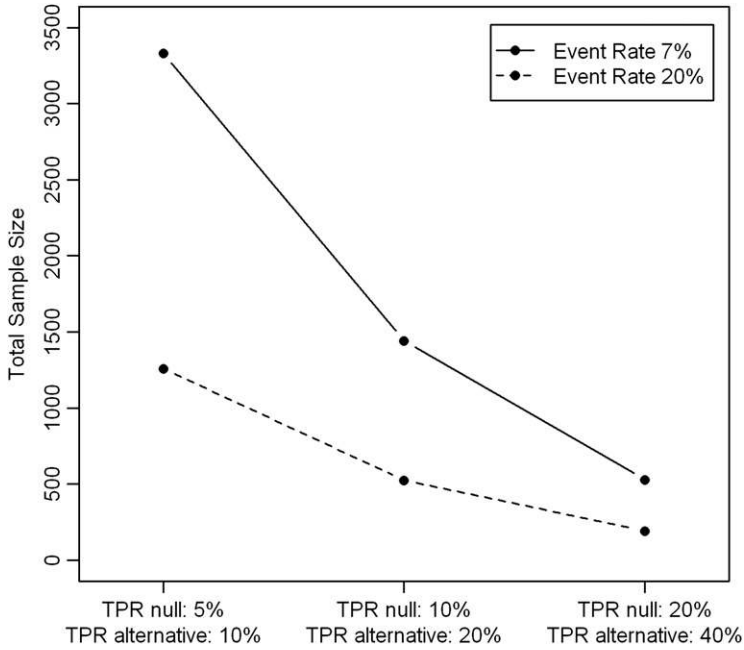

Figure 2.3 ***Sample size required for a FPR of 5%.*** TPR, true positive rate.

patients.[17,18] Standardized placement values for diseased patients are calculated as the proportion of non diseased patients, which have a biomarker value greater than the diseased patient's biomarker value. The standardized placement value does not have a measurement unit that allows for direct comparisons between biomarkers.

5.2. Risk models

For diseases where multivariable risk models have been derived and vali dated, it is of interest to determine if there is any improvement in a model including a biomarker. There have been new developments in this field. A few ideas that we will highlight here are predictiveness curves,[19,20] two new metrics net reclassification improvement (NRI) and integrated discrimi nation improvement (IDI).[21]

5.2.1. Predictiveness curves

Predictiveness curves provide a graphical method of combining risk prediction models and classification methods. The curve is created with the estimated risk of the disease calculated for each individual using the risk prediction model plotted against the percentile value of the biomarker.

Predictiveness curves offer the ability to provide the new risk for an individual based on their inherent risk and biomarker test result. It is useful for assessing the fit of the risk model and the classification performance of the biomarker.[19,20] A horizontal line of disease prevalence is included as a reference for a completely uninformative risk model. Better models will have larger areas, below the horizontal disease prevalence line and above the predictiveness curve, and above the disease prevalence line and below the predictiveness curve. In that regard, predictiveness curves are mirror images of ROC curve.

For example, in Figure 2.4 we have assumed the prevalence rate of AKI is 20% (represented by the horizontal reference line). From the predictiveness curves, we can see that Biomarker #1 has better performance than Biomarker #2. For example, without the knowledge of the biomarker values every individual has a 20% risk of AKI. Biomarker #1 identifies 97% of individuals with a risk of AKI less than 20% and 3% of individuals with a risk of AKI greater than or equal to 20%. Biomarker #2 only identifies 38% of individuals with a risk of AKI less than 20% and 62% of individuals with a risk of AKI greater than or equal to 20%.

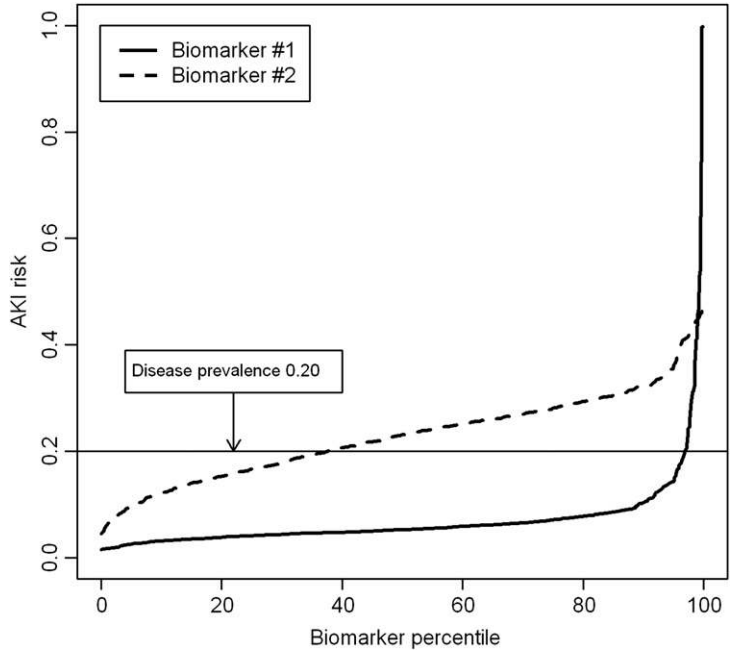

Figure 2.4 ***Example of a predictiveness curve.*** AKI, acute kidney injury.

5.2.2. Net reclassification index (NRI) and integrated discrimination improvement (IDI)

NRI and IDI are two new metrics based on the concept of reclassification tables. This applies to the situation where two models are being compared. For example, comparing the original risk model to the risk model with the biomarker added. If a diseased patient moves "up" a risk category in the new model this is seen as an improvement in classification and any "downward movement" is considered worse reclassification. NRI is the sum of the proportion of individuals with an improvement in classification (diseased patients moving up or non diseased patients moving down) minus the proportion of individuals with worse classification (diseased patients moving down or non diseased patients moving up). The IDI can be used to look at probability differences continuously instead of categorically.[21]

6. SUMMARY

Biomarker development is a phased program and requires several years to develop for clinical use. For each phase of biomarker development, it is important to customize the study design, statistical analysis, and sample size calculation, in order to evaluate the clearly defined research objective. Biomarker classification performance should be quantified with appropriate metrics, such as TPR, FPR and ROC curves. Having a clear understanding of the research methodology and research goals can improve efficiency for successful biomarkers and prevent wastage of resources and effort on failed biomarkers.

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CHAPTER 3

The Role of Metabolomics in the Study of Kidney Diseases and in the Development of Diagnostic Tools

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1. INTRODUCTION

Although functionally and physically separate entities within the body, all of the body's cells are in constant communication with the various fluid compartments of the body. Cell metabolites, peptides and proteins are in constant flux, being alternatively released from cells or taken up by cells from body fluids via a variety of mechanisms: normal excretion, trans membrane diffusion or transport and during the death process when cells release all of their contents. Thus, at least to a certain extent, the biochemical and protein based changes which are occurring within cells and organs are reflected in body fluids.

It was already recognized in ancient Greece that changes in tissue and biological fluids were observed to be coincident with the development of pathology, and thus were capable of serving as indicators of given disease processes. To that end, the so called urine charts were developed and have widely been used since the middle ages.[1] The developments in chemistry in the late 18th century and the emergence of analytical methods, albeit simple, provided the basis for the first clinical chemistry diagnostic tools used in nephrology. In 1795 the nitric acid test for proteinuria was described and only a few decades later already more than 100 organic and inorganic compounds in urine were known.[2] Technological advances in nuclear magnetic resonance spectroscopy (NMR), mass spectrometry and chemo metrics (biostratistical pattern recognition methods) have opened up new opportunities in biochemistry by introducing metabonomics as an approach to study metabolism and its regulation in response to drugs, disease, genetic

and environmental factors.[1] In general, metabolomics based strategies have been developed and employed in order to:

- Identify unknown molecular mechanisms.
- Discover molecular markers that can be used for drug discovery, pre clinical and clinical drug development.
- Develop diagnostic tools.

1.1. Definitions

Metabonomics has been defined as "the quantitative measurement of the multi parametric metabolic response of living systems to pathophysiological stimuli or genetic modification".[3] There are numerous and often conflicting uses of the terms metabonomics and metabolomics in the literature and both words have been used interchangeably. The definitions metabolomics, metabonomics and other related terms are listed in Table 3.1. Since in most cases it is in fact metabolic profiling that is being performed in body fluids or in specific organs (in this case, the kidney), the term metabolomics will be used here for the sake of simplicity.

A biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to therapeutic intervention".[6] On the basis of this definition, biomarkers have been in use since the emergence of clinical diagnostics and include a whole host of procedures, ranging from the mundane, such as measurement of clinical signs and symptoms — blood pressure readings or temperature assessment — to the slightly more sophisticated analysis seen in ECG tracings, to the various refined exami nations available, including imaging technologies like CT or MRI, and ultimately extending to the most modern technologies such as high throughput gene arrays.[7] Since metabolomics is based on technologies that directly or indirectly assess molecular mechanisms, the more focused term 'molecular marker' will be used here instead of the broader term 'biomarker'. A molecular marker can consist of the measurement of a single molecular entity but it can also be a set of several molecular entities, as in a molecular pattern or fingerprint.

1.1.1. Why are metabolomics-based molecular markers expected to be more sensitive and specific than currently established markers used in nephrology?

Clinical diagnostics is usually based on a limited set of biomarkers, often only one parameter that is closely correlated with a functional aspect of the

Table 3.1 Terms and definitions. See also Holmes et al[4] and Nicholson[5]

Metabolome	A quantitative descriptor of all endogenous low molecular weight components in a biological sample such as urine or plasma. Each cell type and biological fluid has a characteristic set of metabolites that reflects the organism under a particular set of environmental conditions and that fluctuates according to physiological demands. The metabolome can be divided into the primary metabolome (as controlled by the host genome) and the co metabolome (dependent on the microbiome)
Co metabolome	Metabolites that can only be formed by the integrated biochemical actions of more than one genome such as the gut microbial metabolism of a mammalian metabolite or vice versa
Metabonome	Theoretical combinations, sums and products of the interactions of multiple metabolomes (primary, symbiotic, parasitic, environmental and co metabolic) in complex systems
Metabolomics	The comprehensive quantitative analysis of all the metabolites of an organism or a specific biological sample
Metabonomics	The quantitative measurement over time of the metabolic responses of an individual or population to a disease, drug treatment or other challenge
Microbiolome	The consortium of microorganisms, bacteria, protozoa and fungi that live commensally or symbiotically with a host
Xenometabolome	Characteristic profile of non endogenous compounds such as drugs, their metabolites and their excipients, dietary components, herbal medicines and environmental exposure

organ in question or with a specific disease process. However, there is not and there will never be a single molecular entity marker that captures the function of the kidney in all its complexity. Although the limitations of the currently most widely used biomarkers for the detection of acute and chronic kidney injury such as proteinuria, creatinine in serum and blood urea nitrogen are well known and have often been discussed, these diag nostic markers remain the standard of care. All of these markers are less than optimal, in large part because they focus on the later stages of kidney injury when therapeutic interventions may be less effective and less likely to result in complete reversal of the injury.[8] In essence, these are often indicators of irreversible or only partially reversible kidney injury. Moreover, these markers tell us nothing of the causation or location of said injury.

Modern analytical technologies allow for the identification of patterns that confer significantly more information than the measurement of a single

parameter, much as a bar code contains more information than a single number. Well qualified molecular marker patterns will yield more detailed and mechanistically relevant measurements than those currently available, ultimately translating into good specificity. The better the specificity of a molecular marker pattern, the greater the reduction in non specific background noise. Reduced background noise can be expected to result in better sensitivity, and thereby an enhanced ability to recognize a disease process while it remains early in the making.

While, for example, creatinine concentrations in serum typically need to increase by 20% before such an increase is considered clinically significant, several signals in a pattern revealing smaller changes in a certain direction may be sufficient to draw reliable conclusions on the basis of their being congruous.[9] In addition a molecular marker that is composed of several qualified parameters that describe and measure different aspects of kidney function will convey more comprehensive diagnostic information and thereby reduce the risk of overlooking disease processes or drug effects that may have been subtly indicated but considered insignificant when only a single parameter marker is used. The main limitation of such a marker system is that it measures only a very specific aspect of kidney function, and if that function is not yet affected, the damage that has occurred will not be acknowledged or observed.

1.1.2. Metabolomic-based molecular markers versus protein and genomic markers – advantages and challenges

Genomics, proteomics and metabolomics, when taken together as a whole, provide a comprehensive framework, also referred to as systems biology, that describes the biochemical function of an organism and its response to challenges. Genomic and phenotypic molecular markers, including proteins and metabolites, have been differentiated. The genotype of a patient defines the risk or probability of reacting to a disease, drug or environmental challenge in a certain way and is static. The phenotype more closely reflects clinical reality at any given moment. In recent years, gene arrays have extensively been used for not only molecular marker discovery but also in drug development and the identification of molecular mechanisms. One of the reasons gene arrays are considered so desirable is the availability of standardized high throughput technologies, while the analytical technolo gies used for proteomics and metabolomics are not yet as mature. Unfor tunately, it cannot be assumed that changes of mRNA concentrations, also known as the transcriptome, translate directly into corresponding changes in

the number of functional proteins. Accordingly, it cannot be assumed that changes in the transcriptome are necessarily associated with changes in signal transduction and cell biochemistry. Therefore, downstream confir mation by analyzing protein concentrations and/or metabolites is usually required.[10] However, the changes of a protein concentration may also not necessarily translate into changes in cell biochemistry and function since protein concentration is not always correlated with activity. Reasons include changes in translational modifications, reaction with oxygen radicals and allosteric regulation by substrates, products and other inhibitors and acti vators. Pathophysiological changes and histological damage is in most cases directly caused by changes in cell metabolism. Thus, metabolomics typically is more closely associated with a disease process or drug effect than proteins, mRNA or genes.[11]

While transcriptomics and proteomics strictly detect endogenous changes, the metabolome communicates with the environment and is an open system. The exact number of metabolites varies at any given time. Metabolic profiles include endogenous and exogenous chemical entities including peptides, amino acids, nucleic acids, carbohydrates, organic acids, vitamins, hormones, drugs, drug metabolites, drug excipients, food addi tives, phytochemicals, toxins, and other chemicals ingested or synthesized by a cell or organism. The metabolome can also be influenced by environment, gut flora and its metabolites, diet and general activities and responses such as stress, hormones, physical injury and exercise[4] (Figure 3.1). In comparison to the larger proteins and mRNA, small molecules such as metabolites can distribute quickly all over the body. Although this will result in comprehensive information and a rather complete picture of the complex interactions of an organism's metabolism and its interactions with the microbiome, environment and other exogenous factors, deconvolution of this information can be challenging.[4]

The number of major metabolites relevant for clinical diagnostics and drug development has been estimated at 1400–3000 molecules.[7,12] Most endogenous metabolites are tied to specific biochemical pathways such as glycolysis, Krebs' cycle, lipid or amino acid metabolism, signaling pathways such as transmitters and hormones and specific pathobiochemical processes such as oxidative stress. Thus, changes in specific metabolite patterns reflect changes in pathways and processes.

Lipids are a specific group of cell metabolites and the term lipidomics has been used to describe the comprehensive identification and quantification of all lipid molecular species in a biological system.[14] Lipids are loosely

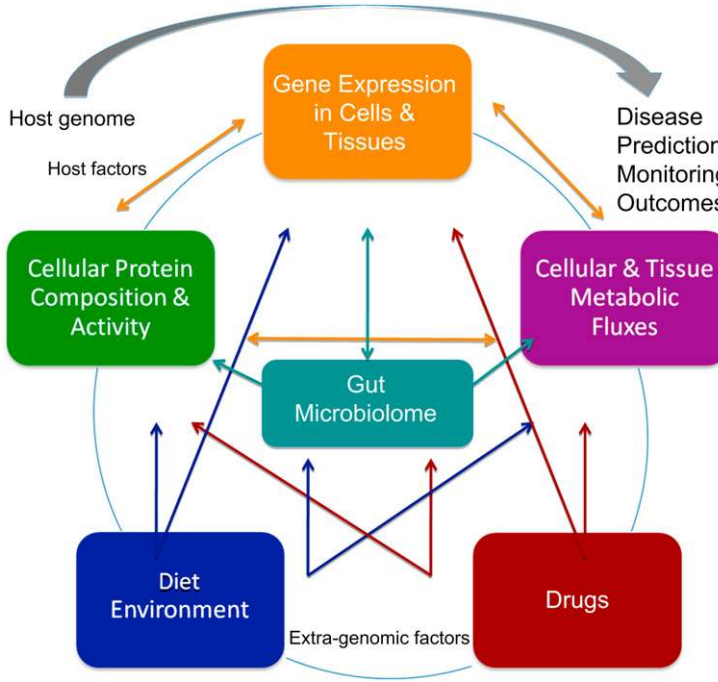

Figure 3.1 ***Interactions between the mammalian system, the microbial metabolome, diet and environment.***[13]

defined as biological compounds that are generally hydrophobic in nature and soluble in organic solvents. Lipids are membrane components, medi ators in cell signaling and are utilized as fuel and energy storage.[15] Their distinct solubility properties often require separate extraction and analysis in metabolomics experiments.[14]

The metabolome is considered the most predictive phenotype and holds the promise to extensively contribute to the understanding of phenotypic changes as an organism's answer to disease, genetic changes, and nutritional, toxicological, environmental and pharmacological influences.[4] Another advantage of metabolomics is that in contrast to genes and proteins, metabolites are often tissue and species independent. This facilitates translation of molecular markers strategies from bench to bedside or vice versa,[12] which is of advantage for drug development and molecular marker qualification (see below). Also, while it may take hours, days and sometimes weeks for protein and mRNA expression to change in response to a chal lenge, metabolic responses can often be measured within seconds or minutes.[4]

2. METABOLIC MAPPING OF THE KIDNEY

The kidney has a wide range of biochemical, physiological and endocrine functions including, but not limited to, the regulation of blood pressure, fluid volume and systemic electrolyte concentrations, the elimination of waste products, the recovery of desired substrates from urine, the metabolism of endogenous compounds and xenobiotics, and the synthesis of hormones such as erythropoietin, renin and 1,25 hydroxy vitamin D_3.[16] The kidney consists of the following major regions (listed from outside in): the cortex, the outer and inner medulla, and the papilla. All of the separate regions of the kidney are associated with unique functionalities and face dissimilar meta bolic challenges. These challenges are in part driven by differences in osmolarity and oxygen tension faced by the various areas of the kidney. Accordingly, enzymes, transporters and other proteins are differentially distributed across the different regions according to the needs of the various functional anatomical structures of the kidney. This has the consequence of developing region specific differences in cell metabolism. Thus, for example, the majority of the kidney's drug metabolizing enzymes, and enzymes involved in the detoxification of radicals are located in the proximal tubule, while regions in an environment with high osmolarity such as the loop of Henle and the collecting ducts are rich in osmolyte transporters and, due to the high energy requirements in such an environment, glycolytic enzymes. For a more comprehensive summary of the distribution of enzymes and transporters in different regions of the nephron, see Niemann and Serkova[16] and Burckhardt and Burckhardt.[17] It has been shown that the region specific differences in metabolite distribution across the kidney can readily be detected through metabolic profiling of kidney tissue samples such as by high resolution magic angle spinning proton nuclear magnetic reso nance (^{1}H NMR) spectroscopy.[16] The following is a brief summary of the regional differences in cell metabolism and metabolite patterns. For a more in depth discussion of renal metabolite distribution patterns relative to kidney metabolomics, see Niemann and Serkova.[16] However, it has to be noted that most information about metabolite distribution in the kidney is based on studies of the rat kidney and that it is not clear to which extent this can be translated to the human kidney.

2.1. Cortex

The cortex is characterized by relatively high expression of mitochondrial oxidative enzymes as well as Krebs' cycle enzymes, while there are relatively

low concentrations of enzymes associated with anaerobic glycolysis such as phospho fructokinase and lactate dehydrogenase. Cortical nephrons contain rather high concentrations of free amino acids, organic acids, choline, glucose and trimethyl amine *N* oxide (TMAO), as well as high concentrations of triglycerides and phospholipids. It has been speculated that this may be related to the significant need for membrane turnover and maintenance generated by the abundance of transporters in the proximal tubules.[16]

2.2. Medulla

The oxygen tension in the inner medulla is significantly lower than in the cortex and in contrast to the cortex that mainly relies on mitochondrial oxidation to cover its energy requirements, cells in the medulla rely on both mitochondrial and glycolytic pathways. Accordingly, glucose, lactate and hydroxybutyrate play a much more important role in the energy metabolism in medulla than in cortex cells. With increasing osmolarity in the inner medulla, the intra cellular concentrations of osmolytes such as betaine, taurine, sorbitol, glycerophosphocholine and myo inositol increase.

2.3. Papilla

The papilla is characterized by a low density of mitochondria and seems to mainly rely on anaerobic energy metabolism which is reflected by the metabolite patterns. In addition, the papilla cells are characterized by high concentrations of osmolytes such as betaine, myo inositol, sorbitol, taurine and glycerophosphocholine.

Due to the aforementioned differences in functionalities and metabo lism, the disturbance of a specific segment of the nephron will lead to characteristic changes in urine metabolite patterns (see also section 7.2). Urinary metabolic profiling therefore presents the possibility of allowing for not only the sensitive detection of disturbances in kidney metabolism, function and extent of injury, but concurrently presents the potential to establish localization of the injury. With that depth of understanding into the process at hand, metabolic profiling offers an insight into the underlying mechanism of injury that we cannot currently generate through any of the non invasive means at use today.

It has been established that the following processes affect urine metab olite patterns: filtration, active secretion and absorption, transport and synthesis of osmolytes, exchange of cell metabolites with urine, oxidative

stress and release of cell contents during injury.[16] With a basic understanding of the processes at work, and the differing metabolic profiles within the regions of the kidney, conclusions can be drawn regarding the location of damage based on the altered levels of metabolites. Thus, an increase of trimethyl amino oxide (TMAO) serves as a marker for medullary injury. Glutaric acid and adipic acid are markers of mitochondrial dysfunction. Glucosuria is a marker of proximal tubular dysfunction. A decrease in citrate, α ketoglutarate and succinate concentrations are rather specific markers for mitochondrial dysfunction in the proximal tubule due to the fact that only proximal tubulus cells possess the ability to compensate for inhibition of their mitochondrial Krebs' cycle by importing Krebs' cycle intermediates from the urine via the sodium dicarboxylate symporter NaDC3.[17] Increased concentrations of dimethyl amine, sorbitol and myo inositol are indicators of papillary damage.[16]

3. NON-TARGETED AND TARGETED METABOLOMICS

The goal of a non targeted assay is to capture as much information as possible. Since the goal is the non biased detection of unknowns, these are semi quantitative at best and are minimally, if at all, validated. In contrast to non targeted assays, targeted assays measure one or several well defined compounds, are validated and are quantitative. Although the quality of the results is much better understood, these assays are limited in terms of their ability to detect unknown effects and are only used when the target of a drug or disease process is at least partially understood. If scarce previous information is available, non targeted assay based discovery strategy is usually a potent first step. Thus, when considered broadly, non targeted assays are hypothesis generating strategies whose results will require follow up with more targeted approaches. The main problem of targeted assays is the possibility of a false negative result. The potential that an effect is missed because it could not be captured by the limited amount of metabolites or proteins included in the assay is a significant concern. In contrast, the major problem with non targeted assays is the false positive. Due to the large number of analytes detected in relationship to the number of samples, signals may be picked up that are random and have no relationship to a disease or drug effect. Hence, there is value in combining targeted and non targeted assays. In such a way, the targeted assays are employed to test a hypothesis and the non targeted approach is utilized to ensure that no important information is missed.

Metabolic fingerprinting describes the unbiased analysis of the metabolome by examination of metabolite patterns in different experimental groups with the subsequent classification of these patterns into a 'fingerprint'.[18,19] Samples can be classified if the metabolite fingerprints differ between groups allowing for sample clustering. In most cases, ^{1}H NMR spectroscopy and mass spectrometry based assays are used for metabolic fingerprinting. In ^{1}H NMR based assays, the chemical shift and area under the peak in mass spectrometry based assays, the mass to charge ratios (m/z) and the signal intensities are used to describe a specific fingerprint. If separation steps such as gas chromatography or high performance liquid chromatography (HPLC) are used to separate compounds before detection, retention times provide additional information for indexing metabolites. Fingerprinting methods benefit from added resolution such as 2D NMR,[20] two dimensional gas chromatography mass spectrometry (2D GC MS),[21] ultra high performance liquid chromatography (UPLC), 2D HPLC and high resolution mass spectrometry.[22,23] The fingerprint of the analyzed sample is then exported for sample classification using multivariate analysis.[24,25] Fingerprinting is solely based on pattern analysis and comparison, the metabolites underlying the signals or peaks are not further identified. Therefore, at this stage not much mechanistic information is gained and the resulting molecular markers cannot be validated or qualified. In order to generate the mechanistic data desired, it is necessary to first pursue statistical analysis and identification of the differences between samples from control and treatment groups or healthy controls and disease groups. Once these data have been established, the metabolites of interest should be identified by database search or further structural identification using analytical technologies such as homo and heteronuclear 2D NMR. A representative workflow is shown in Figure 3.2.

In comparison to completely non biased fingerprinting strategies, semi targeted technologies screen for a multitude of key compounds in specific metabolic pathways such as amino acids, fatty acids, phospholipids, high energy phosphates and NO synthesis pathway. Several assays can be used to screen for changes in known compounds across a range of biochemical pathways. These 'multiplexing' assays typically capture 5–50 compounds and are quantitative or at least semi quantitative. Although these assays allow for much lower throughput than straightforward fingerprinting, this type of semi targeted discovery strategy can give a rather complete picture. Despite the fact that the analytical work load and effort is higher than for fingerprinting approaches, a semi targeted strategy avoids some of the analytical

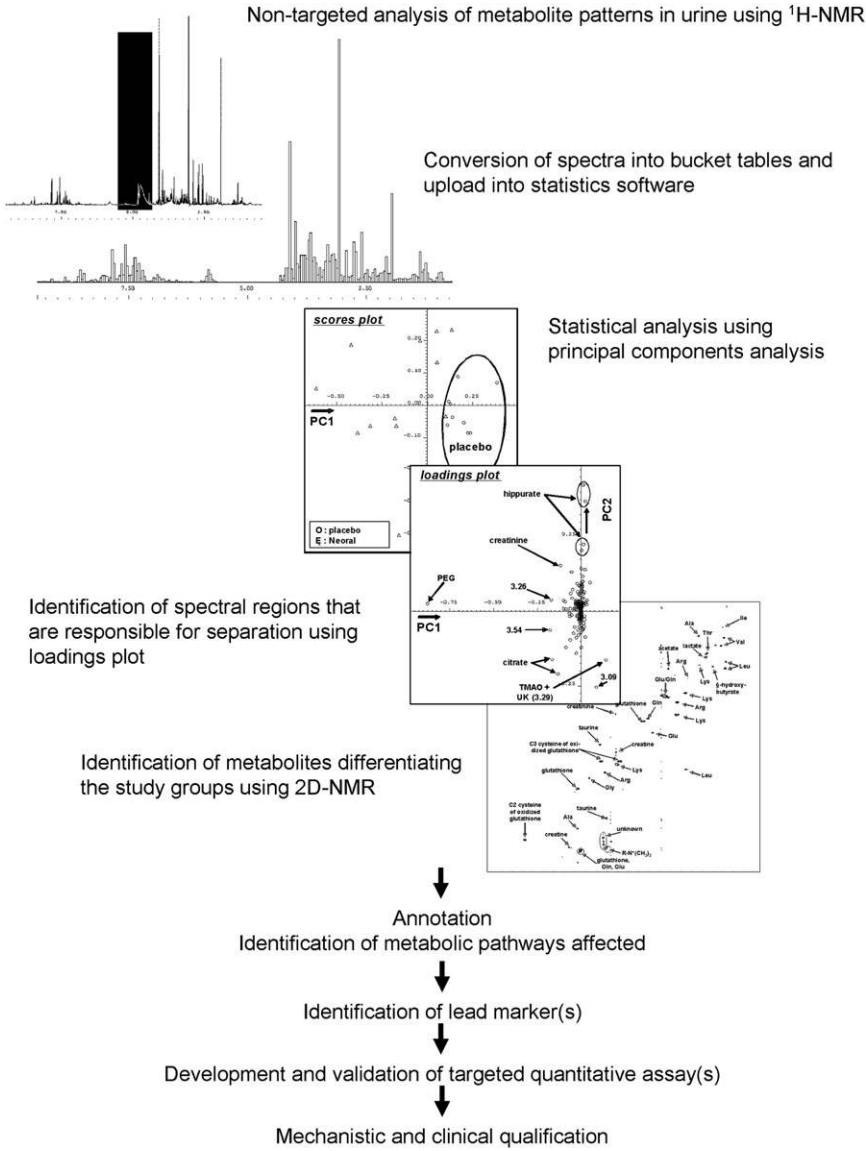

Figure 3.2 ***Representative flow of metabolite marker discovery and development.*** The workflow of a non-targeted metabolome analysis as used in a cross-over, two-period clinical study to compare the effect of a single oral 5 mg/kg ciclosporin dose (Neoral, Novartis, Basel, Switzerland) to placebo (Neoral formulation without ciclosporin) on the kidney in 13 healthy individuals is shown.[26] Metabolome profiling started with the acquisition of a set of ^{1}H-NMR spectra in urine. The spectra were then reduced to histograms ('binning') which represent the area under the curve in a certain spectral region. This created an ensemble of XY-tables (spectral region versus integral), the

and statistical uncertainties associated with completely non targeted data sets. Specifically, semi targeted assays generate higher quality data, are quantitative, and are able to minimize interference and false positives. Another advantage of the semi quantitative approach is that it provides the ability to assess drug or disease effects within a broader range of already known compounds. Thus within one or several assays first mechanistic information will already be available.

It is important to note that at the moment 'global' or 'non biased' analysis of all metabolites is only a theoretical concept.[27] In reality, the available analytical technologies allow for only partial analysis depending on their biophysical principles and the chemico physical properties of the analytes, which reach from strong ions to extremely hydrophilic compounds. Although non targeted metabolomics assays are usually also considered non biased, it has to be kept in mind that the use of different analytical methods will introduce bias simply because of the chemico physical properties of the different compounds, a potentially wide range in concentrations and differences in stability of the analytes. For example, if a GC MS assay is used for metabolic profiling, only compounds will be detected that can be derivatized, will go into the gas phase and can suffi ciently be ionized.^{1}H NMR is not a very sensitive technology; metabolite concentrations are often the limiting factor. The different methods used for non targeted metabolomics are compared in Table 3.2. In most cases, the combination of different metabolomics technologies results in some overlap but will also give significant additional information.

After the molecular marker(s) of interest has (have) been identified, the next step is to establish targeted and validated assays that are capable of quantifying these specific compounds with acceptable total imprecision and

so-called bucket tables. The spectra were analyzed using a principal components analysis (PCA) and partial least squares fit analyzis (PLS) (AMIX software, Bruker, Rheinstetten, Germany). In the PCA, the principal components are constructed in such a way that the first explains most of the variance in the ensemble, the second explains the second most, and so on. The clustering analysis of the scores plots, the PC_1 versus the PC_2, was used to determine if groups of spectra differed from each other. Thus, hidden phenomena that were not obvious from the usual spectral dimension could be discovered. The spectral regions that caused the separation were identified in the loading plots, which form the link back to the spectral dimension. The compounds under the signals that were responsible for the separation of the effects of drug and placebo were identified using 2D-NMR.

Table 3.2 Comparison of technologies used for non-targeted metabolic profiling. See also Xu et al[12]

	Number of metabolites	Sensitivity	Quantitation	Sample prep.	Metabolite ID	Comments
GC MS	+	+++	++	+	+++	Requirement for derivatization excludes compounds that do not react, sample preparation with derivatization can be extensive, low throughput with run times typically between 20 and 60 min, large databases for metabolite identification based on fragmentation patterns such as the NIST database are available
CE MS	++	++	++	+	+	Relatively extensive sample preparation, limited software and databases, rather low throughput
LC MS/TOF LC orbitrap MS LC FT MS LC QTRAP	+++	+++	+	+++	+	Very sensitive, detects the most metabolites (> 1000), ion suppression in the electrospray source limits quantitation, poor separation and resolution of peaks as well as relatively poor reproducibility, limited body of software and databases, sample preparation can be automated, low throughput with HPLC runtimes typically between 20 and 60 min, shorter when UPLC is used

MALDI MS	+++	+++	+	+++	+	Ion suppression limits quantitation, little sample preparation required, can be used for metabolite mapping or imaging of tissue slices, rather high throughput
Infusion nanospray high resolution MS	+++	+++	++	++	+	Infusion in combination with nanospray sources mostly eliminates the ion suppression problems observed with high flow electrospray sources, more extensive sample preparation required than for HPLC or UPLC MS assays, low throughput with 10 40 min infusion times
Sample effusion and atmospheric sample introduction, EESI MS, DESI MS, DART MS	+++	+++	+	+	+	In general less quantitative and suffer from ion suppression in complex biological samples, virtually no sample preparation required, relatively high throughput
NMR	+	+	+++	++	+++	Quantitative, non destructive, low sensitivity, very robust technology, good metabolite capabilities identification using 2D NMR and databases

CE-MS, capillary electrophoresis–mass spectrometry; DART–MS, direct analysis in real time–mass spectrometry; DESI–MS, desorption electrospray atmospheric ionization–mass spectrometry; EESI-MS, extractive electrospray ionization–mass spectrometry; FT-MS, Fourier transformation–mass spectrometry; GC-MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MALDI-MS, matrix-assisted laser desorption ionization–mass spectrometry; MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; prep., preparation; QTOF, quadrupole-time-of-flight; QTRAP, quadrupole linear ion trap mass spectrometry; TOF, time-of-flight mass spectrometry; UPLC, ultra-performance liquid chromatography.

sensitivity. In many cases targeted quantitative assays have been described in the literature or are even established in clinical routine laboratories.

4. THE SAMPLE

In general, metabolomics studies utilize biofluids, cells or tissues.[28] Cells and tissues can be extracted before analysis, but since NMR spectroscopy is a non destructive technology, they can also be perfused and thereby preserved inside an NMR magnet. This is an attractive approach for the study of the time dependency of effects following exposure to a challenge, since metabolic changes can be assessed continuously and in real time. The perfusion of intact organs or tissue slices is also referred to as 'ex vivo' experiments.

4.1. Tissues

Tissues can be samples from animal experiments or patient biopsies. The challenge with the collection of tissues is that as soon as the sample is collected, secondary to the hypoxia incurred as a result of the collection process, there are almost instantaneous metabolic changes occurring. These alterations in metabolism make it difficult to exclude 'after the fact' changes and artifacts from the primary process of interest. To that end, effort has been made to find methods that will immediately arrest metabolic processes during sample collection. This is referred to as quenching.[27] Common approaches are freeze clamping with lower temperature receptacles, immediate freezing in liquid nitrogen and acidic protein precipitation with perchloric or nitric acid. If the latter method is used, stability of the compounds of interest has to be assured.

4.2. Biofluids

A major limitation of genomics approaches is that in most cases clinical diagnostics based on gene chips or arrays will require a biopsy, while phenotypic molecular markers such as metabolites and proteins can be monitored in body fluids. In nephrology, urine is an attractive matrix, since it can be considered a proximal matrix that can be collected non invasively. In contrast to measuring molecular markers in blood, plasma or serum that reflect changes in the systemic compartment, a 'proximal' fluid is defined as a biofluid closer to, or in direct contact with, the site of disease or drug effect.[29] Proximal fluids are local sinks for metabolites, proteins or peptides secreted, shed or leaked from diseased tissue, while once in the systemic

circulation, these get quickly diluted and eventually mixed with metabolites, proteins and peptides from other sources that may complicate location of an injury.

First void urine or spot urine samples are used commonly for metabonomic analyses.[30] Based on NMR profiling of morning and afternoon urine samples, it was found that the effect of diurnal variation on healthy human urine samples is insignificant and that potential differences may rather be caused by diet.[31] First void urine samples are preferred compared to spot urine samples because the influence of lifestyle factors such as diet, physical exertion and stress on the metabolic urinary profiles is relatively minimal in the case of first void urine.[32] However, collection of first void urine samples may be more challenging due to poor patient compliance.[30]

Sample integrity is defined as stability of the analyte(s) in the biological matrix throughout variable environments spanning from sample collection, storage, shipping and further storage up to the last sample analysis.[33] The analytical results and the conclusions drawn from the results can only be valid if the sample that reaches the laboratory is of sufficient quality (the so called 'garbage in, garbage out' principle). In most cases, the typical quality control measures taken during analysis will not catch samples of poor quality. Especially, the time period from sample collection until the sample reaches the analytical laboratory is often poorly controlled and validated. Thus, method development and validation for molecular markers will have to start with the moment the samples are collected and will have to take into account sampling devices and tubes.[25,34,35] Considerations regarding sample tubes should include potential interferences due to compounds leaking from the tubes[36,37] and blood coagulants. EDTA is usually the best choice when it comes to the prevention of clotting of the matrix during long term storage; however, EDTA has the potential to interfere with ^{1}H NMR analyses. Standard measures employed for the stabilization of biofluids for metabolomics analysis span a broad range of methods, including the following: maintaining samples on ice, flash freezing in liquid nitrogen, addition of sodium azide to avoid contamination by bacterial metabolism, addition of antioxidants for markers that are prone to concentration changes due to autoxidation, to immediate extraction. As a general rule, biofluids for metabolomics analysis should always be stored at −80°C in sterile glass or plastic containers with 0.02% azide added as bacteriocide.[23] Blood, serum and plasma samples stored for 1 year and urine samples stored for 9 months under such conditions have been shown to maintain stability.[38]

Metabolic profiling is also possible in vivo using magnetic resonance imaging spectroscopy. In animal models radiofrequency coils have successfully been used to study kidney metabolism.[39,40]

5. ANALYTICAL TECHNOLOGIES

5.1. NMR spectroscopy

NMR based chemical shift imaging technologies have extensively been used for monitoring metabolic changes in vitro, ex vivo and non invasively in vivo. NMR is non destructive, highly discriminatory and can quantify compounds in rather crude samples without the requirement for extensive sample clean up. Sample preparation and the set up of NMR experiments are described in detail in Beckonert et al.[38] Urine samples can be used without further sample preparation; however, strong pH variations between urine samples can lead to signal shifts.[38] Blood, plasma and serum samples will require extraction since otherwise broad macromolecule peaks will interfere with the signals of low molecular weight molecules.[41] Different deproteinization methods have been compared. Acetonitrile precipitation at physiological pH was found to result in the resolution and detection of the most low molecular weight molecule signals.[41] Single metabolites often give several signals in the spectra. Water signals are a problem and have to be suppressed.[42] Typically NMR spectroscopy allows for the simultaneous quantification of 20–50 metabolites.[27] Sensitivity is a limiting factor and often metabolite concentrations in the range of 1–10 μmol/L are required for detection and quantification by NMR. High field NMR spectroscopy[43] and cryoprobes can improve sensitivity with detection limits in the nmol/L range.[44] Still, in comparison to mass spectrometry based methods that are several orders of magnitude more sensitive, rather large sample volumes or numbers of cells (often $>$ 3 million) are required.[27] The sensitivity depends on the natural abundance of the nucleus studied (^{1}H, ^{31}P or ^{13}C) and the potential concentration of isotopes that cell culture, animal or human has been exposed to. ^{13}C NMR provides a greater spectral range in comparison to ^{1}H NMR (200 ppm versus 15 ppm) but the low natural abundance of ^{13}C of 1.1% limits its sensitivity.[43] Most studies have been based on recording one dimensional ^{1}H NMR spectra, but the resulting spectra are usually complex with many overlapping peaks. This can be improved by separation of the hydrophilic and hydrophobic components in a sample by dual step extraction before NMR analysis[45] as well as by using NMR pulse sequences[20] such as J resolved, homonuclear correlated spectroscopy

(COSY), $^{1}H-^{1}H$ total correlated spectroscopy (TOCSY), ^{1}H ^{13}C hetero nuclear single quantum correlation (HSQC), nuclear Overhauser effect spectroscopy (NOESY) and flip angle adjustable 1D NOESY (FLIPSY).[46] 2D J resolved spectra are attractive since they simplify the spectra due to increased resolution in comparison to 1D spectra and metabolites can be quantified even if they are present in concentration 10–100 fold lower than the major components. However, one drawback is that the integrals of the method are strongly influenced by T_2 relaxation during the long T_1 evolution period and hence only relative quantification of metabolites is possible.[47] Magic angle spinning allows for analysis of intact tissue in a non destructive manner. The sample is spun by a rotor at 3–6 kHz at an angle of 54.7° relative to the magnetic field resulting in high resolution, liquid like NMR spectra.[47] This technology can be used for the analysis of tissue biopsies and thus can directly be compared to histopathological findings using the same tissue sample.[48]

5.2. Mass spectrometry

GC MS is still considered the gold standard in metabolite detection and quantification.[27] It is also an established clinical technology to detect inborn metabolic errors in newborns.[48] GC MS has good sensitivity, peak reso lution, reproducibility and robustness.[30] Depending on the analyte, detec tion limits are typically in the picomolar and nanomolar range. The drawbacks are: the rather long run times (usually between 20 and 60 min), the requirement for extensive sample preparation including derivatization and the limitation to volatile compounds. It has also been observed that derivatization may cause artifacts; for example, silylation can convert argi nine into ornithine.[30] While the number of metabolites that can be iden tified in one GC MS run is usually between 100 and 300, deconvoluting software can increase this number to 1000.[27] A representative GC MS ion chromatogram of a urine sample from a healthy volunteer is shown in Figure 3.3. Another strategy to increase the number of metabolites that can be differentiated in a sample is the use of GC/GC TOF mass spectrom etry.[50] In addition to the differentiation of more metabolites, approximately 1200, spectral purity is better than using 1D GC MS. This improves spectral deconvolution and the reliability of peak identification.[51]

Compared with GC/MS, a major advantage of LC atmospheric pressure ionization MS is that samples usually do not require derivatization and that metabolites with a larger range of physico chemical properties can be

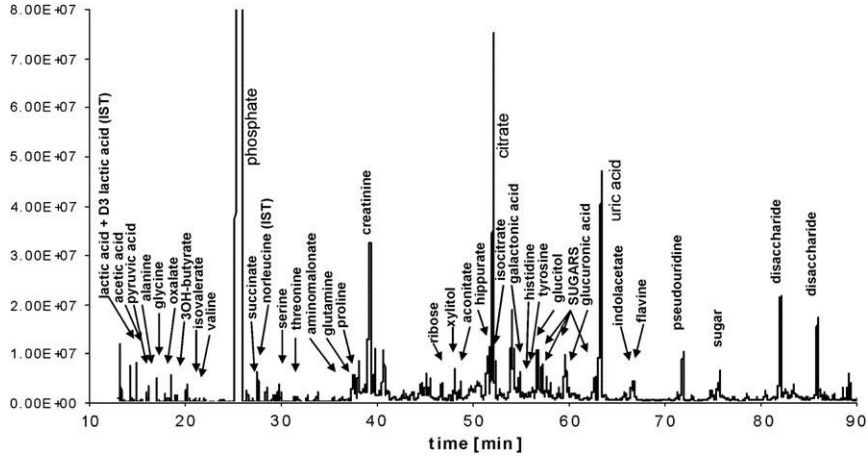

Figure 3.3 ***Representative GC/MS ion chromatogram in human urine from a healthy subject.*** More than 100 compounds can be identified. Only the major peak assignments are shown. The method used was a modification of the assay described by Shoemaker and Elliot.[49]

detected. LC MS assays that are able to detect more than 2000 metabolites in one run have been described.[52] The number and type of metabolites depends not only on the extraction procedure but also on the ionization technology used. It has been suggested that true global metabolomics requires multiple ionization technologies to address the inherent metabolite diversity and therefore the complexity in and of metabolomics studies.[53] A major draw back of mass spectrometry ionization technologies is that they require ionization in a fluid or matrix; ion suppression and/or ion enhancement that may be caused by the interaction of multiple analytes are in the ionization source at the same time.[54] This means that the mass spectrometry signal of an analyte is not only dependent on the concentration of the analyte itself, but potentially also dependent on the concentration and physico chemical properties of other compounds that are ionized simultaneously.

Direct injection mass spectrometry (DIMS) that may involve direct injection using an HPLC system without column separation or direct infusion of the sample into the ionization source using a syringe pump and detection of the different metabolites solely based on their mass to charge ratio is an attractive concept since it allows for high sample throughput.[24,35] In most cases, positive electrospray ionization has been used and, obviously, this strategy benefits greatly from the use of high resolution mass spec trometers.[24] However, ion suppression is a major problem that is almost impossible to control. At the moment nanospray ionization seems the most

viable direct injection mass spectrometry strategy for high complexity samples. Nano electrospray ionization liquid chromatography is performed at flow rates of approximately 200 nL/min. This produces small sub micron sized droplets requiring less evaporation and a greater ability to focus the resulting ions into the analyzer increasing sensitivity and ultimately offering a greater dynamic range.[55] Nano electrospray ionization also reduces the risk of ion suppression commonly associated with electrospray ionization. Infusion chips have successfully been coupled to nanospray electrospray sources for metabolomic profiling in highly diluted samples.[56]

As an alternative to chromatographic separation, sample effusion and atmospheric sample introduction methods have recently become available.[47] These include extractive electrospray ionization (EESI) MS, desorption electrospray atmospheric ionization (DESI) MS and direct analysis in real time (DART) MS.[47] A commonality that these methods share is that they use very little or no sample preparation, but all suffer from the limited ability to quantify metabolites due to ion suppression when complex biological matrices are analyzed. In brief, EESI MS uses two colliding spray sources for ionization and introduction into the mass spectrometer, DESI MS involves a charged and nebulized solvent directed towards the sample and DART MS uses a stream of excited meta stable helium gas and hot nitrogen to ionize the analytes. Due to matrix suppression issues for low molecular weight mole cules, matrix assisted laser desorption/ionization (MALDI) applications have been limited.[55] Desorption ionization on porous silicon (DIOS) seems to have more potential for metabolomics studies since it allows for the detection of small molecules in both positive and negative mode with little background interference.[55]

The most frequently used detectors for LC MS based metabolomics are time of flight, linear ion traps and ultra high resolution detectors such as Fourier transformation mass spectrometers and orbitraps.[55] The linear time of flight (TOF) mass analyzer is the simplest mass analyzer, with virtually unlimited mass range, whereas the TOF reflectron has mass range up to mass/charge ratios of approximately 10,000. TOF instruments offer high resolution, fast scanning capabilities (milliseconds), and accuracy of the order of 3–5 parts per million (ppm). Combination of the TOF analyzer with a quadrupole (QTOF) allows for fragmentation of a metabolite, thus rendering additional information. The ion traps allow for the isolation of a specific ion species and all others are ejected from the trap. The isolated ions can subsequently be further fragmented (MS^n). However, a limitation is that the ratio between precursor mass to charge ratio and the lowest

trapped fragment ion is ~0.3 (the 'one third rule'). Further limitations of 3D ion traps are their inability to perform high sensitivity triple quadrupole type precursor ion scanning and neutral loss scanning experiments.[55] The dynamic range is also limited due to space charge effects when too many ions are in the trap, which diminish the performance of the ion trap. Linear ion traps have advantages over the 3D trap. A larger analyzer volume results in a greater dynamic range and an improved range of quantitative analysis.

Fourier transformation mass spectrometry and orbitraps offer high resolution in the range of 1 ppm or below. This is usually sufficient to allow for specific structural identification of larger molecules based on the exact molecular mass alone. In addition, hybrid instruments such as ion trap Fourier transformation and ion trap orbitrap mass spectrometers are available.

5.3. Other technologies for metabolic profiling

Other technologies that are used for metabolomics are Raman and infrared spectroscopy.[35]

It has been shown that the analysis of the same sample by a combination of multiple technologies such as GC MS and NMR and LC MS will result in a far more complete picture than each of these technologies used alone.[57] The number of 'shared' compounds identified by one method versus another is often less than 50% and indeed may be as low as 20%.[27]

5.4. Chemometrics and databases

Chemometrics is defined as the application of mathematical and statistical methods to chemistry.[58] Chemometric or non quantitative metabolomics does not require the initial identification of compounds. It is solely based on spectral patterns and intensities. Chemometric analyses are necessary in order to develop statistical pattern recognition models, achieve optimal characterization of the samples and detect biomarkers from diverse, highly dimensional omics datasets.[59] The spectra are statistically compared, clus tered, and/or correlated and used to make diagnoses, identify phenotypes and/or to draw conclusions.[25] Common approaches to analyze metab olomics data sets are summarized in Table 3.3.

In quantitative metabolomics, metabolites are identified before statistical analysis is carried out.

Databases are important tools for metabolite identification.[14] The Human Metabolome Data Base is the metabolomic equivalent of GenBank. It is

Table 3.3 Analysis of metabolomic datasets

Quality control and quality assurance	This may include the acceptance and rejection of data or whole datasets based on predefined acceptance criteria. Data are also checked completeness, integrity and correctness and queries are resolved. Once a 'clean' database exists, the database is locked and data can be analyzed. Conclusions drawn from data can only be as good as the quality of that data. This is especially important if data are generated by multiple laboratories and entered at different sites
Data processing	This may include normalization of data data transforms, normalization based on internal standards, baseline corrections, peak alignment, background reduction, missing value corrections, deconvolution of peaks, data binning and data scaling to emphasize smaller concentration metabolites
Data reduction	This may include limiting data analysis to a specific region of interest, removal of data of poor quality, removal of data that are outside the analytical limits or that cannot consistently be replicated, and exclusion of outliers
Unsupervized data analysis	This may include principal component analysis, multiple component analysis, independent component analysis and their subtypes, hierachical cluster analysis, non linear mapping, k means clustering and self organizing maps
Supervized data analysis	In contrast to unsupervized methods, supervized methods will require a training dataset or require that the classes of the samples are already known. Examples of such methods include Fisher discriminant analysis, soft independent modeling of class analogy (SIMCA), artificial and polynominal neuronal networks, partial least square discriminate analysis and support vector machines
Quantification	NMR is inherently quantitative. For GC MS and LC MS assays a calibration strategy is required
Statistical comparison	This may include univariate and multivariate statistics, correlation and regression analysis, analysis of variance (ANOVA) or multiple analyses of variance (MANOVA), and calculation of coefficients of variance. In general, statistical comparison is used in combination with quantitative metabolomics

(*Continued*)

Table 3.3 Analysis of metabolomic datasets—cont'd

Annotation	The results are put into context with existing knowledge about molecular interaction networks such as metabolic pathways and signaling pathways. The metabolite and/or protein changes that are indicated by non targeted discovery technologies may be complex and may present surrogate markers of complex molecular interactions. Current manual curation processes will take far too long to complete the annotations of even just the most important model organisms, and they will never be sufficient for completing the annotation of all currently available metabolome, proteome and genome interactions.[60] Computational strategies are required that include molecular pathway and network analysis tools,[61,62] computational systems biology approaches[63] as well as knowledge based systems that combine reading, reasoning and reporting methods to facilitate analysis of experimental data such as the Hanalyzer software.[64] Software suites such as KEGGarray are capable of integrating data from transcriptomics, proteomics and metabolomics studies[62]

Depending on the nature of the data and the goal of the study some or all of these steps are required. For a more detailed review, see Wishart.[25]

web accessible, provides reference to NMR and mass spectra, metabolite disease associations, metabolic pathway data and reference to metabolite concentrations for hundreds of human metabolites from several biofluids.[25,65]

There are two basic principles in mass spectrometry that allow for the structural identification of a molecule: fragmentation pattern and exact molecular mass. Traditionally, due to the early use of GC MS and the lack of high resolution mass spectrometers, mass spectrometry libraries have iden tified molecules based on low resolution mass, fragmentation patterns and retention times.

5.4.1. GC-MS

Automated analysis software and extensive databases such as the National Institute of Standards and Technology (NIST) database and the Automated Mass Spectral Deconvolution and Identification System (AMDIS) are available.[27,66] The 2008 version of the NIST contains 220,460 GC MS spectra of 192,108 unique compounds. Although these numbers seem extensive, this database contains only a relatively small amount of endoge nous compounds.[30] AMDIS provides deconvolution, quality matching

using advanced spectral matching algorithms, adjacent peak deconvolution and background subtraction, as well as retention index comparison.[66] For more details about other deconvolution software packages and databases used for analysis of GC MS spectra, see Kind et al.[67]

5.4.2. LC-MS

Although atmospheric pressure ionization MS/MS techniques have been used for many years, research libraries of production mass spectra have only reluctantly been created since the mass spectral patterns are less reproducible than GC MS electron impact ionization (EI) mass spectra among instruments from different manufacturers.[67] The fragmentation patterns depend on a large number of factors, many of which are not properly understood, such as ion source designs, ion source potentials, fragmentation gases and mobile phase effects. In the meantime, several atmospheric pressure ionization MS libraries such as the MetLin database have been compiled and successfully been utilized.[55] The use of HPLC retention parameters is complicated by the variety of column stationary phases available and the infinite number of mobile phase combinations which can be used to provide suitable separations. HPLC retention times may also be influenced by column age and column load. Despite such difficulties, retention parameters have been included in LC MS databases and this area has importance in metabolic profiling by LC/MS. The quality of metabolite annotation increases and the false discovery rate decreases with the resolution of the mass spectra and the quality of the database.[25] It has also been determined that today's databases are not capable of comprehensively retrieving all known metabolites.[67] For more details regarding metabolite databases, see Wishart[25,68,69] and Griffiths and Wang.[14]

5.5. Normalization of urine data

Urine presents a very attractive matrix for the metabolomics based studies as discussed previously; however, a variety of difficulties must be overcome in order to gain meaningful information regarding urinary metabolite markers. One initial difficulty that must be surmounted is normalizing urinary samples for differences in dilution. It has been common to normalize samples based on the creatinine concentrations in urine samples.[70] The assumption that creatinine is an acceptable surrogate marker for dilutional leveling may be correct so long as creatinine clearance is normal. However, the urine creatinine concentration is a function of glomerular filtration,

tubular excretion, gender and age and may be affected by creatinine release from other sources such as muscle. Adjustment of urinary molecular marker concentrations based on urinary creatinine in patients with disease processes or drug effects that affect release and handling of creatinine by the kidney will give misleading results.[70] It was shown that the normalization factor has a great impact on statistical and quantitative results.[71] In ^{1}H NMR based metabolomics studies, scaling based on the overall integral represents the standard approach.[72 74] However, integral normalization may not always be the best strategy for metabonomic studies. Especially strong metabonomic changes, evident as massive amounts of single metabolites in samples, significantly hamper the integral normalization resulting in incorrectly scaled spectra. Normalization based on urinary cystatin C concentrations[70] has been proposed for clinical samples. In the case of ^{1}H NMR based metabolite profiles, probabilistic quotient normalization was found to work best.[72] Probabilistic quotient normalization involves calculation of almost probable dilution factors by looking at the distribution of the quotients of the amplitudes of a test spectrum and comparison with a reference spec trum. Zhang et al[74] showed that peak picked and logarithm transformed ^{1}H NMR spectra are preferred. Signal processing and statistical analysis steps seemed to be independent. While variance stabilizing transformation worked best in conjunction with principal component analysis, constant normalization seemed more appropriate for analysis using *t* test. Overall, given the fact that this is a critical issue with significant impact on the results, there is surprisingly little consensus or even discussion. It seems that choice of the appropriate normalization procedures is dependent on context, analytical technology and statistical algorithms.[74]

5.6. Validation of analytical assays, quality control and standardization

The successful translation of a molecular marker into a viable clinical diagnostic test requires the availability of a robust, precise and sensitive assay that is simple, can be automated and is reasonably high throughput.[33]

The validation of analytical assays for the quantification of metabolic markers will have to follow regulatory and other accepted guidances.[75 77] Assay validation typically includes:

- Determination of the lower limit of detection, of the lower limit of quantitation, of the range of reliable response, of the intra and inter day accuracy and imprecision, of the absolute recovery, of dilution integrity.

- Carryover, matrix interferences and ion suppression/enhancement (for LC MS assays) should be excluded.
- The following stabilities need to be established: storage stability, freeze thaw stability, bench top stability, autosampler stability (processed sample stability) and stock solution stability.

Method validation should demonstrate that a particular assay is 'reliable for the intended application' and, thus, the rigor of method depends on the purpose.[78] While a discovery assay used for metabolic fingerprinting can only partially be validated, at least stability of the samples and reproducibility should be ensured. A quantitative assay used as a clinical diagnostic test will require rigorous validation in compliance with applicable regulatory guidances.

Validation of more complex metabolic marker assays can be challenging. In most cases, metabolic markers are endogenous compounds so that an appropriate blank matrix for the preparation of calibrators and quality control samples may not be available. Solutions may include charcoal stripping, the use of corresponding matrices from other species or artificial surrogate matrices. In the case that blank matrices are not available, samples from healthy individuals or animals, depending on the species relevant for molecular marker testing, preferably with low concentrations of the compound of interest, have to be enriched with the reference compounds and the endogenous signal has later to be subtracted.

To compare data across different experiments and among different laboratories, standard reporting structures for metabolomics data are being developed.[79,80] This also extends to sample handling and labeling.[81] The comparison of results among different analytical laboratories and datasets has shown that metabolic profiling using ^{1}H NMR spectroscopy is surprisingly robust[38,82,83] and that most variability could be assigned to sample handling rather than ^{1}H NMR analysis.

6. METABOLIC MOLECULAR MARKER DISCOVERY AND DEVELOPMENT

The goal of a molecular marker development is to take the marker from discovery to a status where it becomes an accepted clinical diagnostic tool and/or outcomes marker for clinical drug development. The key steps are shown in Figure 3.4. To reach this goal, a molecular marker will ultimately require regulatory review and approval before it can be used for the intended purpose. Regulatory approval will require validation and qualification.

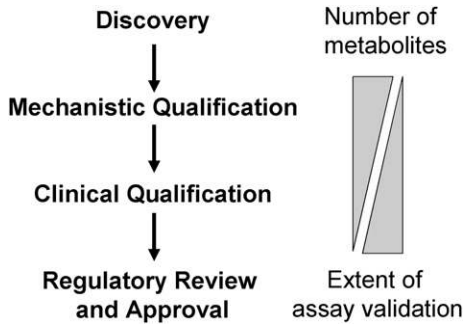

Figure 3.4 ***Development of molecular markers into diagnostic tools for patient management and drug development.*** As of today, most metabolite markers described in the literature have been observed and been suggested based on discovery studies. Rarely, the next steps of molecular and clinical qualification have been taken.

Qualification and validation have sometimes been used interchangeably in the literature, but in a regulatory sense are two different concepts. While validation focuses on the reliability and performance characteristics of the analytical assay used to measure molecular markers,[84,85] qualification has been defined as "a graded, fit for purpose evidentiary process linking a biomarker with biology and clinical endpoints".[78] As this definition indi cates, there are two key aspects to the qualification of a molecular marker:

1. To mechanistically link the molecular marker to the biochemical process underlying a disease or drug effect.
2. To establish a link between the molecular marker and clinical outcomes.

The most important first step of a molecular marker qualification is a clear understanding of what the molecular marker will be used for in a scientific, preclinical, clinical and regulatory context. This will have a critical impact on the extent and depth of the required work. There are three basic strategies that can be used for establishing a mechanistic link between the molecular marker and the biochemical process underlying a disease or drug effect:

1. Leverage of pre existing knowledge.
2. Biostatistical strategies.
3. Experiments identifying the underlying molecular mechanisms leading to changes in the molecular marker.

In most cases, a thorough literature analysis and/or data mining approach will already provide substantial information. The next step is a gap analysis that provides the basis for a qualification plan and then will map out which further in vitro and in vivo studies will be required. Experiments supporting

a mechanistic qualification strategy may include, but are not limited to, the assessment of dose dependency, of time dependency, of recovery, gene knock outs, knock downs and gene silencing. Among the three mechanistic qualification strategies, solely relying on biostatistical evaluations such as algorithms that are available in several current biomarker discovery software packages is the weakest. It has to be kept in mind that most biostatistical methods establish associations and correlations, which may suggest but rarely prove cause–effect relationships. Establishing cause–effect relationships between a drug or disease effect and a molecular marker is the core purpose of a robust mechanistic qualification strategy. Even if a molecular marker is discovered within clinical trials, this does not by any means establish that this marker is clinically relevant. Ultimately, proper qualification of such a marker will generally require a bed to benchtop approach. Often only cell and animal studies allow for such a systematic in depth mechanistic evaluation. Patient populations are often far too complex with many confounding factors to elucidate cause–effect relationships and, if drug effects are studied, well controlled molecular marker qualification studies in healthy volunteers as translational proof of concept can be invaluable.

The next part of a molecular marker qualification is to show that a molecular marker is linked to and/or is a valid predictor of a disease process or drug effect in humans. In addition to sensitivity and specificity, a rigorous clinical qualification should also include the assessment of time and dose dependency. The extent and rigor of these studies will depend on the goal of the molecular marker qualification.[86] Will a molecular marker be used as a clinical diagnostic tool or to support regulatory claims, studies have to go beyond just proof of concept in terms of statistical power considerations, documentation, monitoring and regulatory compliance. Receiver operating characteristic (ROC) curves for the definition of sensitivity and specificity[87] are basic metrics to assess molecular marker performance.[88] In general, area under the ROC curves (AUC ROC) ≤ 0.5 are considered not useful and indicate that the molecular marker cannot discriminate between treatment or disease and the control group. While in the ROC analyses of preclinical animal studies, histology is often used as the gold standard endpoint; established outcome parameters are used in clinical trials. Nevertheless, it is important for the quality of the ROC analysis that the reference outcome parameters are precise and non biased.

In the United States, the use of molecular marker data in regulatory review and decisions is currently based on the FDA guidance "Providing

Clinical Evidence of Effectiveness for Human Drug and Biological Prod ucts".[89] Appropriately qualified, molecular markers can support primary outcomes in a number of different ways; they may help to understand and monitor: mechanisms of toxicity, drug–drug interactions, disease–drug interactions and the effects of genotypes, gender and age. Molecular markers can also be used to stratify patient populations, guide subgroup analyses in such a manner as to bridge safety and efficacy data between different populations. This becomes increasingly important when drugs are consid ered for use in pediatric populations, where it is more difficult to obtain the appropriate clinical trials. As such, the utilization of molecular markers with known correlations between adult and pediatric populations could provide an added measure of security when making this bridge. Molecular markers that progress to the stage where they can be considered appropriate for clinical drug development will require regulatory review and approval.[84,86,88,90]

In many countries, in order for in vitro clinical diagnostic tests and devices to enter the market, these tests or devices must comply with a set of rules and regulations and regulatory review and approval is required.[29]

7. METABOLOMICS IN RENAL RESEARCH AND AS KIDNEY FUNCTION, DISEASE AND INJURY MARKER

As aforementioned, one of the challenges in nephrology today is that there is a limited set of established clinical diagnostic markers that are not very specific, are rather insensitive and detect a disease process or negative drug effect at a later stage when the injury often cannot be fully reversed.[91]

Historically, molecular markers have been established empirically, sometimes throughout years and decades of use in clinical practice and in drug development. Incremental numbers of publications during their period of utilization have established their validity as well as their limitations. Due to the absence of clear rules and guidances, the qualification of a biomarker was mostly accepted based on consensus among clinician/scientists and between clinician/scientists and regulatory agencies. It is reasonable to assume that many of today's established clinical markers would not meet the acceptance criteria and standards in terms of qualification, sensitivity and specificity that are required by regulatory agencies and scientific consensus today.

Metabolomics holds the promise to serve as a potent tool to discover and develop new diagnostic strategies and to develop into a specific and sensitive diagnostic tool itself. In addition, metabolomic strategies can help to better

understand the molecular mechanisms of disease processes and drug toxicities. This knowledge can be leveraged to develop new therapeutic approaches and to develop better and safer drugs.

A good example is a study described by Beger et al[92] assessing the metabolic changes in serial urine samples in 40 children undergoing cardiopulmonary bypass. Twenty one of these children developed acute kidney injury (AKI) defined as an increase of creatinine concentrations in serum 50% or greater from baseline after 48–72 h. The urine metabolite patterns were analyzed using UPLC time of flight mass spectrometry in the negative ionization mode. The urine metabolomes of children developing AKI were distinct and further analysis showed that the dopamine metabolite homovanillic acid sulfate was a major molecular marker indicating AKI in this patient population. Using a cutoff value of 24 ng/μL at 12 h after surgery, a sensitivity of 90% and a specificity of 95% was found.

7.1. Identification of disease, pharmacodynamic and toxicodynamic molecular mechanisms

The problem with targeted research approaches to assess molecular mechanisms is that some information must already exist that allows for generation of a hypothesis. Another limitation is that the approach itself will bias the results – one will only find what one is looking for. It is often not possible to completely understand the results in the context of the complex cause–effect relationships, correlations and interactions of the biochemistry and signal transduction pathways of a cell, an organ or an organism. Metabolomics alone, and even more so in combination with proteomics and genomics, are a 'hypothesis generator' that when combined with molecular, cellular and pharmacological techniques provide a framework for understanding molecular mechanisms.[14] These are critical tools for the mechanistic qualification of molecular markers. It also has to be taken into account that in most cases during today's drug development flow, a molecular target is identified and then often molecule libraries are screened to identify suitable molecules that interact with the target. This means that the mechanism of action is known almost from the beginning. However, toxicities are usually detected for the first time during preclinical animal toxicology studies or even later during clinical development. There is significant value to identify the toxicodynamic mechanism for risk assessment, to assess if the toxicodynamic mechanism is linked to the pharmacodynamic target and/or to identify molecular marker strategies for toxicodynamic monitoring during the clinical phases of drug development.

The identification of the unknown molecular mechanism will have to incorporate a non biased, non targeted screening strategy to generate a hypothesis that will guide subsequent targeted studies and/or to ensure that no important unexpected effects are overlooked.

A powerful metabolomics tool to identify unknown molecular mechanisms is the assessment of fluxes in the metabolic network of a cell, organ or organism.[93] This strategy is also termed 'fluxomics'.[93] It provides a true dynamic picture of the phenotype since it captures the metabolome in its functional interactions with the environment and the genome and provides a link. Although several methods for flux quantification are available, the most reliable strategies are still based on isotope labeled precursors of metabolic pathways, mostly using ^{13}C labeled substrates.[93] Depending on the metabolic pathway, the ^{13}C atoms of the precursor are incorporated into the newly formed downstream metabolites in distinct numbers and specific positions. Each metabolite may have several isotope isomers, this means molecules of the same metabolite with distinct labeling states, so called isotopomers. Isotopomer distribution is accessed by metabolomics platform strategies, most importantly ^{13}C NMR, GC MS, isotope ratio mass spectrometers and high resolution mass spectrometers. The analysis of fluxes and the effects of disease and drugs on these using tracer based metabolomic data requires a priori knowledge of the possible distribution of a tracer within the metabolic network.[94,95] But there are challenges, the size of the studied metabolic network should be restricted, otherwise too many alternative formation pathways will confound data interpretation. As of today, most fluxome analyses have focused on the central carbon metabolism. The number of usable labeled substrates is limited. Substrates that are formed by several alternative pathways may potentially dilute and confuse the analysis. The most widely used substrates are 1 ^{13}C, 1,2 ^{13}C and uniformly labeled U ^{13}C glucose.[94] Software packages for the calculation and interpretation of fluxes such as ^{13}C FLUX have been developed.[95]

The following two examples illustrate how metabolomics can be used to gain further insights into disease mechanisms. To assess the downstream molecular mechanisms in three genetic types of renal Fanconi's syndrome, namely Dent's disease, Loewe's syndrome and autosomal dominant idiopathic forms, urine samples from patients with Fanconi's syndrome, from healthy volunteers and from patients with tubular proteinuria caused by ifosfamide treatment were compared using a combined proteomics and metabolomics approach.[96] Like the protein patterns, cluster analysis clustered Loewe's and Dent's metabonomes together, whereas the autosomal

dominant idiopathic forms and urines from ifosfamide treated patients clustered together. The differences in the urine metabolomes were mainly due to different amino acid patterns (increased concentrations of basic and neutral, but not of branched amino acids in the case of Loewe's and Dent's disease) and differences in *N* methyl nicotinic acid, suggesting the involvement of cation transporters in the proximal tubule.[96] Tayor et al[97] used a GC time of flight mass spectrometry based metabolomics approach to partially qualify a juvenile mouse polycystic kidney disease model. Before there was serological evidence of kidney dysfunction, there were already marked changes in the urine metabolome. Functional score analysis and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database suggested significant early changes in the purine and galactose metabolism pathways. The study also revealed several candidate molecular markers in urine, most notably allantoic acid and adenosine.[97]

7.2. Nephrotoxicity and drug development

Metabolomics strategies to assess drug toxicity have been developed as early as in the 1980s with a focus mainly on hepato and nephrotoxicity. Since then a large knowledgebase has been developed that is beyond the scope of this chapter. For more comprehensive reviews, see Niemann and Serkova,[16] Coen et al,[18] Wishart[98] and Shockcor and Holmes.[99] Metab olomic approaches are useful to:[98]

- identify the target organ or region of toxicity;
- identify the biochemical mechanism contributing to toxicity;
- identify molecular marker profiles of nephrotoxicity in plasma and urine;
- monitor the time course of nephrotoxicity, its dose dependency and its recovery.

These efforts cumulated in the Consortium for Metabonomic Toxicology (COMET), a consortium of five major pharmaceutical companies and the Imperial College of London. The goal of the COMET study was to build expert systems and predictive models of target organ toxicity based upon ^{1}H NMR spectra mainly based on renal and hepatic toxins. COMET led to the creation of a database of 35,000 NMR spectra with conventional histopathology data on mice and rats for 147 model toxins.[18,28,59,98,100] Based on the COMET database, Ebbels et al[100] conducted an analysis including 12,935 NMR spectra from 1652 rats that had received 80 different treatments to build a modeling system for toxicity prediction.

Where predictions could be made, there was an error rate of 8%. The sensitivities to liver and kidney toxicity were 67% and 41%, respectively, whereas the corresponding specificities were 77% and 100%. In some cases, it was not possible to make predictions because of interference by drug related metabolite signals (18%), an inconsistent histopathological or urinary response (11%), genuine class overlap (8%), or lack of similarity to any other treatment (2%). This study constituted the largest validation of the metabonomic approach to preclinical nephrotoxicity and in vivo drug toxicity screening and confirmed earlier observations that the pattern changes of urinary metabolites can be used with good sensitivity and specificity to identify the nephrotoxic compounds. One of the foci of the ongoing COMET2 study is the better understanding of renal papillary necrosis such as caused by non steroidal anti inflammatory drugs and the study will also include clinical samples.[18]

A recent extensive study in which rats were dosed with the nephrotoxins gentamicin, cisplatin or tobramycin in combination with non targeted combined GC MS and LC MS based metabolomics analysis showed that increases in urinary concentrations of polyamines and amino acids could be detected already after the first dose before any histopathological changes occurred.[101] Upon prolonged exposure, a progressive loss of amino acids in urine with a concomitant decrease of amino acid and nucleoside concentrations in the kidney tissue were observed. A nephrotoxicity prediction model based on urinary concentrations of branched amino acids distinguished nephrotoxin treated samples from vehicle controls with 70%, 93% and 100% accuracy after 1, 5 and 28 days of treatment, respectively.[101]

Metabolomics can also determine the site of nephrotoxicity (cortex or medulla).[102–105] The following metabolite signatures in urine have been associated with injury to specific regions of the kidney:[98]

- Proximal straight tubules (via D serine): increase of lactate, phenylalanine, tryptophan, tyrosine and valin.
- Proximal convolute tubules (via gentamicin): increase of glucose; reduction of trimethylamine *N* oxide, xanthurenic acid and kynurenic acid.
- Cortical injury (via mercuric chloride): increased glucose, alanine, valine, lactate and hippurate and decreased citrate, succinate and oxoglutarate.
- Papilla and medulla (via bromoethanamide): increase of glutaric acid, creatine and adipic acid; reduction of citrate, succinate, oxoglutarate and trimethylamine *N* oxide.

The changes of urine metabolite patterns found in several key nephrotoxicity studies in the rat are summarized in Table 3.4. The table interestingly indicates that under the experimental conditions used in these studies, to a large extent the changes in urine metabolite patterns caused by these toxins or drugs is rather determined by the region of injury than by specific drug effects. It is also important to observe that results differed among studies using the same toxins. This may be explained by the use of different instrumentation, analytical strategies, different doses, time of sample collection relative to drug administration, length of treatment, the rat strains studied, diet and the environment at the study site.

Although an attractive concept and although there is promising feasibility data, there are many obstacles that have prevented this technology from becoming a widely accepted drug development tool in the industry and for regulatory submissions.

7.3. Kidney transplantation

Although current immunosuppressive protocols have dramatically decreased and nearly eradicated acute rejection episodes after kidney transplantation, there has been only minimal progress in long term graft survival after kidney transplantation over the last two decades.[119]

While calcineurin inhibitors undoubtedly prolong graft survival, chronic immunosuppressant mediated nephrotoxicity is a significant concern. In cases of established nephrotoxicity, it has been commonly believed that the use of non nephrotoxic immunosuppressants such as mycophenolic acid or the proliferation signal inhibitors (sirolimus and everolimus) allows for the reduction or even discontinuation of calcineurin inhibitors; so called 'calcineurin inhibitor free' immunosuppressant long term maintenance regimens. However, in the effort to prevent calcineurin induced nephrotoxicity, many studies detailing attempts to minimize or wean patients from these medications have shown that improvement in renal function is often obtained at the cost of an increase in the incidence of rejection. Other factors that limit the success and long term outcomes of kidney transplantation are the quality of the transplant kidney, cold storage time and ischemia/reperfusion injury and infections such as CMV and BK virus.

As of today, serum creatinine concentrations are routinely used as a clinical marker for monitoring function of kidney allografts.[91] Once an elevation in serum creatinine concentrations is detected, a biopsy is then

Table 3.4 Effects of selected nephrotoxin on metabolite patterns in the rat

Toxin	Location of injury (histology)	Crea	Citr	Succ	2Oxo	Gluc	Hipp	TMAO	Tau	AA	Acet	Lac	Reference
Adriamycin	Glomerulus	↑	↑↓		↑↓				↑				106
Puromycin aminonucleoside	Glomerulus and proximal tubule	↑	↓		↓	↑		↑	↑	↑	↑		99
Sodium chromate	S1 proximal tubule		↓		↓	↑	↓						106
D serine*	S1 proximal tubule	↓								↑		↑	107
Gentamicin†	S1/S2 proximal tubule	← →	↓	↓	↓	↑	↓	↓		↑	↑	↑	108
DCVHC	S2/S3 proximal tubule	↓	↓	↓	↓	↑	↓			↑	↑		106
DCVC	S2/S3 proximal tubule		↑	↑									106
Hexachlorobutadiene	S3 proximal tubule	↓	↓	↓	↓	↑	↓			↑	↑		106
Mercuric chloride	S3 proximal tubule	↑	↓	↓	↓	↑	↓	↓	↑	↑	↑	↑	109
Mercuric chloride‡	S3 proximal tubule		↓	↑	↓	↑	↑	↓		↑	↑	↑	110
Mercuric chloride	S3 proximal tubule	↓	↓	↑	↑	↑	↑↓				↑		111
Para aminophenol	S3 proximal tubule	↓	↓	↓	↓	↑	↓			↑	↑		99
TCTFP	S3 proximal tubule	↓	↓	↓	↓	↑	↓			↑	↑		106
Uranyl nitrate	S3 proximal tubule	↓	↓	↓	↓	↑	↓			↑	↑		112
Ciclosporin‡¶	S3 proximal tubule	↓	↓	← →	← →	↑	↑↓				↑		113
Ochratoxin‡	S3 proximal tubule	↑	↓		↑↓	↑		← →		↑		↑	114

Cisplatin	S3 proximal tubule				↑		↑		↓					115
Ciclosporin[‡¶]	Proximal tubule and medulla			↑			↑		↓			↑	↑	↑	116
Gentamicin[‡**]	Proximal tubule and medulla		↑		↑				↓						117
Gentamicin[‡††]	Proximal tubule and medulla		↑				↑		↓					↑	117
Doxorubicin	Medulla	↑	↓	↓	↓		↑	↓	↑		↑	↑	↑		118
Thioacetamide	Medulla	↓	↓	↓	↓		↑	↓	↑		↑	↑	↑	↑	106
2 Bromoethanamine	Medulla/papilla		↓	↓	↓			↓	↓		↑	↑	↑	↑	119
2 Bromoethanamine	Medulla/papilla			↑↓					↑↓						106
2 Chloroethanamine	Medulla/papilla			↑↓					↑↓						99

If not mentioned otherwise, metabolite patterns were assessed using ^{1}H-NMR spectroscopy-based metabolomics. Due to the large number of metabolites that can be captured with metabolomics technologies, only a selection of major metabolites can be shown here. For more details, please see the original references. Also the list of studies referenced and toxins that have been tested cannot be considered complete.

Crea, creatinine; Citr, citrate; Succ, succinate; 2Oxo, 2-oxoglutarate; Gluc, D-glucose; Hipp, hippurate; Tau, taurine; AA, amino acids; Acet, acetate; Lac, lactate.

[*]LC-MS-based metabolomics.

[†]GC-MS1- and ^{1}H-NMR-based metabolomics.

[‡]LC-MS1- and ^{1}H-NMR-based metabolomics.

[§]GC-MS1, LC-MS1- and ^{1}H-NMR-based metabolomics.

[¶]10 mg/kg over 28 days.

[¶]High dose of 45 mg/kg for 9 days.

[**]On day 3.

[††]On days 8–9.

procured to differentiate between the possible diagnoses. A Banff graded, two core allograft biopsy remains the gold standard with which all novel diagnostic tools must be compared. However, even biopsies will not necessarily allow for conclusive diagnosis of the etiology of the observed histopathological changes with sufficient confidence. Lesions such as interstitial fibrosis and tubular atrophy, as well as glomerular injury, are non specific responses to injury. Antibody mediated endothelial activation, calcineurin inhibitor toxicity, recurrent disease, chronic inflammation, innate immune mechanisms as well as diabetes mellitus and hypertension have all been invoked as potential etiologies. Unfortunately, serum creat inine is not a sensitive biomarker. It has been shown that up to 30% of grafts with stable creatinine may have smoldering rejection and treatment of this chronic/subclinical rejection may result in improved graft func tion.[120] The key to reducing chronic renal allograft dysfunction is early detection. As mentioned above, the most common strategy to reduce the prevalence and severity of renal allograft dysfunction has been minimizing or discontinuing the doses of calcineurin inhibitors during long term maintenance immunosuppression. This is often performed without fore knowledge of which factors are contributing to chronic allograft dysfunction in any individual kidney transplant patient and without guidance by an appropriate diagnostic strategy. Overall, this frequently results in reduction of the immunosuppressive efficacy of the drug regimen and creates a dilemma. As mentioned previously, another major factor contributing to renal allograft dysfunction is the allograft immune response. Treatment to avoid damage by immunological responses requires enhanced immunosuppressive drug regimens. There is currently no non invasive diagnostic tool available that allows for differentiating between renal allo graft dysfunction due to alloimmune response or immunosuppressant toxicity. The concept of monitoring biochemical changes and of detecting disease processes and immunosuppressant toxicity before significant histo logical or pathophysiological damage occurs and while said process is still potentially reversible, is attractive. Metabolomics can be applied towards:[7,121]

- assessment of transplant kidney quality before and during cold storage;
- monitoring ischemia/reperfusion injury;
- toxicodynamic drug monitoring of immunosuppressants and individu alization of immunosuppressive and other drug regimens;
- detection of acute and chronic alloimmune reactions.

7.3.1. Organ quality, organ storage and ischemia reperfusion injury of kidney transplants

Injury of a kidney transplant in the donor (in the case of cadaveric donors), during explantation, cold ischemic storage and reperfusion after trans plantation may affect the extent of damage by oxidative stress, inflammation and alloantigen dependent factors, all of which may have a negative influence on outcomes of a kidney transplant. Mostly in animal models, ^{1}H NMR based metabolomics has been used to study the effects of donor treatment, explantation techniques, conditions during cold storage, cold storage times and of ischemia/reperfusion as well as pharmacological prophylaxis against ischemia/reperfusion injury.[122 130]

In rat kidney transplants it was shown that after ischemia/reperfusion in kidney tissues polyunsaturated fatty acids were decreased and allantoin, a known marker of oxidative stress, was increased.[130] At the same time blood concentrations of trimethylamine *N* oxide and allantoin were significantly increased. Interestingly, no statistically significant changes in serum creatine concentrations were found.[130] In a porcine transplant model using ^{1}H NMR based metabolic profiling, it was shown that urine concentrations of citrate, dimethylamine, lactate, and blood concentrations of acetate and trimethyl amine *N* oxide in plasma were indicators of ischemia/reperfusion injury.[130] These findings suggested that graft dysfunction is mainly associated with damage to the renal medulla determined by trimethylamine *N* oxide release in urine and plasma associated with dimethylamine and acetate excretion.[130]

In 20 renal transplant recipients, HPLC was used to measure whole blood and plasma concentrations of adenosine triphosphate, adenosine monophosphate, guanosine, inosine, hypoxanthine, xanthine, uric acid and uridine.[131] Hypoxanthine and xanthine concentrations were increased in the renal allograft vein after reperfusion as compared with peripheral vein during the pre and post reperfusion periods. The results suggested that differences in hypoxanthine and xanthine concentrations between renal and peripheral veins reflect metabolic alterations in renal tissue.[131] Overall, these studies indicate that metabolomics is a valuable tool to study and improve transplant kidney quality, cold ischemia conditions, ischemia/reperfusion injury, to assess the effect of pharmacological prophylaxis and interventions and to study the correlation with outcomes.[121]

7.3.2. Immunosuppressant nephrotoxicity

Although immunosuppressants have made organ transplantation possible, immunosuppressive drug regimens have serious side effects that not only may

damage the transplant kidney but may also limit patient survival. These include, among others, an increased prevalence of cardiovascular disease, diabetes, neurotoxicity, cancer and nephrotoxicity. Nephrotoxicity of immunosuppressants is also a relevant problem for transplant patients who have received organs other than kidneys and it has been shown that in these patients the development of immunosuppressant nephrotoxicity also negatively affects long term outcomes.[132] Pharmacokinetic therapeutic drug monitoring and blood level guided dosing of immunosuppressants is common clinical practice; however, this strategy does not seem to be sufficient to prevent chronic nephrotoxicity.[9] Reasons may include that transplant patients usually receive two and more immunosuppressants and that pharmacokinetic drug monitoring does not take the pharmaco/toxicodynamic interactions among different immunosuppressants into account, does not measure drug–disease interactions and does not reflect individual differences of a patient's tolerability to certain drug regimens. Therefore, the concept of toxicodynamic monitoring of transplant patients seems attractive.[9]

Several studies have focused on the effects of immunosuppressants alone and in combination and on kidney tissue and the metabolite patterns in blood and urine (see also Table 3.4). While most of these studies have been purely descriptive and show the urine metabolite pattern changes typical for primary proximal tubular injury, recently a series of systematic studies has been published that also included first qualification steps.[9,26,112,133,134] After treatment of rats with calcineurin inhibitors and their combination with sirolimus or mycophenolic acid (mycophenolate mofetil) for 28 days, glomerular filtration rates were significantly reduced. The decrease of glomerular filtration rates was associated with significant changes in urine metabolite patterns that correlated with the reduction in glomerular filtration rates. The changes of metabolite patterns in urine were associated with a combination of changes in glomerular filtration, changes in secretion/absorption by tubulus cells and changes in kidney cell metabolism.[112] Based on these results, a combinatorial metabolite marker for monitoring immunosuppressant induced kidney dysfunction in rats treated with calcineurin inhibitors was proposed:[9] markers of glomerular filtration (creatinine), reabsorption (glucose), tubulus cell metabolism (citrate, oxoglutarate, lactate), active secretion and kidney amino acylase activity (hippurate), as well as oxidative stress (isoprostanes), and the release of metabolites protective against the protein precipitating effect of uric acid (trimethylamine *N* oxide). An association between immunosuppressant induced

changes in kidney metabolism and urine metabolite patterns was confirmed by proteomics studies that were conducted to mechanistically explain and qualify the urinary metabolite pattern changes.[134] The changes in expression of several enzymes compared to untreated controls explained several of the changes in metabolite patterns observed in urine. The extent of changes in glomerular filtration rates after 28 days was predicted by the extent of metabolite pattern changes in urine after 6 days, even though glomerular filtration rates at that time were not different from baseline, and histological changes were not detectable.[112] In this study after 6 days of treatment, urine metabolite patterns were similar to those reported for agents causing oxidative damage, while pattern changes after 28 days were typical for agents that cause S3 tubular damage.[112] These results matched the histologies showing specific damage of the proximal tubuli. After 28 days, there was also histological damage to glomeruli. These studies suggested the following mechanism causing the characteristic changes in urine metabolite patterns: calcineurin inhibitors directly and/or indirectly (via endothelial dysfunction) derail mitochondrial oxidation causing oxygen radical formation, inhibition of Krebs' cycle and decline of energy production. The proximal tubule cell tries to compensate by activating anaerobic glycolysis and importing Krebs' cycle intermediates from urine via the NaDC1 and NaDC3 transporters.[112,134] In an open label, placebo controlled, crossover study the time dependent toxicodynamic effects of a single oral ciclosporin dose (5 mg/kg) on the kidney was assessed in 13 healthy individuals.[26] In plasma and urine samples, $15F_{2t}$ isoprostane concentrations using HPLC MS and metabolite profiles using ^{1}H NMR spectroscopy were analyzed. The increase in urinary $15F_{2t}$ isoprostane observed 4 h after administration of ciclosporin indicated an increase in oxidative stress. $15F_{2t}$ isoprostaglandine concentrations were on average 2.9 fold higher after ciclosporin than after placebo. Unsupervised metabolome analysis using principal components analysis and partial least square fit analysis revealed significant changes in urine metabolites typi cally associated with negative effects on proximal tubulus cells. The major metabolites that differed between the 4 h urine samples after ciclosporin and the placebo were citrate, hippurate, lactate, TMAO, creatinine and phenylalanine (Figure 3.2), indicating that analysis of urinary metabolites was a sensitive enough marker for detection of the effects of a single ciclosporin dose already shortly after drug administration and that the results in rats translate into at least healthy humans. Creatinine concentrations in serum remained unchanged.[26] A decrease in citrate

concentrations in urine during treatment with immunosuppressants had also been reported by others.[135]

The results of the study by Klawitter et al[112] also suggested that changes in urine metabolite patterns reflected the negative effects of immunosuppressants on kidneys with better sensitivity and specificity than metabolite changes in blood, although it has been reported that immunosuppressants alone and in combination lead to changes of metabolite patterns in the blood of rats[136] and transplant patients.[137 139]

7.3.3. Allo-immune reactions

Foxall et al studied the changes in urine metabolite spectra early after transplantation.[140] In this study no patient showed clinical or histopathological evidence of ciclosporin nephrotoxicity. Urine samples were collected daily for 14 days from 33 patients who underwent primary renal allograft transplantation, and analyzed by 500 and/or 600 MHz ^{1}H NMR spectroscopy. The NMR spectra of urine from patients with immediate functioning grafts were similar with respect to their patterns of amino acids, organic acids and organic amines, whereas the patients with delayed or non functioning grafts showed significantly different metabolite excretion patterns. In longitudinal studies on individual patients there were increased urinary levels of trimethylamine *N* oxide, dimethylamine, lactate, acetate, succinate, glycine and alanine during episodes of graft dysfunction. However, only the urinary concentration of trimethylamine *N* oxide was statistically significantly higher ($P < 0.025$) in the urine collected from patients during episodes of graft dysfunction (410 $\pm$ 102 μM of trimethylamine *N* oxide/mM creatinine) than in patients with good graft function (91 $\pm$ 18 μM of trimethylamine *N* oxide/mM creatinine) or healthy control subjects (100 $\pm$ 50 μM of trimethylamine *N* oxide/mM creatinine). These findings suggest that early graft dysfunction is associated with damage to the renal medulla which causes the release of TMAO into the urine from the damaged renal medullary cells. Urine and plasma samples from 39 patients who underwent renal transplantation were analyzed by proton nuclear magnetic resonance (NMR) spectroscopy. Le Moyec et al[141] found that the most relevant ^{1}H NMR signals for evaluating renal function after transplantation were those arising from citrate, trimethylamine *N* oxide, alanine and lactate when compared to creatinine. The respective variations of these metabolites in urine were associated with ciclosporin toxicity and rejection. Knoflach and Binswanger[142] reported that hippuric acid concentrations in plasma may be a sensitive and early marker of acute

allograft rejection but also a marker for the response of anti rejection treatment. The classification of urine metabolite spectra from 33 kidney transplant patients with normal histology and from 35 patients with rejection as confirmed by Banff graded protocol biopsies taken shortly after the collection of the urine samples resulted in 96.3% sensitivity and 93.1% specificity when samples were analyzed using ^{1}H NMR spectroscopy and in 96.2% specificity and 88.9% specificity when samples were analyzed with infrared spectroscopy.[143] In a more recent study, the metabolite patterns of 15 mid stream urine samples from patients with improving acute cellular rejection of a kidney allograft and 24 urine samples from eight patients without evidence of rejection were analyzed using MALDI FTMS.[144] Seven molecules with mass/charge ratios between 278 and 424 were identified that differentiated the two sets of urine samples with 100% specificity. However, the molecular structures of these molecules were not further identified.[144]

In summary, although metabolomics seems to be a promising concept to provide reliable indications of transplant kidney function, injury and immunosuppressant toxicity, the development of these into clinical diagnostic tools must be considered still in its early stages.[121,145]

7.4. Cancer

Kidney cancer is the sixth leading cause of cancer deaths and represents 3% of cancer incidence. Renal cell carcinoma is usually diagnosed when already symptomatic and by this time one third of the patients already have metastases. Non invasive, sensitive and specific diagnostic tools will facilitate identifying renal cell carcinoma at an earlier stage. The application of metabolomics in cancer research, diagnosis and treatment in general is summarized in Gowda et al[47] and Serkova et al.[146] In addition to ex vivo metabolomic analysis of body fluids or biopsies using NMR spectroscopy or mass spectrometry, non invasive magnetic resonance imaging/magnetic resonance spectroscopical imaging (MRI/MRSI) and positron emission tomography (PET) have been used to assess the metabolism of tumors in vivo. Metabolomics for the diagnosis of tumor and monitoring treatment is a promising concept since tumor metabolism markedly differs from the metabolism of normal cells. In addition to cancer tissue specific metabolite changes, major differences include the energy metabolism with an upregulation of glycolysis and inhibition of the mitochondrial oxidation and the mitochondrial Krebs' cycle that can be promoted by p53, hypoxia inducible factor 1, c Myc and Akt. Choline metabolism is modulated by

growth factor signaling, cytokines, oncogene activation and chemical carcinogenesis. Phosphocholine and total choline concentrations have been found to be increased in tumor tissues.[146]

7.4.1. Urinary metabolite markers of renal cell carcinoma

Kim et al[147] assessed the utility of urine metabolome profiling to detect renal cell carcinoma. In a clinical study, urine from 50 patients with renal cell carcinoma was collected and compared with urine samples from 13 healthy individuals. The urine metabolites were profiled using hydrophilic interaction chromatography–electrospray–linear iontrap mass spectrometry. Spectra between $m/z = 80$ and 800 in the positive and negative mode were recorded. Data were analyzed using cluster, principal components, differential and variance components analyses. Urine samples of patients with renal cell carcinoma could be differentiated from healthy individuals. Interestingly, it was found that these metabolome differences persisted after the tumor was removed. Although this study provided statistical proof of concept, it can only be considered a first step since the metabolites responsible for the separation were not identified. In a pilot study, Kind et al compared the urine metabolite patterns from six patients with clear cell renal cell carcinoma with those of the urine of six randomly selected healthy individuals using three independent analytical techniques, hydrophilic interaction chromatography (HILIC LC MS), reversed phase ultra performance liquid chromatography (RP UPLC MS) and gas chromatography time of flight mass spectrometry (GC TOF MS).[148] The combination of these techniques covered a large part of the urine metabolome by enabling the detection of both lipophilic and hydrophilic metabolites. The results were analyzed by a feature selection algorithm with subsequent univariate analysis of variance and a multivariate partial least squares approach. From more than 2000 mass spectral features detected in the urine, several significant components were detected that enabled discrimination between urine samples from patients with renal cell carcinoma and controls despite the relatively small sample size. A feature selection process condensed the significant features to less than 30 components in each of the datasets. However, none of these metabolites was identified.[148]

7.4.2. Biochemical classification of renal carcinoma biopsy samples

Renal biopsies have mainly been studied using magic angle spinning ^{1}H NMR spectroscopy. As in the case of the analysis of biofluids and tissue homogenates, sample collection, preparations and metabolic stabilities

during the NMR experiment can cause artifacts and affect spectral quality.[149] In a study by Moka et al,[150] paired biopsy samples from the same kidney, one from a region of the renal cell carcinoma, the other from the unchanged cortex region, were collected and immediately stored at −70°C. Eighty milligrams of the samples were used for magic angle spinning NMR experiments including 1D ^{1}H NMR spectroscopy and 2D J resolved, TOCSY and ^{1}H ^{13}C HMQC experiments. The differences included a significant accumulation of lipids as well as higher concentrations of *N* acetylneuramic acid, of *N* acetyl glucosamine and of various amino acids in the samples from the tumor than in the samples from the unchanged cortex. These results were confirmed by a later study using a similar paired sample study design and magic angle spinning ^{1}H NMR spectroscopy.[151] Unsupervised and supervised statistical procedures distinguished between renal cell carcinoma samples and healthy cortex samples with 100% accuracy. This study included also a sample from a renal metastasis of a primary lung cell carcinoma and a sample of a renal collecting duct tumor. Both samples could clearly be differentiated from the cortical tumors based on the metabolite patterns.[151] A more recent study compared 1D magic angle spinning ^{1}H NMR spectra of clear cell and papillary renal cell carcinomas in comparison to normal renal cortex and papilla tissues, respectively.[152] The spectra of human normal cortex and medulla showed the presence of differently distributed organic osmolytes as markers of a physiological renal condition. As found in the earlier studies, the marked decrease or disappearance of osmolytes and the high lipid content was typical for clear cell renal cell carcinoma tissues, while papillary renal cell carcinoma were characterized by the absence of lipids and very high amounts of taurine.[152]

7.4.3. Monitoring of cancer treatment effects

Metabolomics has extensively been used to assess the biochemical response of tumors during exposure to anticancer drugs.[146,153] Studies have mainly focused on cell and animal models. Very little data about the treatment of renal cancers is available. However, there are several studies assessing the nephrotoxic effects of cancer drugs and the metabolome changes in plasma and urine (see also Table 3.4).

7.5. Urine as matrix for non-renal disease and injury

Since most small molecules can pass the glomerular membrane, at least to a certain extent, systemic metabolite changes in blood are reflected in urine.

Urine metabolite patterns can be affected by the gut microbiolome, diet and other factors[13] (Figure 3.1). Analysis of the urine metabolome has been used to study the effects of diet on human biochemistry[154] and the biochemistry of term and pre term neonates.[155,156] Metabolite changes can be used for monitoring extra renal disease processes and drug effects. The monitoring of inborn metabolic errors is an established clinical procedure that is based on the profiling of urine metabolites.[47] Studies in rats using hepatoxins have shown that it is possible to deconvolute metabolite pattern changes in urine caused by liver and nephrotoxicity.[157,158] The detection of bile acids in urine was found to be an indicator of liver function impairment in the rat,[159] but can also be detected in the urine of liver transplant patients (Figure 3.5). To delineate systemic and kidney specific urine metabolite changes in discovery studies, it seems advisable to also monitor metabolite pattern changes in plasma.

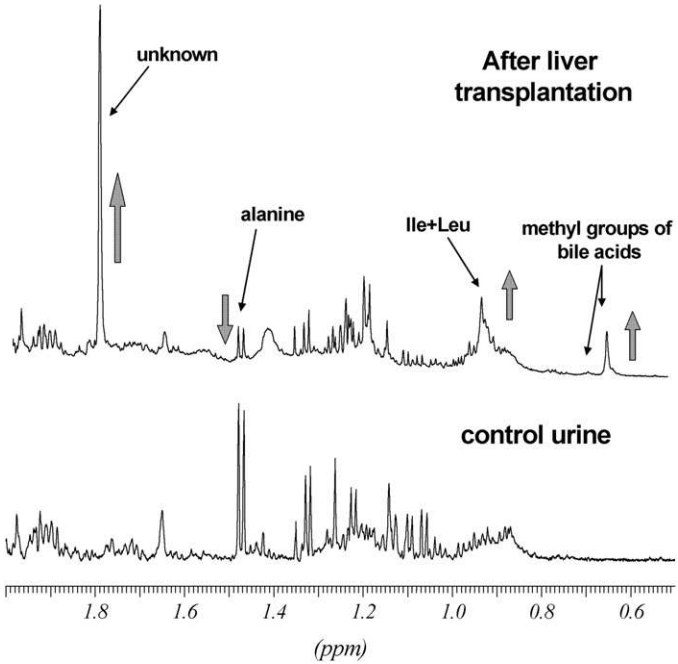

Figure 3.5 ***Representative ^{1}H-NMR spectra of urine of a healthy volunteer compared to urine of a patient who underwent liver transplantation 1 month before urine collection.*** Aliphatic spectral regions are shown. Bile acid urine concentrations are indicators for liver injury.[159] The grey arrows indicate major changes in the urine of liver transplant patients in comparison to healthy subjects.

8. METABOLOMICS AS CLINICAL DIAGNOSTIC TOOL IN NEPHROLOGY

8.1. Why hasn't it worked?

While metabolic marker discovery is relatively easy and fast, the process of actually making them useful is much slower, trickier and requires substantial resources.[160] Molecular markers can be potentially misleading if they correlate with a disease process or drug effect, but there is no cause–effect relationship and they do not reflect mechanistically relevant changes. It has to be considered that many biostatistical methods used for molecular marker discovery are based on correlation analysis, but a significant correlation never establishes a cause–effect relationship. Other typical problems with these procedures and study designs include, but are not limited to:

- Distribution of the data is unknown and not tested. Many procedures assume normal distribution, while biological data are often skewed.
- Due to the complexity of the analyses, studies often have relatively small numbers of observations while hundreds and thousands of parameters are measured and compared. Thus, statistical power is often very low.
- The quality of the data entered into such analyses is often poorly con trolled, if at all. This is especially relevant if data have been collected and analyzed over a period of time and/or by different sites and laboratories.
- Since the metabonome is an open system, differences in diet, exercise and environment need to be taken into account as potential confounders and, if not appropriately controlled, may increase the risk of false positives even further.

Computational and mathematical techniques, such as multivariate analysis or machine learning, can find differences or clusters of differences that distinguish members of one group from the other, at least for a specific sample set. Given enough variables these algorithms may discriminate groups by chance, with often misleading impressive statistical significance.[160]

It is impossible to decide if a molecular marker is a valid surrogate of a disease or drug effect, if the pathophysiological and pathobiochemical links are not understood.[160] The key to establishing this link is mechanistic qualification. The evaluation of molecular mechanisms typically involves animal and cell culture studies. A widely recognized problem is the trans lation of molecular markers from animal models to humans and vice versa. Fortunately, this is less of a problem with metabolites than with proteins or genes. Other than genes and protein, metabolites are often tissue and species independent.[12]

A molecular marker solely based on a metabolic fingerprint without any attempt to understand the underlying molecular changes cannot be qualified and thus will not meet current regulatory standards. Another problem with fingerprinting is that such an assay cannot be validated following current laboratory standards. Therefore, it is reasonable to assume that such a strategy is a powerful discovery tool but, at least for the next few years, will remain clinically irrelevant.

In terms of biomarker discovery, qualification and determination of sensitivity and specificity, it is critical to consider the time dependency of biochemical changes. While the genome is static, the proteome and metabolome are in constant flux.[161] While in the later stages of a kidney injury the biochemical signature often remains unchanged, during the earlier stages, cell and organ biochemistry may change quickly as the injury progresses. This may include compensatory mechanisms, the onset of secondary reactions such as oxygen radical formation and damage, changes in cell function and regulation and the triggering of additional systemic processes such as immune reactions and inflammation. Different stages during the development of a biochemical injury may be characterized by different sets of metabolite markers and thus time dependency and its underlying mechanistic dynamics need to be understood. A good example is the study described by Klawitter et al[112] that showed that ciclosporin caused urine metabolite changes in the rat consistent with oxidative stress of the kidney during the first 6 days, the primary mechanism through which ciclosporin causes nephrotoxicity. But after 28 days of ciclosporin exposure, the urine metabolites had shifted to a pattern typical for S3 tubular damage. Accordingly, the correct timing of sample collection is critical for the success of molecular marker development and the later use of a specific molecular marker in clinical trials and as a clinical diagnostic tool. Although there is tremendous potential in understanding such time dependent metabolic changes and patterns in terms of prediction, early detection and monitoring disease progression, treatment response and organ recovery, this important aspect has often not been taken into account or systematically explored.

Interestingly, most original publications in this field are limited to describing metabolite pattern changes and a fair number of these manu scripts conclude that the discovered metabolite changes may be useful as diagnostic markers. However, with very few exceptions (such as Klawitter et al[112,134]), investigators have followed up on their results and have taken steps towards mechanistic and clinical qualification. As suggested by

Table 3.4, the urinary metabolite pattern changes associated with proximal tubular and medullary kidney damage are fairly consistent. Although similar urinary metabolite pattern changes have been described over and over again, to the best of our knowledge there is no systematic approach to explain why this is the case and to explore the mechanistic reasons for these changes.

Even fundamental issues such as normalization of metabolite concentrations to compensate for differences of dilution in urine samples have not systematically been approached. Even though there is consensus that the use of creatinine concentrations in urine for this purpose can be misleading in certain cases and even though this is a critical and fundamental problem, normalization based on creatinine remains the clinical standard.

Overall and in comparison to urinary protein kidney dysfunction markers, there has been very little systematic effort yet to develop metabolite molecular markers into clinical diagnostic tools. Most of the work has focused on the development of metabolomics strategies for preclinical drug development. This is reflected by the literature with the majority of data published in animal models and still only relatively few publications in humans. The advantages, opportunities and risks of metabolomics as a clinical diagnostic tool or for the development of diagnostic tools are summarized in Table 3.5.

8.2. How will it work?

It has been argued that while transcriptomics and proteomics are important research tools, metabolic profiling will offer the greatest impact on the field of personalized health and as an outcomes parameter[5] (Figure 3.6). One reason is that metabolomics reflects best the interaction between phenotype and environment (Figure 3.1).

Metabolomics allows for a global view of an individual's metabolome and its interactions with the microbiolome, the environment, drugs and disease agents. Profiling the whole metabolome and to extract relevant information using chemometrics has been referred to as a 'top down' approach. This approach is intriguing and in the long term in combination with the development of expert systems possibly the future of medicine. However, today, the amount of information generated by modern non targeted screening technologies is often clinically irrelevant, impractical and can only be meaningful if it assists in drawing clear and valid conclusions. Using truly non targeted screening technologies in clinical decision making is not yet feasible, mostly because of the complexity of the data generated and, as discussed above, the lack of algorithms to convert this

Table 3.5 Strengths, weaknesses, opportunities and threats of metabolomics as a clinical diagnostic tool and as a tool to discover and develop new diagnostic strategies and in drug development[162]

Strengths	Weaknesses
Comprehensive profile of the entire metabolome hypothesis generation and identification of unknown molecular mechanisms	Lack of databases with comprehensive information for metabolite identification
Small molecules in the metabolome are the ultimate manifestation of cellular genomic and proteomic signaling	Lack of software for automated identification and quantitation. Currently available chemometric approaches are still based on many assumptions and the analysis is vulnerable to false positive results
Analytical high throughput screening technologies such as NMR spectroscopy and MS for metabolite measurement are already in place	Minor and potentially toxicologically important metabolites may be overlooked
Development of molecular markers of effect not just exposure	Metabolic cause of toxicity and consequence of damage can be difficult to distinguish
Ability to define the range of normal	Dealing with data where normal encompasses a wide range
Due to the fast response early and time dependent changes can be monitored	Current analytical technologies are probably unable to cover the complete metabolome

Opportunities	Threats
Predictive molecular markers of metabolomic disruption, drug effects and the development of disease states	Will the technology live up to its promise and result in deliverables?
Metabolomic profiles are an open system and can assess and predict interactions between humans and their environment	What to do with the data?
New mechanistic insights from discovery driven research	Technological ability to detect metabolomic changes, but what do changes mean toxicologically?
Use of genetically modified models of disease to understand metabolic mechanisms	What is a 'normal' metabolome for humans?
Use of high throughput systems to rapidly generate vast amounts of data	When is the 'normal' metabolome perturbed in a manner that is toxicologically consequential?
Technology complementary to and integrated with genomics and proteomics (→ systems biology)	How will regulatory agencies use the data?
Personalized 'susceptibility' index	

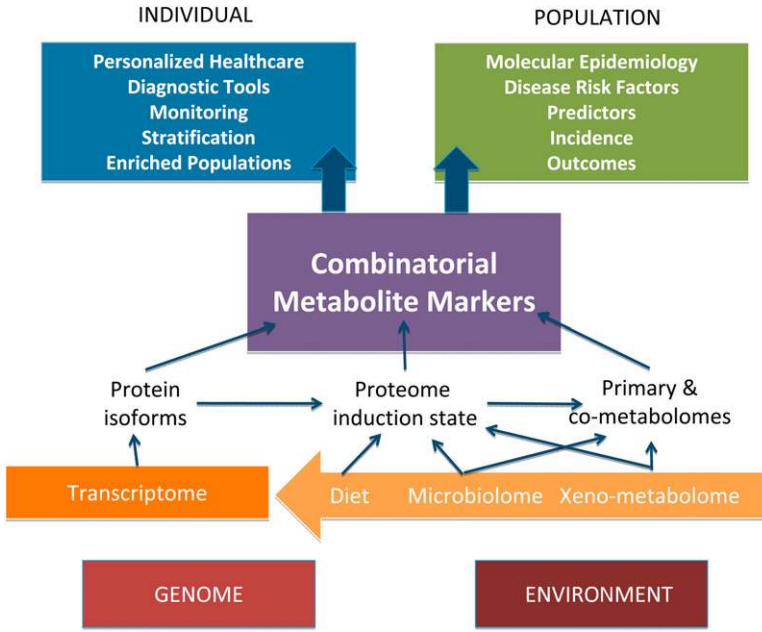

Figure 3.6 ***The role of metabolomics and metabolomics-derived combinatorial metabolite markers for individualized medicine and molecular epidemiology.***

information into robust and meaningful clinical information. Another problem is that most of the hundreds and thousands of data points generated are not relevant to the disease or drug effect. Instead of conveying additional information they only cause random statistical noise including falsely positive results and may mask valid information. However, while non targeted 'omics' technologies are mostly hypothesis generating technolo gies, this information is valuable to develop new targeted diagnostic strategies and tools.[163]

A 'bottom up' approach will start with metabolite markers already established in clinical practice and will look at them not as single markers but will combine them into patterns. New markers that may have been discovered using non targeted metabolomics based discovery may be added. This will result in the development of 'combinatorial biomarkers'. Those are molecular marker patterns that typically consist of 3–10 indi vidual parameters.[164] In general, specific combinatorial biomarker patterns confer significantly more information than a single measurement and, thus, can be expected to have better specificity and sensitivity than clinical chemical and biochemical markers currently used in nephrology. Such

combinatorial markers consist of metabolites that are qualified and will rely on targeted, validated and quantitative assays. As indicated in Table 3.4, as summarized by Niemann and Serkova,[16] Wishart[98] and Shockcor and Holmes,[99] and as discussed above, there is an extensive body of work that results in an almost consistent pattern of urinary metabolite markers indicating injury of specific kidney regions. Several of these studies have also indicated that these metabolite pattern changes precede detectable kidney histology changes and changes of serum creatinine concentrations. As already mentioned, based on rat studies the following combinatorial metabolite marker in urine has been suggested: markers of glomerular filtration (creatinine), reabsorption (glucose), tubulus cell metabolism (citrate, oxoglutarate, lactate), active secretion and kidney amino acylase activity (hippurate), as well as oxidative stress (isoprostanes), and the release of metabolites protective against the protein precipitating effect of uric acid (trimethyl amine *N* oxide).[9] This combinatorial metabolite marker has partially been qualified using a proteo metabonomic approach[134] and it has been shown that it translates into healthy human individuals.[26]

Although the development of urinary protein markers indicating kidney injury seems ahead of the development of metabolite markers,[8,10] there are several advantages to metabolite markers that make them attractive. Many proteins in urine are unstable, samples will require processing at the bedside, and the quality of the sample reaching the analytical laboratory is difficult to control. In contrast, biofluid samples for metabolite analysis are common in clinical practice and usually do not require extensive handling. Another advantage of small molecules is that the development and validation of quantitative analytical assays is rather straightforward. In most cases assays have already been described in the literature or may even already be available in a clinical laboratory.

Based on our current knowledge, combinatorial metabolite markers are an intriguing and very promising concept that will likely lead to markedly improved clinical diagnostic strategies, especially in nephrology, since with urine a 'proximal' matrix is easily available. However, the focus has to shift from finding markers to qualifying their mechanistic and clinical relevance.[160] A full qualification of a metabolite marker is a highly integrated and comprehensive project that requires extensive inter disciplinary expertise, collaborations and resources. Communication tools and infrastructures such as initiatives driven and supported by funding agencies, regulatory agencies and consortia with the pharmaceutical industry will be critical.

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CHAPTER 4

The Role of Proteomics in the Study of Kidney Diseases and in the Development of Diagnostic Tools

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1. INTRODUCTION

Most of the physiological functions within a cell, a tissue, an organ and an organism are mediated by proteins and, hence, proteins are of substantial interest as clinical diagnostic molecular markers.[1] Proteins are the functional output of genes.[2] While the genome is static, the proteome is dynamic, or constantly in flux, and changes in response to external and internal stimuli. Gene sequences and patterns of gene expression are neither complete nor accurate surrogate markers of protein concentrations, their structures or activities.[3] Concentrations and activities of proteins are controlled by processes affecting gene expression such as transcription, mRNA splicing and mRNA stability, processes regulating activity such as protein folding and post translational modifications, allosteric interactions with substrates, products, inhibitors and activators, and processes inactivating proteins through covalent binding or breakdown. In particular, post translational modifications often play a critical role in the regulation of the activity of a protein. After translation, most proteins are modified through the addition of carbohydrates, phosphates, cholesterol synthesis pathway intermediates and other molecules. Post translational modifications are not encoded by genes.

The proteome is defined as the expressed protein and peptide complement of a cell, organ or organism, including all isoforms and post translational variants. While an organism possesses a single genome, it possesses multiple proteomes depending on the cell compartment, type of cell, type of tissue and organ. Proteomes undergo constant temporal changes. Changes can occur within minutes, hours and sometimes days if regulated via translation, but can also occur within seconds at the functional level. This may involve mechanisms such as phosphorylation, substrate and co substrate interactions, allosteric inhibition and activation, reaction with radicals and proteolytic cleavage. The term peptidome has been used for the peptide subset of the proteome.[4]

Proteomics has been defined as the 'systematic analysis of proteins for their identity, quantity and function'.[5] Thus, the term proteomics summarizes the procedures required for analysis of a proteome. While typical protein analysis involves the assessment of an individual protein, proteomics investigates populations of proteins rather than a single protein.[1]

Until the 1990s, enzymatic or chemical evaluation, such as Edman degradation of highly purified proteins, constituted the mainstream methods for the determination of amino acid sequences of polypeptides and proteins. Protein profiling started with the introduction of 2D gel electrophoresis in 1975.[6] However, it was not until the introduction of mass spectrometry, the availability of protein databases, search algorithms and other informatics procedures during the last 20 years that identification of proteins cut from 2D gels became routine.[7] The almost explosive development of modern proteomics technologies over recent years was associated with the completion of the human genome project and the availability of genome sequence databases, the progress in mass spectrometry technologies including the development of 'soft' ionization technologies, such as electrospray and matrix assisted laser desorption ionization, as well as advances in bioinformatics.[8] At present, mass spectrometry in combination with library searches has evolved as the backbone of proteomics and allows for the simultaneous structural identification of multiple proteins in complex mixtures.[9]

Clinical proteomics has focused on the discovery of novel drug targets as well as the discovery of diagnostic and prognostic disease biomarkers.[8] It also aims at providing the clinician with tools to accurately diagnose, monitor and predict treatment effects for patients, thus enabling individualized patient management when properly utilized. The key is that such protein marker based strategies hold the promise of being highly sensitive, specific and predictive, and, overall will outperform the currently established clinical diagnostic tests.

1.1. Why are molecular marker strategies considered predictive?

Most kidney injuries leading to end stage renal disease are characterized by silent and progressive courses and non specific symptoms that, in their early stages, often remain undetected by current clinical diagnostic tools.[10] The quality of diagnostic tools is determined by their sensitivity and specificity. The sensitivity and specificity of chemical and biochemical molecular

markers that are traditionally used in clinical diagnostics, preclinical and clinical drug development is sometimes poor. The reasons include, but are not limited to, the fact that often the following assumptions were made: (A) one marker detects all disease processes/drug effects targeted against a specific organ, and (B) one marker fits all patient populations and age groups. Also, when these more traditional markers were established in the clinic, the mechanisms of diseases or drug effects, in many cases, were not well understood. Molecular markers did not have to undergo the rigorous validation and qualification procedures that are required by current regu latory guidelines today and have, historically, been introduced as diagnostic tools into the clinic based on scientific consensus. A good example of this is creatinine concentrations in serum. Although generally considered a marker of glomerular filtration in the kidney, it is now known that creatinine is also actively secreted in the proximal tubulus and even reabsorbed by the kidney.[11] Serum creatinine is not specific for the kidney but can also increase in the case of muscle damage and it is gender and age dependent. Furthermore, its sensitivity is poor and a rather large amount of glomeruli needs to be destroyed for the serum creatinine to increase by 20%, the value that is considered clinically significant.[12] This can result in a critical delay in therapeutic interventions. This becomes problematic when the disease process or drug toxicity primarily targets other parts of the kidney and glomeruli are only damaged at a later stage by secondary processes, such as inflammation. There has never been, and there will never be, a single molecular marker that is able to adequately assess all aspects of the kidney's function and detect all types of kidney injury with adequate sensitivity. Although these shortcomings of serum creatinine as a molecular marker for kidney injury are well documented, it still remains the primary marker for preclinical and clinical drug development.

Poor sensitivity and specificity relate directly with poor predictive value. To better understand how molecular markers can be more predictive, it is important to look at the stages of kidney injury caused by a disease or a drug. This is illustrated in Figure 4.1. The development of a disease process or drug injury can roughly be divided into three stages: a genetic, biochemical and symptomatic stage.[15]

A genetic predisposition may increase the risk for an individual to develop a disease, modify the efficacy or tolerability of a drug, or influence its tissue distribution and pharmacokinetics; however, in most cases, other factors, such as diseases, drugs, nutritional status and/or environmental factors, will also be required to trigger a pathological biochemical process.

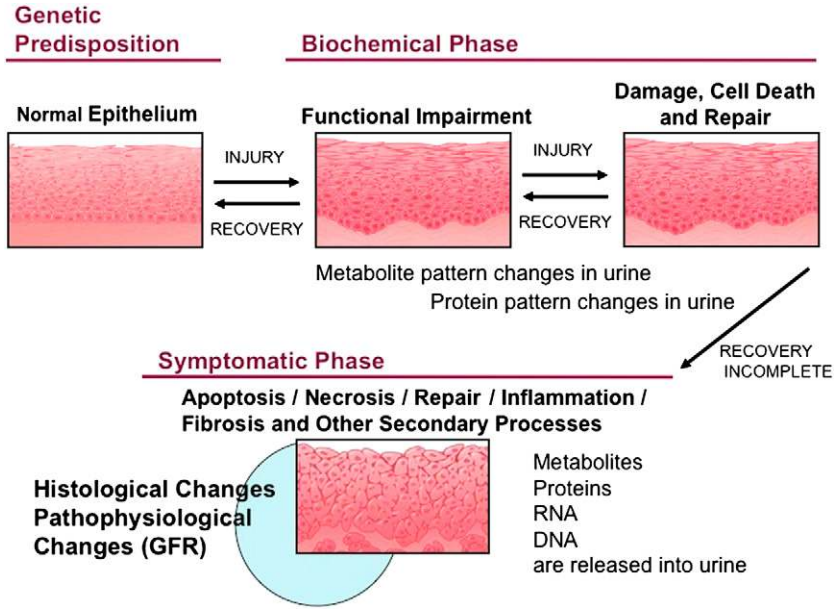

Figure 4.1 ***Time-dependency of kidney tubular epithelium injury and molecular markers in urine.***[13,14] Injury will affect cell function before histological and pathophysiological damage can be detected. At an early point in the process, this is reflected in protein and metabolite patterns in urine, as absorption and excretion are altered, repair proteins are formed and cells release proteins into urine. The resulting extent of urine metabolite and protein pattern changes depends on the intensity of the injury and how many cells/tubules are affected. Proteins that have been found to be changed in urine and that may serve as early kidney injury markers are listed in Table 4.5 and are shown in Figure 4.4. As increasing numbers of cells die by necrosis and/or apoptosis, the biochemical phase of injury will progress towards the symptomatic phase. These cells will release at least some of their contents, such as metabolites, proteins, RNA and DNA, into the urine. Cell death will also trigger secondary reactions such as inflammation and fibrosis. Once this occurs, a complete recovery may no longer be possible. The injury results in histological changes and kidney function will be reduced. It is not until the symptomatic phase that currently established diagnostic markers such as serum creatinine concentrations and blood urea nitrogen will significantly change.

During the biochemical stage, changes in gene expression, protein expression and biochemical profiles occur, but the cells and organs are still able to compensate for this. At this stage, an injury process should be detectable if sufficiently sensitive assays are available. During the biochemical phase, no notable histological damage has occurred, and the disease process may be fully reversible if an appropriate therapeutic intervention is available.

In the symptomatic stage, biochemical changes on a cellular, organ or systemic level can no longer be compensated for. This leads to pathophysiological and histological changes that define the symptoms of the injury process. Most established outcome metrics used presently during preclinical and clinical drug development detect injury processes in their symptomatic stage. The concept of monitoring biochemical changes and detecting an injury process before detectable histological or pathophysiological damage occurs is attractive. If the cause–effect relationships between protein expression, biochemical changes, the symptoms of a disease and a drug effect or toxicity are known, then detecting specific changes in protein and cell biochemistry patterns has the potential to predict development of the symptomatic injury.

Technologies such as genomics/transcriptomics, proteomics and biochemical profiling (metabolomics) have the potential for the development of molecular marker strategies that allow for monitoring early changes in cell signal transduction, regulation and biochemistry with high sensitivity and specificity and, therefore, can detect an injury process at a much earlier stage than currently established clinical diagnostic markers.

2. NON-TARGETED AND TARGETED PROTEOMICS

2.1. Non-targeted

Non targeted proteomics approaches try to evaluate a whole proteome. This concept embraces the acknowledgment that the complexity of protein networks and their interactions can only be assessed and fully understood if information covering all aspects of a biological system is available. By definition, targeted approaches that evaluate only one or several specific pathways are biased and may miss critical information. Non targeted approaches, however, are non biased and seek to capture as much information as possible. Nevertheless, given the large numbers and varying abundance of different proteins in biological samples, as of today there is no single experimental approach that enables the visualization of a complete proteome.[2] The three basic pillars (Figure 4.2) of mass spectrometry based non targeted proteomics are:[16]

- the front end fractionation of complex mixtures;
- mass spectral data acquisition; and
- protein identification and characterization by database searching.

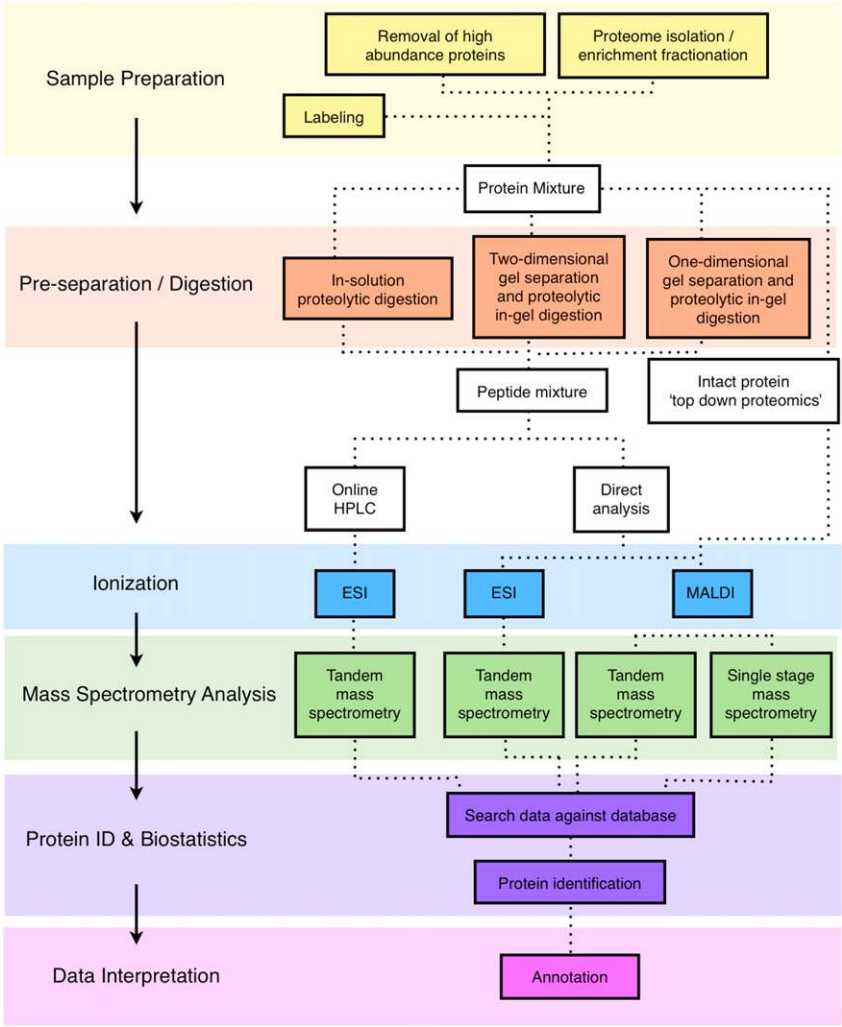

Figure 4.2 ***Proteomics sample analysis.***[17] Proteomics analysis is a multiple step procedure that typically involves sample preparation, pre-separation and/or digestion, ionization, mass spectrometry analysis, protein identification, biostatistics and annotation. Proteomics strategies can be divided into 'bottom up' and 'top down' approaches. Bottom up approaches are most frequently used and involve digestion of the proteins of interest and, after mass spectrometry analysis, identification of proteins using database searching based on the detected peptides. Top down proteomics does not involve a digestion step and analyses the intact proteins. As discussed, both strategies have their advantages and limitations.

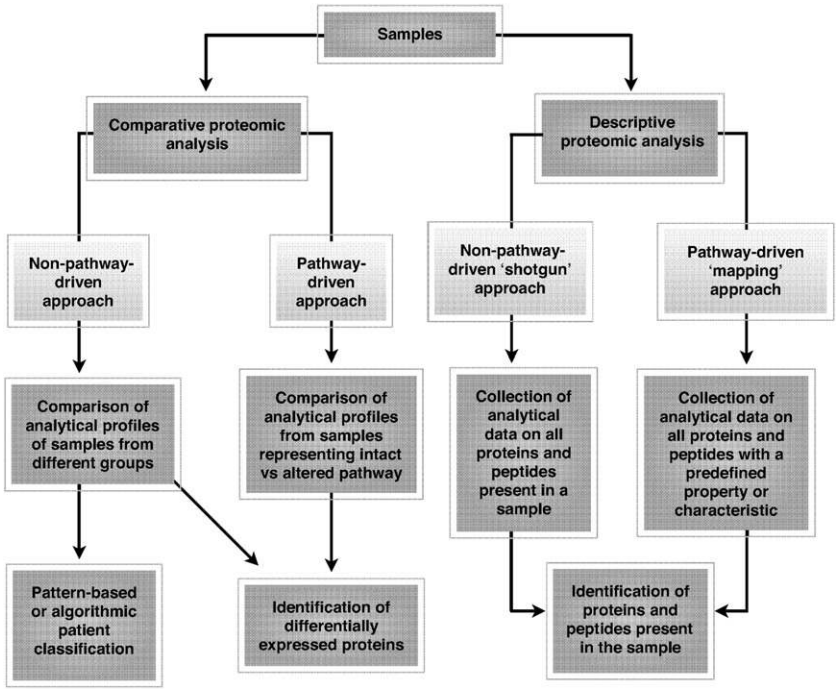

Figure 4.3 ***Main proteomics strategies.***[2] The goal of comparative approaches is to detect differences between samples and, therefore, requires semi-quantitative comparison. Descriptive studies are usually qualitative and provide information about which proteins are present in a defined sample. In either approach, study designs can be pathway- or non-pathway-driven. Pathway-driven studies are targeted — they focus on selected specific pathways, a protein interaction network or a specific sub-population of proteins. Some previous knowledge or a hypothesis is required. By contrast, no prior biological knowledge is used in the design of non-pathway-driven or non-targeted studies. Global analysis is undertaken (although steps are usually taken to reduce sample complexity) and the data generated can be regarded as hypothesis-generating. Most clinical protein marker discovery studies have been non-targeted and comparative, and they identify proteins differing between study groups. Often such studies do not produce protein identities, but generate algorithms to classify samples on the basis of protein separation profiles ('fingerprinting'). The output in descriptive studies is a list of proteins. This list typically represents the catalogue of all proteins detectable with a particular technology.[2]

Proteomics inherently is a hypothesis generating discovery technology. Proteomic studies can be classified as comparative studies that try to establish quantitative or qualitative protein differences between samples and descriptive studies that focus on the identification of proteins[2] (Figure 4.3). In both cases, the study designs can be either pathway driven (targeted) or

non pathway driven (non targeted). Most clinical molecular marker discovery studies have no pre determined hypothesis of which pathways or proteins might be of interest. Non pathway driven studies are often conducted with the goal to offer new insight into previously unknown mechanisms. Attempts have been made to utilize the protein patterns to detect a pathobiochemical process without further protein identification and mechanistic qualification. This approach of pattern based patient classification is also called 'fingerprinting'. Although straightforward, given the biological variability of a proteome and the many potential confounding factors in complex patient populations, fingerprinting usually has poorly controlled risks. Furthermore, it is difficult to validate and qualify such diagnostic approaches to an extent that they will be acceptable for approval by regulatory agencies.[18] By contrast, pathway driven studies seek to achieve more in depth mechanistic or functional insight.[2] They focus on specific proteins or protein networks and usually use a more targeted strategy.

2.2. Targeted

Targeted assays do not seek to capture a whole proteome, but assess a set of known proteins that typically have common pathways, protein network or context such as inflammation or kidney dysfunction markers. In many cases, immunoassays are used for this purpose and only those proteins can be detected, against which antibodies are included. Thus, the use of targeted assays for research purposes requires pre existing knowledge about a disease process or drug effect, or at least a hypothesis. Limitations are the availability of antibodies, their specificity and the sometimes poor batch to batch reproducibility of more complex assays.

In clinical proteomics, after the proteins of interest are identified, there may no longer be a need to assess the whole proteome because the desired information can be obtained by measuring a set of well defined and qual ified proteins. Another advantage of targeted assays is that these usually require less sample preparation, are quantitative, can be validated, are rela tively high throughput and can be run using instrumentation that may already be readily available in a clinical laboratory such as ELISA readers, multiplexing platforms or mass spectrometry.

An ideal case scenario would be the availability of quantitative targeted protein arrays that contain the whole human proteome and that can be scanned in a high throughput fashion, similar to those already available for

genome array analysis. Unfortunately, the information currently known about the human proteomes is insufficient, there is more than one relevant proteome, and the technology for building such comprehensive protein chips is not yet available.

3. PROTEINS AND THE KIDNEY

Kidney research has mainly focused on two proteomes: the kidney and urine. Although the kidney extensively communicates with the blood compartment, blood or plasma proteomes have only been of minor interest, simply because proteome changes originating from the kidney are quickly diluted and mixed with protein populations from other organs. This may create opportunities for a more systemic and holistic analysis, but it also complicates the interpretation of such data.

The renal proteome is made up by multiple cell types that comprise the kidney. The kidney can be viewed as an assembly of subproteomes of lesser complexity than the whole, released by or contained in kidney cell compartments such as plasma membranes, nuclei, cytosol and mitochon dria.[19] Proteomic studies have sought to improve our understanding of kidney function, attempted to map proteins in the cortex of the human kidney[20] and compared protein expression in the cortex and medulla of the rat kidney. The function and regulation of specific cell populations in the glomerulus, proximal tubule, thick ascending loop of Henle and inner medullar collecting duct of the kidney have been studied using cell culture models. They have also been studied after isolation of the cells and tissues of interest using sieving or micro dissection techniques. For a comprehensive review see reference.[7]

Structures in the cortex mainly reabsorb water, electrolytes, glucose and amino acids and they produce hormones that regulate blood pressure (renin), hematopoiesis (erythropoietin) and calcium homeostasis (1,25 dihydroxy vitamin D_3).[21] The inner medulla is mainly responsible for concentrating urine and is characterized by high osmolarity and relatively low oxygen tension. In contrast to the relatively leaky proximal tubule, the inner medullary collecting duct of the mammalian nephron is characterized by low sodium permeability and by a large transepithelial resistance.[22] A tight epithelial barrier is critical for the control of sodium excretion. To maintain functionality and cellular viability, inner medulla cells have a unique metabolism that ensures the maintenance of intracellular ATP concentra tions (high expression levels of the γ subunit of Na^+/K^+ ATPase),

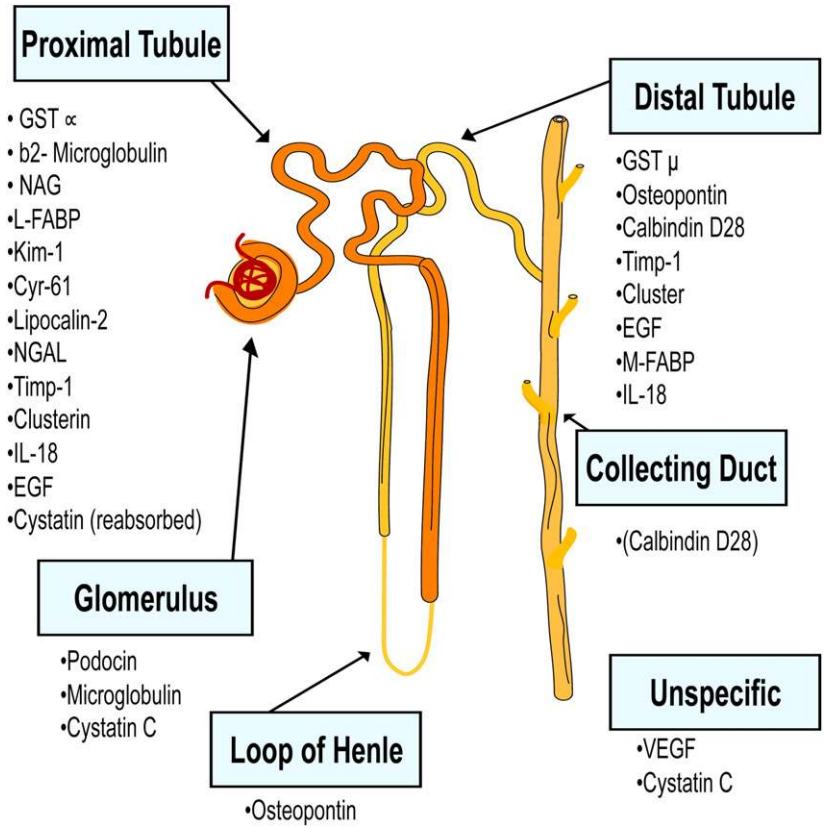

Figure 4.4 ***Protein markers of kidney injury and their mapping to the nephron.*** Potential marker proteins frequently mentioned in the literature are shown. Thus, this list should not be considered complete. The mapping represents the most abundant locations; however, in the case of some proteins, this may be an over-simplification. For more information about these proteins, please see Table 4.5, page 142.

intracellular osmolyte concentrations (NUP88) and tight junction integrity (MUPP1).[22] As indicated, these distinct functions require the expression of specific sets of proteins. Exact knowledge of these distinct proteomes will not only allow for characterizing the type of injury and yield information regarding the associated mechanisms, but also for locating the injury. As shown in Figure 4.4, patterns of protein kidney injury markers can be mapped in the nephron.

As urine can harbor proteins from all kidney subproteomes, and the protein composition of urine is perturbed by kidney injury or disease, the urine proteome can subsequently signal the status of kidney health as well as

the onset, nature and location of injury and dysfunction.[19] Even though an intact glomerular membrane will prevent larger proteins from entering the so called 'primitive' urine, the urine of healthy individuals contains a significant amount of peptides and proteins. These proteins originate from three main sources: extra renal (such as filtration of plasma proteins), the kidney and the lower urinary tract.[23] It has been estimated that 49% of the proteins in urine are soluble proteins that enter the urine via glomerular filtration or tubular secretion, such as Tamm–Horsfall protein, 48% are urinary sediment proteins (mainly due to sloughing of epithelial cells, shedding of microvilli and apoptosis of epithelial cells resulting in cell membrane fragments) and 3% stem from urinary exosomes.[24] However, the handling of proteins by the kidney involving the complex glomerular slit diaphragm and podocytes as well as the role of proximal tubule epithelium in protein secretion, breakdown and reabsorption is not fully understood yet.[25,26]

The human urine proteome probably contains more than 100,000 proteins and peptides of which 5000 are considered high frequency and have been observed in more than 40% of the individuals examined in different studies.[27–29] However, not all of these have been identified. In a recent 2D electrophoresis study 1118 protein spots were reproducibly found in normal urine samples. Two hundred and seventy five of those were characterized as isoforms of 82 proteins.[30] Although there is extensive knowledge regarding the handling of small molecules by the kidney, there is surprisingly little data regarding the handling of proteins. Using proteomics technologies, plasma and urine proteomes, which were considered the input and output pro teomes, were studied.[31] After removal of proteins secreted downstream of the kidney, 2611 proteins were found in plasma and 1522 proteins were found in urine. These could be separated into three subproteomes: plasma only (2280 proteins), plasma and urine (394 proteins) and urine only (1128 proteins). It seemed reasonable to assume that the plasma only subproteome was derived mainly from soluble proteins and proteins in solid plasma components that do not pass through the glomerular membrane. The plasma only proteome also contained proteins that had a molecular weight of < 30 kDa that, based on their molecular weight, should have been filtered, but were probably retained due to their charge, shape, interactions or associations with other proteins. The plasma and urine subproteome probably contained soluble proteins that were filtered from plasma or secreted by the kidney. The urine only subproteome was most likely constituted by soluble proteins that were released into the urine by epithelial

Table 4.1 Sources of urinary proteins

Source	
Soluble proteins	
Glomerular filtration of plasma proteins	Normally present (< 150 mg/day) Defects in glomerular filter increases high molecular weight protein concentrations in urine such as albumin Defects in proximal tubule reabsorption or abnormal production of low molecular weight plasma proteins increase low molecular weight proteins such as β_2 microglobulin
Epithelial cell secretion of soluble proteins	Via exocytosis (e.g. epidermal growth factor) or glyosylphosphatidylinositol anchored protein detachment (e.g. Tamm Horsfall protein), proteolytic fragments
Interstitial processes, cell injury and other cells	Leakage of proteins during injury such as inflammation, immune reactions, necrosis, apoptosis and repair; products of prostate gland
Solid phase components	
Whole cell shedding of epithelial cells	Increased cell number during diseases such as acute tubular necrosis (renal tubular cell shedding) and glomerular diseases (podocyte shedding)
Plasma membranes and intracellular organs	Non specific nephrotoxic, necrotic and apoptotic processes
Exosome secretion	Normal process[33]
Other cells and bacteria	During certain disease processes: red blood cells, white blood cells, tumor cells, bacterial infections

Epithelial cells include all epithelial cells along urinary tract from podocytes to urethral epithelia. Based on O'Riordan and Goligorsky[23] and Pisitkun et al.[32]

secretion or shedding and/or were from solid phase components in urine[31] (see also Table 4.1).

It is well established that aging induces morphological changes of the kidney and results in reduced kidney function. This includes the glomerular filtration rate that declines 20–25% during the age range of 40–80 years and the ability of the kidney to concentrate urine. In a study based on 324 apparently healthy subjects between the ages of 2 and 73 years of age, the low molecular weight proteome in urine was assessed using capillary electrophoresis mass spectrometry.[34] Five thousand protein signals could be separated, and 325 of them showed age dependent differences. Most of these changes were associated with the development of the kidney before

puberty. Forty nine proteins were found to be associated with aging in adults and several of these were associated with proteolytic activity and uromodulin targeting. Interestingly, several subjects did not have urinary protein patterns that matched their age and this may have reflected unde tected chronic disease processes.[34]

4. THE PROTEOMICS SAMPLE

As discussed above, urine is an attractive matrix. It is considered a 'proximal' matrix, a biofluid that is close to, or in direct contact with, the site of disease.[18] Proximal fluids are local sinks for metabolites, proteins or peptides secreted, shed or leaked from the tissue of interest. The nephron is capable of filtering smaller proteins and reabsorbing proteins. Proteins are distinctly formed and distributed throughout the different parts of the nephron, the reasons for which are defined by differences in function, availability of oxygen and osmolarity. Thus, changes in protein patterns in the urine allow for localizing the injury in the kidney (Figure 4.4).

The gold standard for the quantification of proteinuria has been 24 hour urine collection, but 24 hour urine collection is time consuming, incon venient and dependent on the patient's compliance.[24] Collection of midstream urine is widely considered the standard for urine proteomics analysis.[35] While no difference has been observed between first void and midstream urine in males, there was marked variation in females, most likely due to bacterial contamination of first void urine.[36] However, this may also depend on which disease is targeted with the analysis. Prostate cancer markers were found in higher abundance in first void than in mid stream urine,[37] indicating that urine collection protocols affect the results of pro teomics studies and need to be assessed during method development.[38]

Urine as a source of protein markers has several advantages and disad vantages.[28] Urine can easily be obtained non invasively in relatively large quantities and there are no limits for how often urine can be collected from the same patient. Typically, urinary peptides and proteins are water soluble. Thus solubilization that can pose major problems in the proteomics analysis of cells and tissues is not a problem with urinary peptides and proteins. Urinary proteins are usually small with molecular weights of less than 30 kDa and can be analyzed with time of flight mass spectrometers without enzymatic digestion to peptides ('top down proteomics'). In most cases they are also stable since urine stagnates in the bladder at body temperature for hours – degradation and proteolytic processes are often complete by

the time the urine is collected.[28] The addition of protease inhibitors to stabilize urine samples for proteomics analysis is not recommended anymore.[38,39] By contrast, activation of proteases constitutes a major problem in the collection of blood samples for proteome analysis. Nonetheless, urine is considered one of the most difficult proteomic samples to work with due to its highly variable contents, dilution due to fluid intake, and pH, as well as the presence of various proteins in low abundance or modified forms.[28]

It has independently been shown that the urine proteomics samples can be stored for up to 6 h at room temperature, up to 3 days at +4°C and several years at −20°C,[28,36,39–41] but this may not be the case for individual proteins, peptides[13] and urinary exosomes that have been described to be less stable.[33] Long term storage at −80°C seems to be a safer approach.[33,42] When frozen samples were thawed, an initial loss of minor protein signals was observed.[43] Hereafter, urine samples for proteomics analysis were generally found to be stable for at least three freeze–thaw cycles, but marked losses of proteins were found if samples were frozen and thawed more often.[36,38,43] Overall, it is recommended to avoid freeze–thaw cycles whenever possible.[35]

A challenge is that the dynamic range of protein concentrations in body fluids spans several orders of magnitude and urine is no exception to this rule.[18,28,44,45] Most analytical approaches assessing the urine proteome include an initial sample preparation step enriching the proteins of interest. A common strategy utilized is the removal of high abundance proteins that confer little diagnostic information using techniques such as column purification (size exclusion, ion exchanger, affinity columns), selective surfaces, immunodepletion and equalizer beads.[28,46] Immunodepletion has the inherent risk of also losing proteins of interest by co-depletion[28] that may be caused by protein–protein interactions independent of the desired specific antibody interactions. The preparation of urine samples for proteomic analysis has systematically been studied and is described in detail by Kushnir et al,[46,47] Thongboonkerd et al,[48,49] Pieper[49] and Khan and Packer.[50]

4.1. Kidney tissues and cell culture

The basic principle of the preparation of a tissue sample is that the heterogeneity should be diminished as much as possible and that the sample should be pure and relevant. The first step in the proteome analysis of tissue samples is homogenization. Homogenization methods used for proteomics

purposes can be divided into five major categories: mechanical, ultrasonic, pressure, freeze–thaw and osmotic or detergent lysis.[51] It is critical to protect the samples from proteolysis during processing[39] – the most common protective measures are protein denaturation and the addition of protease inhibitors. The next steps include the removal of contaminants, such as salts, detergents, abundant proteins, lipids, polysaccharides, nucleic acids and other contaminants, and protein enrichment, using precipitation, centrifugation, prefractionation, electrophoretic, antibody based proce dures and/or chromatographic techniques.[51] The sample preparation approach also depends on the intended analysis. For comprehensive reviews, see Bodzon Kulakowska et al,[51] Ahmed,[52] Matt et al[53] and Hu et al.[54]

Cell cultures are of interest for mechanistic and molecular marker qualification studies. It is assumed that a cell on average expresses 10,000 proteins.[51] If a cell culture contains multiple types of cells, then this number is higher. The preparation of cell cultures is simpler than that of tissue and often involves direct lysis of the cells in the dish after removal of the cell culture medium as a first step. After solubilization, the sample is transferred and sonicated. The following steps may involve those described for tissue sample preparation.[51 53] Tissue samples, cells and purified samples should be stored long term at 80°C.

It always should be kept in mind that the results of proteomics analysis may be influenced by sample preparation (e.g. 2D gels, enzymatic digestion and isolation of cell organelles), the selectivity of separation technologies preceding mass spectrometry analysis (e.g. activated surfaces and ion exchangers) and ionization methods.

5. ANALYTICAL TECHNOLOGIES

To date, more than 228,000 human proteins have been described in the literature.[55] The number of different components of the human proteome that has been estimated adds up to approximately one million, vastly exceeding the number of different genes in the human genome.[56] As already mentioned, this is because of single nucleotide polymorphisms and post translational modification. Currently, more than 300 different post translational modifications have been described. Post translational modifications, such as phosphorylation, can be temporary and diseases can influence protein reactions, for example, through radical formation, as well as somatic mutations.

Table 4.2 gives an overview of the major proteomics technologies. Proteomic approaches can broadly be separated into techniques that are based on separation and detection of the intact proteins ('top down' pro teomics) and techniques that involve digestion of the proteins into peptides and analysis of the resulting peptide patterns ('bottom up' proteomics).[16,58] Bottom up proteomics is the most common approach. After digestion of proteins into peptides, peptide mixtures are usually separated by high performance liquid chromatography (HPLC) and analyzed utilizing mass spectrometry (Figure 4.2). The peptide patterns are analyzed using database searches and protein hits are identified based on the peptide patterns. Some disadvantages of the bottom up approach is that the ability to quantify proteins is limited, unless labels are used, and post translational modifica tions may get lost or are undetected. Top down proteomics can detect post translational modifications and provides a 'bird's eye' semi quantitative view of a proteome.[16] The simplest form of top down proteomics is 2D gel electrophoresis. In most cases, mass spectrometry based top down proteo mics involves high resolution mass spectrometers and works best for proteins < 100 kDa and less complex proteomes.[16]

The combination of different proteomics methods may result in some overlap but also will give significant additional information.

5.1. 2D gel proteomics

2D gel electrophoresis is based on the separation of individual proteins contained in a proteome by isoelectric point (first dimension) and then by molecular weight (second dimension). The intact proteins are visualized and quantified by staining and densitometry. Thus, 2D gel electrophoresis is a top down proteomics technique. 2D gels can resolve 1500−3000 proteins. By spreading the pH range across several gels, also known as zoom gels, between 5000 and 10,000 proteins can be resolved.[59] 2D gels of different proteomes that run on separate gels are compared and the intensities of stained protein spots are analyzed using statistical procedures. Next, relevant protein spots are cut out, destained, digested and the peptides are eluted from the gel matrix. The result is a peptide mixture from substantially purified proteins which is further analyzed by peptide fingerprinting using MALDI TOF or nano LC ion trap mass spectrometry, then followed by database search. 2D gels and subsequent mass spectrometry based identifi cation is best suited for samples of limited complexity where specific proteins that need to be characterized do not require high throughput.[9]

Table 4.2 Proteomics technologies

Technology	Description	Advantages	Disadvantages
2D electrophoresis	Separation by isoelectric point and size. Proteins are stained after separation and compared between gels	Widely available. Easiest form of 'top down' proteomics. Separation of charge reflects post translational modifications	Low abundance, molecules < 10 kDa as well as large, basic and hydrophobic proteins such as membrane proteins are difficult to detect
DIGE	2D electrophoresis with fluorescent labeling of proteins before separation in gel. Two proteomes (e.g. treatment and control) as well as an internal standard can be separated on the same gel	Improved spot alignment, improved reproducibility, better quantification and spot abundance in comparison to 2D gels	Low abundance, large and hydrophobic proteins are difficult to detect, requires three color imaging system and other additional equipment compared to 2D electrophoresis
LC MS	Proteins are digested before separation by HPLC. The HPLC can be 1D (also after protein spots are cut from gels) or multidimensional using column switching. HPLC separation can also be done offline	Sensitive and more likely to see low abundance proteins and other proteins than 2D electrophoresis. Can easily be automated. Allows for protein identification in combination with database search	Quantification and measurement of post translational modifications require additional tools. Not very quantitative. Reassembly of tryptic peptides into molecules can lead to incorrect results

ICAT/iTRAQ	LC MS with isotopic labeling of the peptides after digestion. Like DIGE, this allows for simultaneous analysis of several proteomes. The labeled peptides are chemically identical but have predictable mass differences. The abundance of the differently labeled peptides is detected in the mass spectrometer by comparison of the intensity of the same peptides with different labels	Relative quantification of low abundance and hydrophobic samples. Since several proteomes are analyzed simultaneously, peak alignment is less of a problem	The number of direct comparisons is limited. Quantification and measurement of post translational modifications will require additional tools
SELDI	Proteins are bound to affinity surfaces on a MALDI chip. Different surfaces are available. Bound proteins are detected in the mass spectrometer (usually time of flight)	Samples can be enriched for specific low abundance proteins. High throughput platform for protein marker discovery	Proteins that do not bind to the selected surfaces will not be detected. Thus, this tech nology is more biased than those discussed above. Not intrinsically quantitative. Large amount of variability between laboratories has historically been a problem.

(*Continued*)

Table 4.2 Proteomics technologies—cont'd

Technology	Description	Advantages	Disadvantages
			One of the reasons is that the manufacturer combined it with a low resolution TOF instrument and its susceptibility to interferences
Capillary electrophoresis MS	Separation of proteins by elution time in capillary electrophoresis and by size in mass spectrometer, highly sensitive, low sample volume	Reproducible and sensitive. Good technology for protein marker discovery	Limited to proteins < 20 kDa
Protein binding arrays	Proteins or antibodies printed on a microchip or bead in a multiplexed format	Sensitive and rapid. Allows for semi quantitative comparison among samples	Specificity is variable and difficult to control. Is bias based and depends on the proteins or antibodies bound. It does not detect proteins that do not bind to the array

2D, two-dimensional; DIGE, difference gel electrophoresis; ICAT, isotope-coded affinity tags; iTRAQ, isobaric tag for relative and absolute quantitation; LC-MS, liquid chromatography-mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; SELDI, surface-enhanced laser desorption/ionization.
Based on Janech et al,[7] Domon and Aebersold,[9] Decramer et al[28] and de Hoog and Mann.[57]

Inherent problems include certain groups of proteins such as membrane proteins that usually are not captured, the reproducibility among separations run on different gels and achieving the correct alignment of corresponding protein spots.

The latter problem has been improved by the introduction of difference gel electrophoresis (DIGE).[60,61] Two or more proteomes are stained with different fluorescent dyes. The proteomes and internal standards are then pooled and simultaneously separated on the same 2D gel. After separation, the different proteins are visualized separately using respective discrete excitation and emission wavelengths. Fluorescent labeling, inclusion of internal standards and simultaneous separation have not only improved reproducibility, but also the reliability of semi quantitative comparison of proteomes.[7]

5.2. LC-MS

Mass spectrometry based protein identification relies on the digestion of protein samples into peptides using a sequence specific protease such as trypsin.[57] Trypsin cleaves at the C termini of arginine and lysine residues. Based on the occurrence of these two amino acids in proteins, an average of ten peptides is expected for a stretch of one hundred amino acid residues.[62] There are several advantages of analyzing peptides rather than proteins by LC MS:

- If proteins have been separated by 2D gels, peptides are easier to elute from the gels than proteins.
- The molecular weight of proteins alone is often not sufficient infor mation to identify a protein.
- Proteins are heterogeneous and do not necessarily possess a single molecular weight.
- The peptides add structural information.
- Peptides fall into the effective mass range of most mass spectrometers that is between 1 and 5 kDa, unless a time of flight mass spectrometry detector is used.

Mass spectrometers consist of the following components: an inlet, an ionization source, sections to focus, separate, select and fragment ions and a detector.[63] The most common inlets and ionization methods used in proteomics are HPLC (one or multidimensional) in combination with electrospray ionization or matrix assisted laser desorption/ionization (MALDI). Mass spectrometers measure the mass to charge ratio of an ion. This is achieved by manipulating ions in an electric and/or magnetic field or

by measuring time of flight. In contrast to small molecules that, in most cases, are singly charged (this means that the mass to charge ratio reflects their molecular weight), large molecules are usually multiply charged. The intensity of the signal caused by a specific molecule reflects the abundance of the ion. As already mentioned, a problem with ionization in a liquid phase or matrix such as electrospray ionization and MALDI is that the abundance of ions varies with ionization efficiency which may depend on other molecules that are in the ionization source at the same time (ion suppres sion, ion enhancement). Therefore, mass spectrometry cannot be consid ered a quantitative technology for proteins when complex mixtures are analyzed.[63] In addition to quadrupole and time of flight mass spectrome ters, ion traps and hybrids such as quadrupole time of flight (QTOF), quadrupole linear ion traps (QTRAP), ion trap orbitrap, ion trap cyclotron resonance Fourier transformation mass spectrometers (FTMS) are used (Table 4.3). Much of the uncertainty of peptide identification is directly related to the accuracy of the mass spectrometer used. The more accurate the mass, the less potential false positive matches in a database search are possible. High resolution mass spectrometers such as FTMS and orbitraps greatly increase the confidence in peptide and, subsequently, protein identification.[63] In addition, the better resolution of peptides with similar masses allows for the detection of more signals when compared with lower resolution mass spectrometers.[9]

Tandem mass spectrometry is the basis of shotgun proteomics or MudPIT (multidimensional protein identification technology) approaches, a strategy that attempts to analyze the complete proteome of a cell, tissue or organism in a single experiment.[62,64,65] Although different approaches have been described, they all have the same basic strategy in common:[66] the proteins in the sample are digested usually using trypsin, the resulting peptide mixtures are subjected to one , two and three dimensional fractionation (online or offline), and the peptides from the last separation step are usually separated using reversed phase chromatography and analyzed by tandem mass spectrometry, in most cases, including a linear ion trap.[62] The MS/MS spectra are assigned to peptide sequences and software tools using search algorithms assign the detected peptides to proteins.[67,68]

While 2D gels will separate proteins with different post translational modifications, the identification of modified proteins in complex mix tures using LC MS/MS remains a challenge. Among the known post translational modifications, glycosylation and phosphorylation are most

Table 4.3 Comparison of mass spectrometry detectors used for proteomics

	3D-ion trap	Q-TOF	TOF-TOF	FTMS	Orbitrap	QQQ	QTRAP
Mass accuracy	+	+++	+++	++++	++++	++	++
Resolving power	+	++	+++	++++	++++	+	+
Sensitivity	++	+	+++	++	+++	++++	++++
Dynamic range	+	++	++	++	+++	++++	++++
Ionization source	API (MALDI)	API MALDI	MALDI	API	API	API	API
MS/MS capabilities	MS^n	yes	yes	yes	yes★	yes	MS^n
Protein identification	++	++	+++	++++	++++	+	+++
Quantification	+	+++	++	+++	+++	++++	++++
Throughput	++	++/+++†	++++	++	++	++	++
Detection of post translational modifications	+/+++‡	+	+	+	+	+	+++

API, atmospheric pressure ionization source such as electrospray ionization and atmospheric pressure chemical ionization; FTMS, Fourier transformation ion cyclotron resonance mass spectrometer; MALDI, matrix-assisted laser desorption/ionization; QQQ, triple stage quadrupole mass spectrometer; Q-TOF, quadrupole-time-of-flight mass spectrometer; QTRAP, triple stage quadrupole/linear ion trap mass spectrometer; TOF-TOF, tandem time-of-flight mass spectrometer. +, possible or low; ++, good; +++, high; ++++, excellent.

† With MALDI.

‡ In combination with electron transfer dissociation (ETD).

★ Only in combination with collision cell.

Based on Domon and Aebersold.[9]

important; however, these modifications are labile and can already be lost in the ionization source under normal electrospray ionization conditions.[69] This becomes even more of a problem when collision energy is applied. The result is cleavage of the labile modification and detection of the peptide fragment lacking the modification. There are two scenarios: if a neutral species is lost after cleavage, only the peptide backbone can be detected and if the lost species is charged, two signals can be detected. One signal again corresponds to the peptide backbone and the second signal in a low mass range is the post translational modification specific fragment ion, also referred to as reporter ion. Even in this case, information regarding the location of the modification is lost. Precursor ion scanning and neutral loss scanning in combination with mild fragmentation conditions have been used to assess post translational modifications.[69]

Although LC electrospray MS analysis is the most frequently used analysis technology to date, MALDI remains a valuable technology that, in addition to being high throughput, is an alternative ionization method that often yields complementary protein hits.[9]

5.3. Labeling technologies for LC-MS analysis

So called isotopic tags allow for quantification of proteins using LC MS.[70] Isotope coded affinity tags (ICAT) contain three functional regions — an affinity purification region, a peptide binding region and an isotopically distinct linker region. Typically, a biotin tag is used for affinity purification and a thiol specific binding moiety covalently links the reagent to cysteine in the target peptide. The linker region is isotopically labeled with ^{12}C or ^{13}C.[71] Thus, after reacting with differently labeled tags, the same peptides remain chemically identical but can be distinguished by the mass spec trometer based on their different tag masses. This allows for labeling of two samples. The samples are mixed and analyzed simultaneously in one run, eliminating the problem of peak shifts and alignment that may occur when samples are independently analyzed and improving quantification. The disadvantage of ICAT is that it is restricted to labeling peptides that contain cysteine and, thus, less peptides for protein identification may be available. Another approach that has similar advantages and disadvantages as ICAT is proteolytic ^{18}O labeling. A protease and $H_2{}^{18}O$ are used to generate labeled peptides.[72] Isobaric tag for relative and absolute quantitation (iTRAQ) labels all peptides and thus increases the confidence in protein identification

by labeling a larger number of peptides per protein of interest. Samples are trypsin digested, labeled and then the iTRAQ labeled peptide samples of between four and eight proteomes are combined, fractionated using a strong cation exchange column and analyzed using nano LC MS/MS.

While ICAT and iTRAQ can be applied to serum and other biofluids, cell homogenates as well as tissue samples, stable isotope labeling with amino acids in cell culture (SILAC) has specifically been developed to detect proteome differences in cell cultures. The cells are incubated with isotopically distinct forms of amino acids until the complete proteome contains amino acids with a specific label. Proteomes with different labels can then be mixed and analyzed simultaneously.[73,74]

5.4. Other mass spectrometry-based technologies

Surface enhanced laser desorption/ionization (SELDI) is a variation of MALDI and has been widely used in discovery studies to identify new protein molecular markers.[28] The basic principle is that the sample is exposed to chips with different active surfaces that enrich certain groups of proteins. These are then eluted onto a MALDI plate and analyzed using a time of flight mass spectrometer. SELDI can be automated. Although the system is highly integrated and easy to use, it is prone to producing artifacts.[75–77] The most likely reason is that the time of flight mass spectrometer that was included in the SELDI system lacked appropriate resolution.[28] Also, due to the selectiveness of the activated surfaces, only a fraction of the proteome is analyzed and potentially critical information may be missed. A significant source of variability and artifacts is that binding of proteins to the active surfaces is not very robust and is easily influenced by even small variations in pH, salt concentrations and interfering compounds. Due to these shortcomings, SELDI has lost its importance.

A limitation of common fragmentation technologies is that they result in cleavage of post translational modifications before the protein or peptide backbone is cleaved; however, cleavage of the backbone with post translational modifications attached will yield valuable structural information. Electron capture and electron transfer dissociation are technologies that are complementary to classical fragmentation, and they tend to result in fragmentation more evenly distributed over the entire peptide backbone. This makes them particularly useful in localizing post translational modifications.[9,78]

5.5. Non-targeted microarrays

Microarrays technology can be used for the targeted quantification of proteins, but can also be used as a non targeted discovery tool. Most tar geted microarrays are based on immobilized antibodies, while non targeted microarrays are often referred to as protein microarrays. Protein function assays are based on immobilized recombinant proteins, peptides or libraries hereof as well as antibodies. They are used for screening for novel substrates, for enzyme activities, for protein–protein interactions, for protein–lipid interactions and for protein–small molecule interactions. Arrays with up to 8000 human proteins are available.[79] In reverse phase assays cell proteomes are coated on the array after extraction and then the immobilized proteins are screened using antibody based detection. For more details, see Korf and Wiemann.[56]

5.6. Technologies for targeted proteomics

After the molecular markers of interest have been identified, the next step is to establish targeted and validated assays that are capable of quantifying these specific compounds with acceptable total imprecision and sensitivity. It was recently reported that there are 105 FDA cleared or approved tests for the quantification of proteins and additional tests for 65 proteins and 32 peptides have been listed in the Directory of Rare Analyses[80,81] – for a complete list, please see Anderson.[80] Assuming that the human genome has 20,500 protein coding genes,[82] this means that the assays approved and cleared by the FDA and listed in the Directory of Rare Analyses cover less than 1% of the human proteome. The typical analytical platforms for such assays are enzy matic assays, antibody based assays, HPLC/UV, LC MS and GC MS. For the targeted analysis and quantification of proteins, in most cases, antibody based (80% of all approved) assays[80] and enzymatic assays are used; however, MS based assays are gaining importance for the quantification of proteins.

Targeted strategies measure well defined molecular markers to detect pattern changes. To achieve this goal, the analytical strategies have to be at least semi quantitative or quantitative.

Enyzme linked immunosorbent assays are frequently used for the quantitative measurement of proteins.[83] Most of these assays are single analyte assays. Multiplexing assays are targeted assays that can test multiple analytes in a single test using a single sample. In principle, current protein multiplexing assays are simultaneous ELISA microassays coated adjacently on a surface. This surface can be an array or a bead.

The basic principle, briefly, is:[83] microspot arrays can be based on several flat surfaces such as polylysine and aminopropylsilane, epoxysilane treated glass and other surfaces that may be covalently or non covalently linked. Spot sizes are between 50 and 250 μm; 50–300 spots, or even more, can be printed in pre defined geometric patterns into a 96 well plate. Fluorescence or chemiluminescent labels are used for detection. The spots in each well are resolved by micro imaging. These assays can be developed on a microarray surface and microarray analyzers and software can be used for analysis. Among antibody microarrays, analog to sandwich ELISAs, are the most quantitative; however, they require a second antibody.[57,79] Today, most commercially multiplexed sandwich microarrays quantify cytokines and chymokines.[56,57]

Bead assays are microspheres with a diameter of approximately 6 μm that can be color coded to assign individual addresses for up to 100 different populations mixed in solution. In a typical set up, each bead population is coated with an analyte binding capture agent that can be either DNA probes or antigen/antibody capture for protein assays. These populations of coupled beads are then mixed into a solution to form an array. Fluorescent labels can be used to detect signals. The analyzer sorts out the populations based on the color code that reads the signal on the bead.

Interestingly, in comparison to ELISAs using the same antibody/antigen combination, microspot assays were found to be more sensitive and have a wider linear range.[83] They also have better sensitivity and specificity than conventional ELISAs in low analyte samples.

In comparison to microspot assays, the signal intensity of beads is much lower since the beads are dispersed throughout the entire volume of the assay fluid that typically is 50–200 μL for a 96 well format assay. Another reason is that only one half of the bead surface area is excited and the bead does not always flow past the detector with the excited half optimally exposed.[83] Also, as previously indicated, bead based assays are limited to about 100 analytes that can be measured simultaneously.

In addition to protein microarrays, two frequently used technologies are the Luminex xMAP and the Mesoscale Discovery electro chemiluminescence detection system. The Luminex xMAP technology is based on polystyrene bead sets encoded with different intensities of red and infrared dyes and coated with a specific capture antibody against one of the analytes of interest. Interrogation of the beads by two lasers identifies the spectral property of the bead and thus the associated analyte, in addition to the R phycoerythrin labeled secondary antibody against the specific

analyte. The Mesoscale Discovery assay platform uses plates fitted with up to 10 carbon electrodes per well, each electrode being coated with a different capture antibody. The assay procedure follows that of a sandwich ELISA, with any analytes of interest captured on the electrode being detected with an analyte specific ruthenium conjugated secondary antibody. Upon electrochemical stimulation the ruthenium label emits light at the surface of the electrodes, allowing the concentration of the analyte to be determined relative to the particular electrode.[84]

Current protein multiplexing assays vary in three key characteristics:[83] the number of analytes that can simultaneously be measured, the type of platform and qualitative versus quantitative differences. Due to differences in sensitivity, specificity, robustness and dynamic ranges, some technologies are more appropriate for quantitative assays than others and quantitation depends on the development and validation of an appropriate calibration and quality control strategy. Protein multiplexing assays are limited by the number of suitable antibodies that are highly specific and bind their cognate antigens with comparable binding constants.[28] Another limitation is the wide concentration differences in cellular proteins that, as already mentioned above, cover several orders of magnitude. Therefore, a single multiplex assay can only semi quantitatively compare proteins that are present in a cell or body fluid in the same concentration range.

In the vast majority of protein detection platforms, the binding event of a protein to a specific recognition molecule must be detected with a signal transducer. In ELISAs, protein microarrays and quantum dot [85] detection platforms, the readout is based on a fluorescent or colorimetric signal.[86] Inherent autofluorescence or optical absorption of the matrix of many biological samples or reagents may become a limiting factor. Similarly, nanowires,[87] micro cantilevers,[88] carbon nanotubes[89] and electrochemical biosensors[90] rely on charge based interactions between the protein or tag of interest and the sensor, making each system dependent on conditions of varying pH and ionic strength. Since the matrices of even complex bio logical samples usually lack a detectable magnetic background signal, a magnetic field based detection platform for protein detection in clinical samples has been described[86,91] that is matrix insensitive yet still capable of rapid, multiplex protein detection with resolution down to attomolar concentrations and a wide linear dynamic range.

Proteins can also be quantified using multi reaction mode (MRM) LC MS/MS assays. Typically, the specific peptides of the candidate protein

molecular markers have already been identified. Sample preparation may be based on protein precipitation, antibody based protein enrichment, removal of high abundance proteins, size exclusion chromatography and/or liquid solid extraction. Proteins are then digested and the specific peptides are analyzed using LC MS/MS.[92] Absolute quantification is possible by utilizing synthetic isotopically labeled versions of the specific peptides.[18,93] The heavy isotope labeled peptide can be used as a calibrator or as an internal standard. In most cases, the isotope labeled peptide is added after digestion of the proteins with trypsin, and the digest is then separated by reversed phase HPLC.

For selection of the best ion transitions for MRM, a number of criteria should be considered.[94] The target peptides should not exhibit any enzyme missed cleavage sites, and they should not be susceptible to post translational modification, unless it is the purpose of the assay to quantify those. They should be of a size that accommodates the mass range of the triple stage quadrupole instrument, usually in the range from 7 to 30 amino acids, and they should uniquely identify the protein of interest. Each MRM transition requires an optimized set of mass spectrometry parameters for maximum sensitivity. Usually studies use at least two peptides per protein and up to two different charge states for each of the parent ions in combination with two different fragment ions for each peptide. This means that such a protein quantification will be based on at least eight MRM transitions. Due to this, designing and validating hundreds of individual peptide transitions for the quantitative analysis of complex samples is extremely difficult. In silico algorithms are available.[94]

Recently, a semi automated assay for the enrichment and MRM LC MS/MS analysis of using specific antibody based capture of individual tryptic peptides from a digest of whole human plasma has been described — the stable isotope standards and capture by anti peptide antibodies (SISCAPA) method.[95] This method uses a simplified magnetic bead protocol and a novel rotary magnetic bead trap device. Following offline equilibrium binding of peptides by antibodies and subsequent capture of the antibodies on magnetic beads, the bead trap permitted washing of the beads and elution of bound peptides inside a 150 μm inner diameter capillary that forms part of a nanoflow LC MS/MS system. The bead trap sweeps beads against the direction of liquid flow using a continuous succession of moving high magnetic field gradient trap regions while mixing the beads with the flowing liquid.[95]

Due to the specificity of MRM, LC MS/MS assays are capable of simultaneously quantifying multiple proteins.[92] The simultaneous quantification of up to 45 proteins in plasma samples using MRM LC MS/MS has been reported.[96] Again, one of the challenges with multiplexing is the potentially large concentration differences of proteins in the matrix of interest.[97,98] The advantages of the quantification of proteins using LC MS/MS over immunological assays are that LC MS/MS does not require the availability of antibodies, that it does not depend on the quality of antibodies and that the quality of the data is easier to control. Interferences can usually easily be detected by inspection of the ion chromatograms while potential cross reactivity or other interference with an antibody reaction is more difficult to detect.

5.7. Database searches, biostatistics and annotation

Accurate, consistent and transparent data processing and analysis are critical parts of the general proteomics workflow in general and for molecular marker discovery in particular.[99]

Although sophisticated and powerful data analysis tools are available today, it should not be forgotten that the quality of the result is determined by the quality of the analyzed samples and the quality of their analysis. It is only when sufficient quality can be assured that meaningful results and conclusions can be expected.

Proteomic data analysis typically includes the following steps described below.[99]

5.7.1. Data processing

This includes signal processing, ensuring that high sensitivity, resolution and mass accuracy are fully retained and exploited during downstream data analysis[99] and conversion of signals into appropriate and preferably standardized data formats.

5.7.2. Peptide identification

Assignment of MS/MS spectra to peptide sequences. This is achieved by using a database engine such as Sequest, Mascot, Comet, Xltandem or Spectrum Mill. In general, the underlying algorithms are matching and scoring the experimental MS/MS spectra with predicted mass to charge ratios of fragment ions of peptide sequences derived from protein databases.[99] High quality experimental data allow for more effective searches.

5.7.3. Validation

False discovery rates (false positives) are a major problem in proteomics and can be caused by: (1) the statistical process used to identify significant protein signal differences, and (2) the algorithms used for identifying the structures of such proteins. For example, 2D gels from treatment and controls or from different treatment groups are usually compared using multiple Students' t tests with a significance threshold of 0.05%. This means that, theoretically, 5% of protein spots may be falsely identified as different.[100] False discovery rates can be reduced by more robust experimental design, improved quality of samples and analysis, the use of technologies that allow for a direct comparison of proteomes such as DIGE and labeling[100–102] and the use of appropriate sample sizes.[103] Another major source of errors is protein identification. This is caused by the fact that several peptides may be common to more than one protein. Thus, it is important to assess the validity of the protein assignment and to associate a probability with the identification. Naturally, if more peptide matches for a specific protein can be identified, then there is greater confidence in its correct identification. Statistical procedures are available that estimate the rates of false positive and false negative errors. The PeptideProphet is an example of an algorithm that has been developed to achieve this goal.[104] Alternative approaches such as 'reversed database' searches have been explored.[105] In addition, presently available databases are still fraught with problems such as redundancies, inconsistencies in nomenclature, fused genes and inappropriately translated introns.[106] Overall, it has to be noted that a positive 'hit' and its associated proposed structure can only be viewed as a hypothesis. Important 'hits' should always be confirmed using independent technologies such as Western blot.

5.7.4. Quantification

Quantification and protein identification may not be possible in the same experiment. Quantification is carried out at the peptide level. There are two major strategies:[99] stable isotope labeling of the proteomes and comparison of corresponding peptides across multiple LC MS runs of individual proteomes based on the relative signal intensity in the full mass spectra. While after stable isotope labeling multiple proteomes are compared in the same analytical run, the unlabeled approach requires more rigorous control of the analysis and data collection conditions, including control of instrument drifts during multiple analytical runs (mass calibration, elution times) and

normalization of ion abundances to compensate for differences in instrument performance (e.g. accumulating contamination of the ionization source).

5.7.5. Annotation

Putting the results into context with existing knowledge about molecular interaction networks such as metabolic pathways and signaling pathways is an important last step. Reference databases including information about the urine proteome are publicly available.[107 108]

Analysis tools for tandem mass spectrometry based proteomics are summarized by Nesvizhskii et al.[109]

5.8. Normalization of urine data

Protein concentrations in urine depend on the dilution of urine samples and, thus, will require normalization.[24] Originally, creatinine filtration into urine was believed to be constant and has been used extensively to compensate for variation in urine dilution.[110] It has become evident, however, that creatinine concentrations in urine can be affected by tubular excretion, aging, gender and disease processes. As an alternative strategy, normalization based on urinary 'housekeeping' peptides that are ubiqui tously present in human urine, and seem to be more robust than creatinine, have been used and recommended for normalization.[111,112] These are only single studies and there does not seem to be general consensus. Given the fact that this is a critical problem, there has been surprisingly little discus sion, and more systematic studies assessing this important issue are lacking.

5.9. Validation of analytical assays, quality control and standardization

Since non biased proteomics approaches are not truly quantitative, valida tion is limited to assessing sample stability and reproducibility. It has been estimated that the technical variation in 2D gel electrophoresis typically is associated with a coefficient of variance of 20–30%.[113] In the case of immuno based assays, the interaction of the antibody with the analyte will require validation including potential cross reactivities and interferences with other drugs, metabolites or matrix components. It has to be noted that disease processes may interfere with immuno based assays, for example, by changing the hematocrit, increasing endogenous compounds, such as lipids and bilirubin, and formation of antibodies, such as rheumatoid factor.[114 116] Such interferences may not be noticed if an assay is exclusively

validated based on samples from healthy subjects. A potential problem especially with more exploratory type semi quantitative immuno based multiplexing assays is also the batch to batch comparability of the anti bodies. A semi quantitative comparison of results generated at different times or in different laboratories may not always be possible. It is important to note that the analysis of macromolecules is inherently more variable. Based on generally accepted guidelines for the HPLC/UV and LC MS analysis of small molecules, an assay is only acceptable if, except at the lower limit of quantitation, inter day precision is $\leq 15\%$ and inter day accuracy is within $\pm$ 15% of the nominal value. Accordingly, an analytical run of study samples is accepted if at least two thirds of the quality control samples fall within 15% of their nominal value. For the quantification of macromole cules using immuno based assays, regulatory agencies have accepted limits of $\pm$ 25% and $\pm$ 30%.[117]

It must be realized that current regulatory guidelines have been written mostly with the quantification of single drug compounds in mind and may be too rigid for emerging multiplexing technologies.[84] In fact, it has been suggested that biomarker methods should not be classified as 'good laboratory practice' assays; nor should they be validated by the same guiding principles developed for drug analysis by HPLC/UV or LC MS.[117,118] The challenge with multiplexing assays is that several compounds are quantified simultaneously and that it is not possible to optimize the assay for each compound to the extent that is possible for analysis of single compounds. On the other hand, there is potential benefit in the additional information conveyed by molecular marker assays because the risk—benefit ratio must be evaluated using different criteria than with standard assays designed to measure single drug compounds. This has been recognized by regulatory agencies. United States FDA guidelines have suggested that 'further research is needed to establish the validity of available tests and determine whether improvements in biomarkers predict clinical benefit'.[119]

Proteomics studies in similar patient populations with the same diseases have generated different protein fingerprints and have identified different sets of potential protein markers. The reasons may include differences in sample handling, preparation and analytical technologies for proteome analysis. In fact, most proteomics based publications cannot be compared, thus greatly reducing their value.[4] The problems with inter laboratory comparability of data was further emphasized by a cross validation study involving 27 proteomics laboratories conducted by the Human Proteome

Organization Sample Working Group.[120] An equimolar test sample containing 20 highly purified recombinant proteins and tryptic peptides of 1250 kDa size was distributed to the test laboratories. Only seven laboratories reported all 20 proteins correctly and only one laboratory reported all of the 1250 kDa tryptic peptides. Missed identifications (false negatives), environmental contaminations, database matching and curation of protein identification were identified in this study as the major problems with this process.[120] Interestingly, after the problems had been identified, laboratories that missed proteins during the original analysis identified all 20 proteins correctly during a second round, emphasizing the importance of experience and expertise in this complex field.[102]

The task of truly understanding proteomes and their association and mechanistic relationships with the different stages of disease development is monumental and cannot be carried out by a single laboratory. Therefore, sharing of proteomics datasets and their deposit into databases will be important and will greatly facilitate generating a larger understanding of the proteome and to translate this proteomics data into clinical benefits.[121] Today, consortia play a critical role in the development, qualification and acceptance of a molecular marker. Consortia depend on pooling and comparison of data from different sites.[122,123] The quality of such databases and the validity of the decisions derived from such data greatly depend on the quality of the individual datasets. A critical tool is the cross validation of the laboratories involved in the measurement of molecular markers to control data quality and to ensure comparability. Since successful cross validation for single analytes can already be a challenging task, it can be expected that consistent measurement of more complex analyte mixtures in multiple laboratories is even more difficult.[102,120]

Standardization will be required to ensure comparability of data. This will also have to include stringent quality control to avoid the 'dilution' of databases by inferior datasets.[4,107] Since the correct identification of proteins is greatly improved with the quality of the spectral data, such quality criteria should include minimum requirements for mass accuracy and mass spectrometry resolution. First steps towards standardization of sample collection, processing and proteomics analysis have been taken at consensus conferences and through publication of guidelines,[124–127] as well as by the establishment of the Human Kidney and Urine Proteome Project (HKUPP).[128,129] Proteomics associations, such as the Human Proteome Organization (HUPO),[130] support proteomics efforts through systematic research in sample handling, technologies, procedures, protocols and defining

standards.[57,131,132] Standards also include study design, infrastructure requirements, minimum information about a proteomics experiment,[133] minimum reporting requirements, standard data formats, common sets of vocabularies and ontologies, annotations and validation guidelines.[134]

6. PROTEOMICS IN RENAL RESEARCH AND AS A MARKER FOR KIDNEY FUNCTION, DISEASE AND INJURY

The use and role of proteomics in the discovery of clinical markers and as a potential clinical diagnostic tool have extensively been reviewed before.[2,24,135,136]

6.1. Identification of disease, pharmacodynamic and toxicodynamic molecular mechanisms

The ability to characterize subcellular, cellular and organ proteomes in an unbiased fashion has led to important insights into biological processes and signal transduction pathways.[17] There are two fundamentally distinct concepts in proteomics. First, the concepts as described above are most important for molecular marker discovery in nephrology and can be termed expression based proteomics. Expression based proteomics seeks to describe the proteome at a given moment and its changes in response to disease or drug challenge. Ultimately, it only results in correlative rela tionships and its use as a clinical molecular marker will require more in depth mechanistic qualification. Second, functional proteomics seeks to assess the interactions of proteins and the interactions within and among protein networks. In many cases, functional proteomics studies complement genetics and functional genomics studies that often lead to a gene product with a putative biochemical function, but a poorly characterized biochemical mode of action.[17] Functional proteomics allows for the iden tification of interacting proteins and for mapping proteins to specific biochemical pathways and protein networks. The methods employed include, but are not limited to, affinity purification, the binary yeast two hybrid approach, phage display technology, protein arrays, tandem affinity purification tags and computational prediction models, some of which are based on the known three dimensional structure and binding motifs. For a more detailed overview, please see Köcher and Superti Furga[17] and Sanderson.[137]

Activity based protein profiling for the functional annotation of enzymes uses site directed, small molecule based covalent probes that can

be used in native biological systems.[138] These probes are designed to target a subset of the proteome with shared principles of binding and/or reac tivity.[138] These probes consist of three components: a binding group that directs the probe towards the target protein, a reactive group (electrophilic or photoreactive) for covalent labeling and an analytical tag. Typically, samples are affinity purified and proteins with the covalent tags are identified by mass spectrometry and database search.[138]

Post translational modifications determine the functionality of most eukaryote proteins and, thus, are critical for understanding the mechanistic role of proteins and the functionality of protein networks. Post translational modifications are covalent processing events that change the properties of a protein by proteolytic cleavage or by the addition of a modifying group to one or more amino acids.[139] Post translational modifications may deter mine a protein's activity state, localization, turnover and interactions with other proteins. Standard proteomics approaches are usually not suitable for identifying or mapping post translational modifications and specific puri fication procedures, analytical technologies or databases that have been developed. For detailed reviews, see Mann and Jensen,[139] Witze et al,[140] Ruttenberg et al[141] and Hoffert and Knepper.[142]

Proteomics has been used to study the role of proteins – protein functions, interactions and protein networks – in the physiological func tions of kidney cells and to study disease mechanisms in the kidney. The most important studies include the:

- role of calmodulin in glucose uptake in human mesangial cells;[143]
- role of proteins in the physiology and function of glomerular cells;[144]
- role of proteins in the function of the renal tubule;[145]
- adaptive response of the tubule to acidosis;[146]
- adaptation of cells in Henle's loop to osmotic stress;[147]
- role of proteins in the physiology of collecting duct cells;[148]
- regulation and function of aquaporin 2 in collecting duct cells;[149–154]
- adaptation of collecting duct cells to osmotic stress;[155,156]
- evaluation of molecular mechanisms underlying renal fibrosis;[157,158]
- evaluation of molecular mechanisms of nephropathies;[159–162]
- cytotoxicity of calcium oxalate monohydrate.[163,164]

The use of proteomics for evaluating molecular mechanisms underlying physiological processes in the kidney and their response to disease and xenobiotic challenges provides the basis for protein marker discovery and qualification.

6.2. Acute and chronic kidney injury

Today, kidney biopsies are still the gold standard for the diagnosis of chronic and/or acute kidney diseases.[165] It has been discussed that proteomics analysis of urine samples can be considered a non invasive biopsy. Indeed, data in the literature suggests that, in the future, in many cases, urine may replace kidney biopsies or at least may provide guidance for when a biopsy should be collected. Renal biopsies have their limitations. They require an invasive procedure that usually involves hospitalization, re sampling is difficult, depending on a patient's habitus it may be impossible to collect a biopsy and, although they are helpful as a diagnostic tool, biopsies do not always provide guidance in terms of treatment and prognosis. Urine pro teomics may overcome several of these shortcomings. Urine is easily available, is obtained non invasively and does not require hospitalization, can be frequently sampled, can be collected from any patient unless anuric, can be used to closely guide treatment and can be used to monitor treatment efficacy, tolerability, disease progression or recovery.[165]

Proteomics has been used to establish and qualify animal models, understand renal disease mechanisms, for molecular marker discovery in animal models[166–168] and in clinical studies. Clinical discovery studies have mostly focused on the urine proteome. Representative studies are summarized in Table 4.4.

Proteomics, in many cases in combination with transcriptomics studies, have suggested several promising novel molecular markers for the clinical investigation of acute, and possibly chronic, kidney injury.[13,178] Many kidney proteins appearing in urine during injury are either proteins that are usually reabsorbed in the proximal tubules, released by cell damage, leaked into the urine during inflammation or immune reactions or are repair proteins that are formed and released during the healing process. The most important urinary proteins that have been described as kidney injury markers in the literature are summarized in Table 4.5. Most of these can be measured by ELISA or protein multiplexing assays,[13] and for some, such as neutrophil gelatinase associated lipocalin (NGAL), assays on analytical platforms established in clinical laboratories are either already available[246,247] or are currently under development. Although Table 4.5 lists single molecular markers, the rational design of a panel of these markers seems superior to the analysis of individual biomarkers. If designed correctly, analysis of such a panel will also result in information regarding the nature and location of kidney injury (see Figure 4.4, page 111) and repair processes.

Table 4.4 Selected clinical proteomics studies for the discovery of renal disease protein markers

Diagnostic target	Study population	Matrix	Analytical technology	Identified correlations	Reference
Steroid resistant/ steroid sensitive nephrotic syndrome; minimal change disease, focal segmental glomerulosclerosis	Pediatric and adult patients, 19 subjects in remission, 19 with relapse, 5 with orthostatic proteinuria	Urine	SELDI TOF	Five peaks were found that distinguish steroid resistant from steroid sensitive patients (mass/charge 3917, 4155, 6330, 7037 and 11117) The peak with mass/charge 11117 was identified as β_2 microglobulin No other proteins were identified	169
Steroid resistant/ steroid sensitive nephrotic syndrome	25 patients with idiopathic nephrotic syndrome, 17 control patients	Urine	SELDI TOF	A protein with the mass of 4144 daltons was identified as the most important qualifier The structure of this protein was not identified Nephrotic syndrome patients were distinguished from controls with 92.3% sensitivity and 93.7% specificity 100% of the steroid resistant and sensitive patients were classified correctly	170
Fanconi syndrome	7 pediatric patients with cystinosis, 6 patients with isofosfamide induced Fanconi syndrome, 45 patients with other renal diseases	Urine	CE MS	24 peptides and proteins in the urine samples from Fanconi syndrome patients differed significantly from the controls Structure of 9 of these 24 peptides were successfully identified using an iontrap orbitrap hybrid mass spectrometer Patients with Fanconi syndrome were identified with 89% specificity and 82% sensitivity	171

Contrast nephropathy	12 patients that underwent therapeutic cardiac catheterization and required a radiocontrast agent, two urine samples were collected: before and after the procedure, 31 controls	Urine	DIGE, linear trap mass spectro metry	Compared to pre procedure, 39 protein spots were increased, 17 were decreased 21 of these 56 spots could be identified, all of which represented proteins derived from albumin Among these proteins known to activate the complement pathway were found	172
Diabetic nephropathy	3 patients with diabetic nephropathy, 5 healthy individuals	Urine	DIGE, SELDI	99 spots differed between urine of patients with diabetic nephropathy and healthy individuals 63 spots were higher, 36 lower Protein structures were identified Alpha$_1$ antitrypsin was identified as the most promising marker This was confirmed using an alpha$_1$ antitrypsin ELISA in a different group of patients (19 diabetic patients, 20 healthy individuals)	173
Diabetic nephropathy	4 groups: patients with type 2 diabetes and no micro albuminuria ($n = 45$), type 2 diabetes with micro and macro albuminuria ($n = 38$), proteinuria due to non diabetic disease ($n = 34$), healthy controls ($n = 45$)	Urine	SELDI, protein arrays	In contrast to diabetic patients with proteinuria, a highly abundant protein with a mass/charge of 6188 was present in urine of the other groups A protein with a mass/charge of 14766 was selectively excreted in diabetic patients with proteinuria A protein with a mass/charge of 11774 was selectively excreted in the urine of diabetic and non diabetic patients with proteinuria	174

(*Continued*)

Table 4.4 Selected clinical proteomics studies for the discovery of renal disease protein markers—cont'd

Diagnostic target	Study population	Matrix	Analytical technology	Identified correlations	Reference
				The peak with a mass/charge of 11774 was identified as β_2 microglobulin, the peak with a mass/charge of 14766 as UbA52, a ubiquitin ribosomal fusion protein, and the peak with mass/charge of 6188 was identified as a processed form of ubiquitin UbA52 concentrations in urine were considered the most promising protein marker	
Diabetic nephropathy	Nested case control study including 14 patients with type 2 diabetes and 14 controls (training set) and 17 patients with type 2 diabetes and 17 controls (validation set)	Urine	SELDI	SELDI detected 714 unique urine protein peaks Of these a 12 peak set correctly predicted diabetic nephropathy with 93% sensitivity and 86% specificity Proteins were not identified Urine proteomic profiles identified norm albuminuric individuals with type 2 diabetes who will subsequently develop diabetic nephropathy	175
Diabetic nephropathy	Type 2 diabetic patients without proteinuria ($n = 10$), with microalbuminuria	Urine	DIGE, QTOF	195 protein spots unique to the urine of diabetic patients were found, representing 62 unique proteins	176

	($n = 13$), with macro albuminuria ($n = 13$) and controls ($n = 10$)			These proteins belonged to several functional groups such as cell development, cell organization, defense response, metabolism and signal transduction 7 proteins were found to be progressively upregulated with increasing albuminuria and 4 proteins exhibited progressive down regulation The majority of the marker candidates were glycoproteins	
Diabetic nephropathy	44 type 1 diabetic patients with more than 5 years of diabetes, age matched control group	Urine	CE MS	Overall more than 1000 different polypeptides (800 Da to 66.5 kDa) were found in urine 54 polypeptides were only found in diabetic patients Another set of 88 polypeptides were either present or absent in patients with albuminuria beginning (albumin to creatinine ratio > 35 mg/mmol) Polypeptides were not further characterized	177

2D, two-dimensional; CE, capillary electrophoresis; DIGE, differential gel electrophoresis; iTRAQ, isobaric tags for relative and absolute quantification; MudPIT, multidimensional protein identification technology; QTOF, quadrupole time-of-flight mass spectrometry; SELDI, surface-enhanced laser desorption ionization; TOF, time-of-flight mass spectrometry.

Table 4.5 Important protein markers of kidney dysfunction[13,14,178,179]

Protein	Description	PSTC	References
Calbindin	Calbindin D is a vitamin D dependent calcium binding protein of 28 kDa that is found predominantly in the epithelial cells of the distal tubules of the kidney Nephrotoxic drugs and diseases involving the distal tubule have been shown to change calbindin concentrations in urine		181 184
Clusterin	A glycoprotein first isolated in Sertoli cells Is present in most tissues Is synthesized after tubular injury and protects the tubule Urine concentrations correlate with tubular damage	Yes	185 187
Cystatin C	13 kDa extracellular inhibitor of cysteine proteases. Serum concentrations are independent of gender, muscle mass and age Is freely filtered, reabsorbed and catabolized by the proximal tubule; there is no active excretion Urinary cystatin C concentrations are elevated in patients with tubular injury	Yes	188,189
Cystein rich Protein (Cyr 61)	Is a heparin binding protein that is secreted and associated with cell surfaces and extracellular matrix Was found to be secreted in the straight proximal tubulus only a few hours after injury It must be considered a limitation that urinary concentrations were found to decrease over time although kidney injury was progressing		190,191
Epidermal Growth Factor (EGF)	EGF is a 53 amino acid peptide that is produced by the ascending portion of Henle's loop and by the distal convoluted tubule It seems to modulate tissue response to injury in kidneys with tubulo interstitial damage		192 195

α glutathione *S* transferase (α GST)	Cytosolic enzyme in the proximal tubule The appearance of α GST is due to leakage of cytosolic content into the urine, dying cells or due to shedding of viable or apoptotic cells into the urine		196 199
π glutathione *S* transferase (π GST)	Cytosolic enzyme in the distal tubule and collection duct Is released into the urine likely via the same mechanisms as α GST Has been used together with α GST to differentiate between proximal and distal tubule damage		200,201
Interleukin 18	IL 18 is a pro inflammatory cytokine and its 24 kDa precursor is cleaved in the proximal tubule Urinary concentrations predict delayed transplant kidney function and acute kidney injury and correlated with its severity Seems most sensitive to ischemic injury and seems less (or not) affected by nephrotoxins, chronic kidney disease and urinary tract infections The association between urinary and blood IL 18 concentrations is unknown		179, 202 204
Kidney injury molecule 1 (KIM 1)	A type 1 trans membrane protein not detected in normal kidney tissue Is expressed at very high levels in cases of dedifferentiated proximal tubulus cells, after ischemic or toxic injury and in cases of renal cell carcinoma A soluble form of cleaved KIM 1 can then be detected in urine	Yes	205 207
Liver type Fatty Acid Binding Protein (L FABP)	Liver fatty acid binding protein is a 14 kDa protein that is normally expressed in the kidney proximal convoluted and straight tubules Increased urinary L FABP concentrations were found in patients with acute kidney injury, non diabetic chronic kidney disease, early diabetic nephropathy, idiopathic focal glomerulosclerosis and polycystic kidney disease A challenge is that due to its size L FABP can be filtered, but is mainly taken up by the proximal tubule; there is some evidence that plasma concentration may not affect urine concentration		208 211

(*Continued*)

Table 4.5 Important protein markers of kidney dysfunction[13,14,178,179]—cont'd

Protein	Description	PSTC	References
Microalbumin	Established molecular marker defined as urinary albumin concentrations between 30 and 300 mg/L Although originally believed only to be a measure of intra glomerular pressure and/or structural changes of the glomerular basement membrane, there is evidence that glomerular membranes normally leak albumin and that albumin is retrieved by the proximal tubulus and thus may also be a marker of proximal tubule function	Yes	212 214
β_2 microglobulin	It is the 11.8 kDA light chain of the MHC I molecule expressed on the surfaces of nucleated cells Its monomeric form is filtered and reabsorbed in the proximal tubule Has been shown to be an early marker of tubular dysfunction	Yes	215 217
N acetyl β glucosaminidase (NAG)	NAG (> 130 kDa) has proximal tubule lysosomal enzyme sensitivity; subtle alterations in the epithelial cells in the brush border of the proximal tubule result in shedding of the enzyme into urine Increased NAG concentrations in urine have been found after exposure to nephrotoxic drugs, in patients with delayed renal allograft function, with acute kidney injury, with chronic glomerular disease, with diabetic nephropathy and following cardiopulmonary bypass		218 221
Neutrophil gelatinase associated lipocalin (NGAL)	NGAL is a lysosomal enzyme that seems to play a role in apoptosis, triggers nephrogenesis by stimulating the conversion of mesenchymal cells into kidney epithelium and, in the kidney, is mainly located in the proximal tubule Its size is about 25 kD and it is protease resistant; it is filtered by the kidney and its plasma/urine concentration relationship will require further clarification There is evidence that NGAL may be useful as a sensitive and predictive marker of ischemia/reperfusion, acute kidney injury, nephrotoxicity and chronic kidney disease		222 224

Osteopontin	Is synthesized at highest levels in bone and epithelial tissues ('44 kDa bone phosphoprotein') Is found at relatively high concentrations in urine and is believed to act as an inhibitor of mineral precipitation and stone formation In human and rodent kidneys, expression is limited to the thick ascending loop of Henle and distal convoluted tubules Was found upregulated in rodent models after kidney injury such as ischemia/reperfusion and drug nephrotoxicity	225 228
Retinol binding Protein	A 21 kDA protein that is synthesized in the kidney and is involved in vitamin A transport It is freely filtrated and reabsorbed in the proximal tubule Plasma and urine concentrations may be associated and vitamin A deficiency may cause false negatives	216,229
Podocin	Podocin is a stomatin family member and is an important component of the glomerular slit diaphragm complex which co localizes and interacts with nephrin and CD2AP in the lipid rafts of the podocyte foot process cell membrane Damage to the podocyte releases podocin into the urine Its mRNA in urine has also been shown to be a molecular marker of kidney dysfunction	230 233
Tissue Inhibitor of Metalloproteinase 1 (TIMP 1)	TIMP 1 (28.5 kDa) is an inhibitor of matrix metallo proteinases and is expressed in the proximal tubule TIMP 1 mRNA and protein is upregulated in different models of renal disease and human sclerotic glomeruli	234 237

(*Continued*)

Table 4.5 Important protein markers of kidney dysfunction[13,14,178,179]—cont'd

Protein	Description	PSTC	References
Trefoil factor 3	Mainly expressed in the gastrointestinal mucosa; the trefoil protein family is typically involved in cell protection and repair; in the intestine, trefoil factor 3 protects the mucosa from the degradation effect of HCl by stimulating the goblet cells to synthesize mucin Although the exact role of trefoil factor 3 in the kidney is not yet published, it can be expected to have a protective role in the kidney	Yes	238 240
Vascular endothelial growth factor (VEGF)	Vascular endothelial growth factor (VEGF) is an important stimulator of angiogenesis; circulating and urinary VEGF levels have been suggested as clinically useful predictors of tumor behavior VEGF is also a mediator during inflammation Urinary VEGF seems to be of advantage over plasma since venepuncture activates platelets and may release cytokines, including VEGF, artificially elevating measured VEGF levels VEGF concentrations in urine were found to be associated with alloimmune processes against kidney transplants VEGF may be involved in remodeling after injury leading to increased urinary concentrations		241 245

PSTC, Predictive Safety Testing Consortium; see also European Medicines Agency, Committee for Medicinal Products for Human Use.[180]

6.3. Nephrotoxicity and drug development

Presently, regulatory agencies still rely primarily on traditional markers such as creatinine concentrations in serum, blood urea nitrogen, kidney histology and estimated glomerular filtration rates to assess a drug candidate's nephrotoxic potential during preclinical and clinical drug development. The problem is that these are very insensitive markers with rather poor predictive value. As already mentioned, an increase in serum creatinine considered clinically relevant will require significant kidney damage.[178] Serum creatinine is not a specific marker, depends on many other factors and will be delayed in rising if glomerular filtration is not the primary target of a disease or toxicity process. Likewise, changes in histology and blood urea nitrogen are rather late markers that require significant kidney damage before they can be appreciated. There is consensus that there is an urgent need in drug development for better molecular markers that have better sensitivity and specificity.[248] There is evidence that metabolomics and proteomics based kidney injury markers are more sensitive, specific and predictive than the currently established markers.[249 251]

The use of proteomics in toxicology has also been referred to as 'toxicoproteomics'.[250] Toxicoproteomics is a promising concept for two reasons:

- While at this time in drug development the drug target, and therefore the pharmacodynamic mechanisms, are known early in the process, toxicity is usually detected during the later stages of preclinical development or, in many cases, during clinical development. The challenge is that the unknown toxicodynamic mechanism ('mechanistic toxicology') remains to be identified.[251] Proteomics is a powerful strategy to achieve this goal.
- Proteomics can be used for marker discovery or as a molecular marker itself that can be used to support drug development.

As of today, proteomics has been used to evaluate the toxicity of the following xenobiotics:[250] 4 aminophenol, cisplatin, ciclosporin,[252,253] dichlorovinyl L cysteine,[254,255] gentamicin, puromycin and uranium.[256] For a comprehensive review of toxicoproteomics, see Merrick and Witzmann.[250]

The United States FDA and European Medicines Agency (EMEA) recently approved a set of seven urinary proteins as biomarkers of nephrotoxicity that were submitted by the Predictive Safety Testing Consortium (PSTC) in collaboration with multiple pharmaceutical companies to the

Voluntary Exploratory Data Submission (VXDS) committee of the United States FDA.[180] These biomarkers are for regulatory use in certain preclinical settings[180] and are discussed in Table 4.5. These markers are urinary total protein, albumin, β_2 microglobulin, cystatin C, kidney injury molecule 1 (KIM 1), clusterin and trefoil factor 3. Data indicating that these markers add information to serum creatinine and blood urea nitrogen and that, as indicated by receiver operating characteristics, six of the seven out performed one or both of the established clinical markers, were submitted. The submission was supported by data of up to 14 day GLP (good labo ratory practice) toxicology studies in rats, validation reports of the analytical assays and a review of the scientific literature; however, several limitations were acknowledged in the EMEA/FDA document.[180] These include a lack of data demonstrating that these molecular markers can be used for monitoring the evolution of kidney changes over time, can be used for monitoring the reversibility of the injury and kidney recovery, can be transferred to other species and can be recommended for general use for monitoring of nephrotoxicity in a clinical setting.[180] Incremental qualifi cation potential was acknowledged and will require the submission of additional data. It is interesting to note that several kidney function markers listed in Table 4.5 were not included and also that the choice of molecular markers is essentially focused on the proximal tubulus and does not allow for mapping of the damage to a specific location in the kidney.

Despite the limitations, approval of these kidney injury protein markers has been considered a 'door opening safety biomarker success story'[257] and is a good example of how molecular marker tools for drug development can be expected to be developed, reviewed and approved in the near future.

6.4. Kidney transplantation

Over the past 30 years, 1 year outcomes after kidney transplantation have markedly improved. Despite this success, however, long term graft survival has not changed significantly. The survival half life for renal allografts from deceased donors is approximately 8–12 years and the pathogenesis of these graft losses is multifactorial. The early detection of injury to the transplant kidney is critical to minimize permanent injury and to maintain long term function.[258] Among many factors that can damage a transplant kidney, allograft immune reactions, infections such as BK virus, recurrent or de novo glomerulopathy and immunosuppressant nephrotoxicity are most impor tant.[259] These contribute to chronically progressive scarring processes and

ultimately allograft dysfunction. Currently, serum creatinine is still the gold standard biomarker for monitoring kidney transplant patients. Unfortu nately, the rise of creatinine in serum is a late event and occurs when kidney function is already severely and often irreversibly impaired. As previously discussed, serum creatinine also lacks specificity and a subsequent biopsy must be procured. But even biopsies are not necessarily conclusive. Biopsies face many dilemmas: they sample only a fraction of the kidney but injury processes are often patchy, different injury processes may present similar histological changes, and histology analysis and grading does not use objective metrics.[260] Urine represents an average of the processes occurring at a given moment in the kidney and proteomics metrics is objective and non biased. Therefore, the development of new molecular marker strategies for the specific and early detection of anti allograft immune processes and immunosuppressant toxicity has generated substantial interest in the field of transplantation. The proteomes of interest are plasma and serum, mostly used for the detection and monitoring of alloimmune processes, kidney biopsies, to provide better discrimination between underlying injury processes and to complement histology, and urine, for the monitoring of kidney injury caused by immune processes, disease processes and drug toxicities.

There are two approaches to monitor alloimmune reactions against a renal allograft: one is to monitor aspects of the immune system, which includes alloimmune recognition and activation pathways as well as the effector pathways of inflammation. For a comprehensive list of individual markers, please see Gwinner.[261] The other approach is to monitor kidney injury markers. Rejection is a complex, heterogeneous and variable process. Often insufficient sensitivity and specificity in the use of single rejection markers has led to the concept of combining markers. This is not limited to the mathematical fact that this will increase the overall sensitivity and specificity. From a biological perspective, appropriately designed sets of markers can also capture variation in the rejection process better.[261]

The effect of kidney injury on urine, kidney biopsies and plasma pro teomes has been studied in animal models[262] and in multiple clinical trials using non targeted proteomics. Representative studies are summarized in Table 4.6. In addition, targeted approaches based on one or several of the protein kidney injury markers described above (listed in Table 4.5) have shown promising results.

Proteomics has also been used to gain a better mechanistic under standing of immunosuppressant toxicity. It is interesting to note that after being the pillar of most immunosuppressive drug regimens for almost thirty

Table 4.6 Identification of proteomics-based biomarker for diagnosis after kidney transplantation in clinical studies

Diagnostic target	Study population	Matrix	Analytical technology	Identified correlations	Reference
Acute renal allograft rejection	17 patients with and 15 patients without rejection	Urine	SELDI TOF	5 polypeptides of 6.5, 6.6, 6.7, 7.1 and 13.4 kDa were identified that allowed for classification Those peptides/proteins were not further identified Sensitivity 83%, specificity 100%	263
Acute renal allograft rejection	19 patients with different grades of rejection (Banff 1997 Ia to IIb), 10 patients with urinary tract infection, 29 patients without rejection, 66 non transplant subjects	Urine	CE TOF	17 urinary polypeptides discriminated between renal transplant patients and non transplant patients 10 between urinary tract infection and samples without infection or rejection (control) 16 between renal allograft patients with and without rejection 10 between acute allograft rejection and urinary tract infection 1 protein differentiating between healthy subjects and renal allograft patients, a fragment of collagen alpha 5(IV) protein, was identified	264
Acute renal allograft rejection	23 patients with and 22 patients without rejection, 20 healthy subjects	Urine	SELDI TOF	7 polypeptides of 2.0, 2.8, 4.8, 5.9, 7.0, 19.0 and 25.7 kDa were identified that allowed for classification	265

				Acute rejection could be distin guished from stable renal allograft patients with sensitivity of 90.5 91.3% and specificity of 77.2 83.3% A protein of 78.5 kDa was found that distinguished between renal allograft patients and healthy subjects. Sensitivity and specificity were 100%	
Acute renal allograft rejection	18 patients with and 22 patients without rejection, 5 patients with tubular necrosis, 5 patients with glumerulopathy, 5 non transplant patients with urinary tract infections, 28 healthy subjects	Urine	SELDI TOF	Patients with rejection showed prominent peak clusters in regions of m/z = 5270 5550, 7050 7360 and 10530 11100 In urine from normal subjects, those clusters were missing 82% from the stable transplant group and 6% from the acute rejection group did not show those clusters The peptides/proteins in the clusters were further structurally identified by Schaub et al[267] and were found to be mostly associated with β_2 microglobulin	266

(*Continued*)

Table 4.6 Identification of proteomics-based biomarker for diagnosis after kidney transplantation in clinical studies—cont'd

Diagnostic target	Study population	Matrix	Analytical technology	Identified correlations	Reference
Acute renal allograft rejection	34 samples were collected from 32 renal transplant patients, 17 of these samples were from 15 patients with acute rejection	Urine	SELDI, protein chip arrays	45 protein peaks of interest were identified 16 of these peaks showed promise as candidate molecular markers to detect acute rejection 13 of these proteins (3.4, 4.1, 6.5, 6.6, 6.7, 7.0, 7.1, 7.3, 7.5, 7.8, 8.0, 10.8 and 13.4 kDa) were present in the majority of urine samples during rejection, but absent in non rejec tion samples 3 proteins (9.0, 9.7 and 9.8 kDa) were present in non rejection urine samples, but were absent in samples collected during rejection Urine samples collected during rejection could be distinguished from those without rejection with a sensitivity of 91.3%	268
Renal allograft rejection	Patients with biopsy confirmed acute rejection ($n = 12$), chronic rejection ($n = 12$), stable graft function ($n = 12$) and healthy individuals ($n = 13$)	Serum	MALDI TOF	18 differential peptide peaks were selected as potential molecular markers for acute allograft rejection 6 differential peptide peaks were selected as potential molecular markers for chronic rejection	269

				The peptides were only identified by their m/z and not further characterized A classifier algorithm recognized 82.6% of acute rejections and 99.0% of chronic rejection episodes correctly	
Renal chronic allograft dysfunction	32 patients with chronic allograft dysfunction, 14 with interstitial fibrosis, 18 with chronic active antibody mediated rejection, 18 controls: 8 stable renal transplant patients and 10 healthy individuals	Urine	MALDI TOF	14 proteins ions were identified that discriminated between the samples from patients with interstitial fibrosis and chronic rejection 100% of both patient groups were identified correctly These proteins were characterized only by their mass/charge and were not further identified	270
Stable kidney transplant patients	Serial urine samples from healthy individuals, kidney donors before and after surgery ($n = 20$), recipients immediately after surgery, kidney transplant patients 1 month to 4 years after transplantation ($n = 16$)	Urine	MALDI TOF, iTRAQ	Several protein peaks were detected that were associated with trans plantation (mass/charge: 3370, 3441, 3385, 4303, 10350, 11732) The protein with a mass/charge of 11732 was β_2 microglobulin, none of the other proteins was identified Although there were differences in the urinary protein patterns among individuals, longitudinal comparison	271

(*Continued*)

Table 4.6 Identification of proteomics-based biomarker for diagnosis after kidney transplantation in clinical studies—cont'd

Diagnostic target	Study population	Matrix	Analytical technology	Identified correlations	Reference
				of protein patterns in the same individuals over time suggested that individual urine protein patterns are remarkably stable It was concluded that the longitudinal follow up of urinary protein patterns in individual patients may be a sensitive biomarker	
Renal allograft rejection	Patients with biopsy proven interstitial fibrosis and tubular atrophy, stages 0 ($n = 8$), I ($n = 8$) and II/III ($n = 8$)	Urine	2D DIGE, MALDI TOF, CE linear ion trap	62% of the urinary proteins detected were identified using mass spectrometry and database searches 44% were secreted, 17% membrane, 13% plasma and 10% cytoplasmatic, 3% cytoskeletal and 1% mitochondrial proteins 19 proteins with differential concentrations depending on the stage of renal graft injury were found Among those were β_2 microglobulin, MASP 2, α 1B glycoprotein, leucine rich α 2 glycoprotein 1, α 1 antitrypsin, immunoglobulin lambda light chain, transferrin and Zn α 2 glycoprotein	272

Chronic allograft nephropathy	Two cohorts with a total of 77 renal allograft patients with mild or moderate/severe chronic allograft nephropathy as confirmed by biopsy	Density purified blood cells	MudPIT in combination with linear ion trap	The study used a proteogenomic approach 302 proteins unique to mild and 509 proteins unique to moderate/severe chronic allograft nephropathy were detected and identified using data base searching	273
Differential diagnosis of kidney dysfunction after renal allograft	2 sets of Banff'97 graded biopsies. Set 1: 4 Banff 0, 4 Banff 1 and 5 Banff 2,3; set 2: 4 Banff 0, 5 Banff 1, 10 Banff 2,3	Renal biopsies	MudPIT in combination with linear ion trap	Genome wide expression analysis was conducted in parallel Proteins were mapped to multiple pathways including immune response, inflammatory cell activa tion and apoptosis as observed during chronic rejection Extent of changes increased with the severity of renal allograft injury	274

2D, two-dimensional; CE, capillary electrophoresis; DIGE, differential gel electrophoresis; iTRAQ, isobaric tags for relative and absolute quantification; MALDI, matrix-assisted laser desorption/ionization; MudPIT, multidimensional protein identification technology; SELDI, surface-enhanced laser desorption ionization; TOF, time-of-flight mass spectrometry.

years, the basic mechanisms of calcineurin inhibitor toxicity are still not fully understood. This is even more surprising if considering that calcineurin inhibitor toxicity constitutes a serious limitation of the use of these drugs in transplantation and for the treatment of immune diseases. Proteomics has proven to be a powerful approach to elucidate the underlying molecular mechanisms. The following two studies are representative examples. Puigmulé et al. studied the effect of ciclosporin on HK 2 cells, a model of human proximal tubule cells, which is the major target of ciclosporin nephrotoxicity.[252] A total of 38 proteins was found changed, relating to protein metabolism, response to damage, cell organization, cytoskeleton, energy metabolism, cell cycle and nucleotide metabolism. Klawitter et al treated rats with the calcineurin inhibitor ciclosporin, the proliferation signal inhibitor sirolimus and a combination of both drugs for 28 days.[253] Drug exposure was in the target range of transplant patients. Sirolimus is not considered nephrotoxic alone, but it synergistically enhances ciclosporin nephrotoxicity. The effects of the immunosuppressants were evaluated using a proteo metabonomic strategy. Ciclosporin affected the following cell processes and related proteins (partially confirmed by Western blot): calcium homeostasis (e.g. regucalcin, calbindin), cytoskeleton (e.g. vimentin, cal desmone and actin binding protein), hypoxia and mitochondrial function (prolyl 4 hydroxylase, proteasome, NADH dehydrogenase) and cell metabolism (e.g. kidney aminoacylase, pyruvate dehydrogenase, fructose 1,6 bis phosphate). Several of the changes in protein expression were associated with corresponding changes in metabolite concentrations in urine and explained why these changes occurred. Sirolimus alone also changed protein expression in the kidney and enhanced the effects of ciclosporin. This study demonstrates the value of combining proteomics and metabolomics to mechanistically qualify urine metabolite markers and the value of a proteo metabonomic approach to study and predict toxicodynamic drug–drug interactions.[253]

6.5. Cancer

Renal cell carcinoma is the most common cancer of the kidney. The main histological subtypes are clear cell, papillary and chromophobe renal cell carcinoma.[275,276] Renal cell carcinomas are often diagnosed at a later stage when approximately 40% of the patients already have local or advanced metastasis. The prognosis of patients with metastatic disease is poor with a 5 year survival of less than 10%. Renal tumors are a challenge for the

pathologist since most common benign and malignant renal tumors cannot easily be distinguished.[275,276]

There are many opportunities where proteomics can contribute to the diagnosis, treatment and monitoring of renal cancer patients. Today, histological diagnosis, staging, detection of relapse and monitoring of therapeutic response require either invasive procedures or the use of radi ology and cross sectional imaging.[275,277] Ideally, a comprehensive set of protein tumor markers would have the following characteristics:[278]

- is secreted or shed by the malignant cells;
- can be detected in an easily available body fluid;
- is detected as soon as the tumor becomes active;
- is detected by a simple, robust and sensitive assay;
- can diagnose a tumor with high specificity during the early stages;
- detects the re occurrence of a tumor;
- establishes and monitors therapeutic success;
- correlates with the clinical stage of a tumor;
- is objectively measured and independent of the experience level of the examiner;
- predicts clinical outcome.

Proteomics in combination with tumor cell lines and animal models have been used to better understand tumor biology and treatment response. Clinical proteomics, with the goal of discovering potential protein markers, have been based on biopsy samples as well as urine, serum and plasma. To date, most proteomics studies in renal cancer have focused on tissue samples and clear cell carcinomas. Okamura et al compared the proteomes of samples surgically obtained from the clear cell carcinoma and adjacent normal kidney ($n = 29$ patients).[279] Proteomes (in both samples) from the same samples were directly compared using a labeling approach, peptides were analyzed using MALDI TOF in combination with database search based protein identification and key hits were confirmed by Western blot and quantitative real time PCR. In comparison to the normal tissue, 34 proteins were found at markedly higher concentrations and 58 at lower concentrations in the clear cell tumor tissue. Among the identified proteins, galectin 1 and CNDP2 occurred with high frequency in the tumor tissue.[279]

Relatively few studies have used biological fluids such as urine, albeit results have been encouraging. The combination of serology with proteo mics technologies represents a powerful tool to identify protein markers of renal cell carcinoma.[277,280,281] A good example is a study described by Sakissan et al.[281] The immunogenic protein expression profile of the human

renal cell carcinoma cell line CAL54 was assessed using 2D gel electrophoresis combined with immunoblotting using sera from healthy individuals and patients with renal cell carcinoma. Pro metalloproteinase 7 was identified as a potential marker. An immunoassay was developed, and the sera of 30 healthy individuals, 40 control patients and 30 clear cell carcinoma patients were analyzed. A sensitivity of 93% and a specificity of 75% were found.[281]

Comprehensive lists of proteomics studies and potential protein markers of renal cell carcinoma in different matrices are included in Banks et al,[275] Seliger et al[277] and Kashyap et al.[278]

The urine proteome is also of interest for the discovery of molecular markers for urothelial, ovarian and prostate cancer.[37,40,112,282–284]

6.6. The effects of extra-renal proteome changes on the urine proteome

Since some proteins can cross over from blood into urine, the urine proteome is also affected by extra renal changes. This can provide diagnostic opportunities such as the measurement of VEGF in urine as a tumor marker[242] and the detection of potential protein markers of coronary artery disease in urine.[285] But this also must be considered as a confounding factor, such as changes in the urine proteome caused by cigarette smoking,[286] age,[34] gender and even circadian changes, just to name a few examples. Although the urine proteome can be changed by extra renal factors, this, surprisingly, has not been taken into account in many clinical proteomics studies even if they were based on complex patient populations. This emphasizes the requirement for appropriate qualification studies before a protein marker can be used as a clinical diagnostic tool.

7. PROTEOMICS AS CLINICAL DIAGNOSTIC TOOL IN NEPHROLOGY

During the last decade, proteomics research and clinical proteomics have been expected to lead to new disease and diagnostic markers that translate into new and improved clinical tests[287] (Table 4.7); however, many published clinical studies have led to confusion and constructive discussions about the suitability of technologies, such as protein arrays and SELDI, in clinical practice instead. It is interesting to note that most molecular marker discovery studies are descriptive and have substantial design flaws, such as being hopelessly underpowered and/or not appropriately taking potential confounding factors into account. Many publications provide only mass/

Table 4.7 Purposes of in vitro diagnostic markers[287]

Marker type	Purpose
Acute marker	Used when an acute disease event occurs Helps in the process of differential diagnosis Assists in decision making regarding best treatment option
Screening marker	Identifies the diseased, preferably in a still asymptomatic stage, within a population Assists in decision making for initiation of treatment; early treatment usually correlates with high probability of treatment success Is generally applied in population subgroups with increased risk and disease frequency
Primary risk assessment marker	Assesses the risk that a healthy individual could suffer from a disease, drug effect or environmental challenge in the future
Secondary risk assessment marker	Used to determine how a disease may develop Used to determine the risk that a patient could suffer recurrent disease or secondary complications
Disease staging/ classification marker	Diagnoses and classifies different disease stages
Treatment response stratification marker	Predicts the probability to respond to a drug Predicts tolerability of a drug treatment Assists in the selection of the most effective drug regimen with the best risk/benefit ratio
Treatment or therapeutic monitoring marker	Monitors the long term efficacy of a drug treatment Monitors for the development of potential chronic toxicodynamic effects May guide dosing or long term adjustment/individualization of drug effects
Compliance marker	Provides information on treatment compliance

charge values of peaks of interest without any further protein identification or long lists of protein hits as generated by database searches without further confirmation or qualification. Only rarely have further steps been taken to further develop such markers for clinical diagnostics. Meanwhile, there are regulatory guidelines on how to develop molecular markers into markers that can be used to guide drug development and what is required to develop a marker into a clinical diagnostic tool.[18,122,123,288] Regulatory guidelines clearly distinguish between validation and qualification. As already mentioned, while validation confirms the validity of the analytical assay and that the key performance parameters are within acceptable ranges, qualification is the key to molecular marker development and can be a complex

and daunting task that may require substantial resources. As in the promising field of metabolomics, clinical proteomics research has to shift focus from pure discovery to mechanistic and clinical qualification of the marker discovered to establish its clinical indications, collection schedule, limitations, robustness, sensitivity, specificity and potential predictive value using appropriately validated analytical assays to ultimately meet regulatory acceptance criteria.[18,288]

The idea of building expert systems based on non biased proteome analyses that will generate a holistic view of a patient's plasma and urine proteome, and their combination with other 'omics technologies such as genomics, transcriptomics and metabolomics that will allow for a systems biology based approach to medicine is attractive. It is not unrealistic to expect that this is where the future of medicine lies, especially since this will open the doors for predictive, preventive and individualized medicine; however, the current utilization of truly non biased proteomics as a clinical tool is unrealistic. Even though substantial progress has been made over the last several years, we do not yet have any technologies that will enable a non biased proteome analysis. While the human proteome has more than 200,000 proteins that can be in different states of folding, post translational modification, interaction and allosteric regulation, current proteomics technologies can only capture a few thousand simultaneously at best. Current proteomics approaches have also suffered from the wide dynamic range that is required to measure the whole proteome with regulatory proteins often hidden by highly abundant proteins.[17] As already mentioned, the typical high abundance proteins are removed to unmask those of lower abundance that are considered of more significance for detecting patho biochemical processes. This means that the methods themselves introduce significant bias into proteome analysis. True non targeted proteomics is still a complex multi step process and, besides more targeted approaches, no clinically feasible high throughput technology is available.[8,289] The other problem is that no computational approaches are presently available that convert the highly complex data into clinically useful specific and robust information. One of the reasons is that there is still a significant lack of understanding in the biological meaning of specific changes in protein patterns. This is due to our current lack of understanding of the intra and inter individual variability of pathways, plasma and urine proteomes,[8] as well as where the limits of normal and pathological states are. In addition, valid interpretation will require detailed knowledge of protein interaction patterns.[3] The changes that are caused by a disease or drug are often of high

complexity and will not only affect the primarily targeted pathways, but due to compensatory regulation and cross talk at the cellular, organ and systemic level, as well as secondary processes such as inflammation and oxidative stress, may affect a multitude of different pathways.[26] The current knowl edge of pathways, protein interactions and networks is still incomplete and proteomics itself has proven to be a valuable tool to expand knowledge in this area.

A more realistic approach than non targeted proteomics at the moment is the targeted analysis of known arrays of protein markers that are well qualified.[288] Analyses of such 'combinatorial' markers can be realized using antibody and mass spectrometry based protein multiplexing platforms as described above. In general, specific combinatorial protein patterns will confer more information than the measurement of a single protein and, thus, can be expected to have better specificity and sensitivity. It can be anticipated that such combinatorial markers are composed of 5 to 20 proteins.[288]

As described for metabolite (metabolomics) molecular markers, protein marker discovery, qualification and determination of sensitivity and speci ficity needs to take the time dependency of the proteome changes into account. While the end stage of an injury is usually static or only slowly changing, during the early stages of injury development, the proteome changes can be relatively rapid and extensive. It is important to understand the dynamics of the mechanisms associated, since this will determine sample collection strategies. In addition, it may be necessary to develop several sets of molecular markers that are specific for certain stages of a disease process.

After discovery, a critical part of the development of protein markers into clinical diagnostic tools is the mechanistic and clinical qualification required for regulatory approval.[18] Clinical qualification is based on the determi nation of specificity and sensitivity in clinical trials, usually using receiver operating characteristic (ROC) curves.[290] These assess the performance of the molecular markers often compared to gold standards. For the devel opment of molecular markers, it will be assessed to which extent a certain molecular marker pattern will be successful in predicting the development of a certain symptomatic disease like end stage kidney disease. The problem is that these end stage injuries may alternately be caused by distinct underlying biochemical mechanisms that ultimately cause the same symp toms. Several of these distinct and alternate biochemical processes may not even be fully understood yet, and may require a more detailed classification of the symptomatic disease process. Alternatively, during later stages

symptomatic injuries caused by different drug toxicities and diseases often involve the same pathobiochemical and pathological mechanisms such as mitochondrial dysfunction, the formation of oxygen radicals, necrosis, apoptosis, inflammation and other immune reactions. The further a path ological process progresses, the more difficult it may be to find specific molecular marker changes. One of the problems with the gold standard outcome being less specific than the molecular marker is that there is no 1:1 relationship between a molecular marker and the predicted clinical outcome. Several molecular marker patterns that are caused by distinct biochemical disease processes that ultimately lead to the same symptoms may be valid predictors of a single clinical outcome. Such a scenario will lead to good specificity — a specific marker pattern will be able to reliably predict a certain clinical outcome; however, sensitivity might be poor since the same outcomes caused by other distinct biochemical processes may be missed. Following current practices and regulatory guidances this may lead to the rejection of a valid highly specific molecular marker while, ironically, a less predictive and specific molecular marker that is a surrogate for later and more common disease processes may be acceptable.

Also, there is only poor consensus in terms of definition of the 'end stage disease' endpoint. For example, there are more than 30 different definitions of acute renal failure, or, now, acute kidney injury, in the published liter ature.[13,291] Therefore, it will be difficult to establish sensitivity and speci ficity for a candidate protein marker if the gold standard itself is potentially inconsistent.

Proteomics plays an important role in molecular marker discovery and qualification during their development into potential clinical diagnostic tools.

As evidenced by the regulatory approval of a protein kidney injury marker panel for preclinical rat drug toxicity studies, proteins in urine as diagnostic markers are starting to have an impact.[14,180] It is reasonable to expect that proteins and the analysis of protein panels, especially in urine, will play an increasingly important role as clinical diagnostic tools in nephrology in the near future.

Even if proteomics and systems biology based expert systems may still be unavailable for some years, it is likely that nephrology will be among the first to benefit from progress in proteomics. This is due to the fact that urine, a proximal and non invasive matrix that has a selective proteome, which is in direct communication with, and, to a large extent, reflective of biochemical processes in the kidney, is non invasive and readily available.

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CHAPTER 5

Biomarkers in Acute Kidney Injury

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Acute kidney injury (AKI) is common and the absolute incidence of AKI has increased during the last decade.[1,2] Between 5% and 20% of critically ill patients in the intensive care unit (ICU) have an episode of AKI. Up to 4.9% of critically ill patients in the ICU will require renal replacement therapy. AKI requiring renal replacement therapy in the ICU has a high mortality of over 50%. The commonest causes of AKI are septic shock, ischemia and nephrotoxins.

Acute kidney injury (AKI) has been defined conceptually as a rapid decline in glomerular filtration rate (GFR) that occurs over hours or days.[3,4] In the recently developed RIFLE criteria for AKI, AKI is defined as a 50% increase in serum creatinine over baseline[4,5] (Figure 5.1). The RIFLE

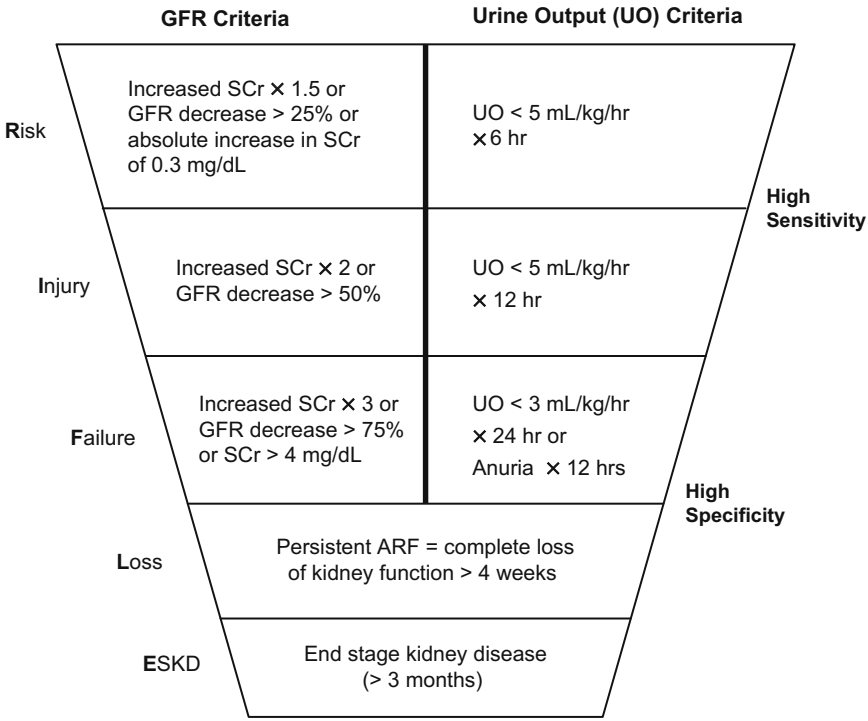

Figure 5.1 ***The RIFLE classification of acute kidney injury (AKI).*** The RIFLE classification of AKI divides AKI into the five stages depending on kidney function as determined by serum creatinine (SCr) and urine output (UO). The RIFLE criteria have been validated in multiple studies. Most biomarker studies in AKI have used the RIFLE or AKIN classification of AKI.

Table 5.1 The acute kidney injury network (AKIN) classification of AKI

Stage	Kidney function	Urine output
Stage 1	Increase in serum creatinine $\geq$ 0.3 mg/dL or increase to $\geq$ 150 199% (1.5 to 1.9 fold) from baseline	$<$ 0.5 mL/kg/h for $\geq$ 6 h
Stage 2	Increase in serum creatinine to 200 299% ($>$ 2 to 2.9 fold) from baseline	$<$ 0.5 mL/kg/h for $\geq$ 12 h
Stage 3	Increase in serum creatinine to $\geq$ 300% ($\geq$ 3 fold) from baseline or serum creatinine $\geq$ 4 mg/dL with an acute rise of at least 0.5 mg/dL or initiation of RRT	$<$ 0.3 mL/kg/h $\geq$ 24 h or anuria $\geq$ 12 h

A change in serum creatinine of 0.3 mg/dL is used to define the presence of AKI and identify patients with Stage 1 AKI (analogous to RIFLE-Risk). In the AKIN classification of AKI, a time of 48 h over which AKI occurs (compared to 1–7 days for the RIFLE criteria) was proposed. Patients receiving renal replacement therapy (RRT) were classified as Stage 3 AKI (RIFLE-Failure). Most biomarker studies in AKI have used the RIFLE or AKIN classification of AKI.

classification of AKI divides AKI into the following stages: (1) Risk; (2) Injury; (3) Failure; (4) Loss of function; and (5) End stage kidney disease[3 5] (Figure 5.1). The term AKI replaces the term ARF (acute renal failure), and ARF is restricted to patients who have AKI and need renal replacement therapy. The RIFLE criteria have been validated in multiple studies.[3 5] The Acute Kidney Injury Network (AKIN) has also developed a classification of AKI.[6] The AKIN group attempted to increase the sensitivity of the RIFLE criteria by recommending that a smaller change in serum creatinine (0.3 mg/dL) be used as a threshold to define the presence of AKI and identify patients with Stage 1 AKI (analogous to RIFLE Risk).[6] In the AKIN classification of AKI, a time of 48 h over which AKI occurs (compared to 1–7 days for the RIFLE criteria) was proposed. In addition, patients receiving renal replacement therapy (RRT) were classified as Stage 3 AKI (RIFLE Failure). Most of the studies referenced in this chapter have used the RIFLE (Figure 5.1) or AKIN (Table 5.1) definitions of AKI.

1. SERUM CREATININE IN ACUTE KIDNEY INJURY

Serum creatinine and blood urea nitrogen (BUN) have typically been used to diagnose AKI. Creatinine is a small molecule of 113 daltons. It is distributed in the body throughout the body water. It is generated in muscle

from the non enzymatic conversion of creatine and phosphocreatine. The reasons why serum creatinine is not sensitive or specific for the diagnosis of AKI will be outlined below.

Interference with the creatinine assay may give false serum creatinine values.[7] The classic method of creatinine measurement is the Jaffe reaction that uses a colorimetric method that detects creatinine as well as non creatinine chromogens in the serum. In the Jaffe reaction, creatinine acts directly with the picrate ion under alkaline conditions to form a red orange complex. Up to 20% of the color reaction may be due to substances other than creatinine, e.g. glucose, uric acid, ketones, cephalosporins, furosemide, hemoglobin, paraproteins, paraquat and diquat.[7] Plasma ketosis and ceph alosporins may result in an increase in serum creatinine due to interference with the picric acid assay for creatinine. Very high bilirubin levels can cause falsely low serum creatinine. The kinetic alkaline picrate method is the most widely used method for creatinine determination in clinical laboratories in the United States. This method reduces interference from non creatinine chromogens.

Serum creatinine may change due to non renal factors independent of kidney function, e.g. age, gender, race, muscle mass, nutritional status, total parenteral nutrition and infection.[7,8] Vigorous prolonged exercise may result in increased serum creatinine due to an increase in muscle creatinine generation. Ingestion of creatine supplements may increase serum creati nine. Ingestion of cooked meat may increase serum creatinine, as during cooking creatine in meat is converted to creatinine which is absorbed by the gastrointestinal tract. Restriction of dietary protein may result in a decrease in serum creatinine. Serum creatinine may change due to renal factors that are independent of kidney function. For example, several medications, e.g. trimethoprim, cimetidine and salicylates, alter the tubular secretion of creatinine leading to changes in serum creatinine independent of GFR.[7,8] In addition, serum creatinine is not sensitive to the loss of kidney reserve as evidenced by the small change in serum creatinine after the loss or donation of one kidney with a normal remaining kidney.[9] Alterations in serum creatinine may lag several days behind actual changes in GFR.[8,10]

BUN is also suboptimal for the diagnosis of AKI. In addition, BUN is dependent on non renal factors independent of kidney function, e.g. protein intake, catabolic state, upper gastrointestinal bleeding, volume status and therapy with high dose steroids.[7,11–13] Thus alterations in serum creatinine and BUN in AKI are not particularly sensitive or specific for small changes in GFR.

A biomarker that is released into the blood or urine by the injured kidney and is analogous to the troponin release by injured myocardial cells after myocardial ischemia or infarction, may be a more sensitive and specific marker of AKI than BUN and serum creatinine. In addition, earlier detection of AKI with a kidney specific biomarker may result in earlier nephrology consultation, more optimal dosing of antibiotics, avoidance of nephrotoxic agents and even earlier specific therapies to repair the damaged kidney. Earlier diagnosis of AKI may identify patients with mild AKI that have increases in serum creatinine in the normal range that may not be recognized by clinicians, for example an increase in serum creatinine from 0.4 to 0.8 mg/dL. An ideal biomarker of AKI would allow the early detection of kidney injury before an increase in serum creatinine and/or BUN, would differentiate AKI from acute glomerulonephritis or acute interstitial nephritis, would predict the need for dialysis, mortality and long term kidney outcome and would be able to monitor the effects of an intervention or treatment.

Major interventional trials in AKI, e.g. anaratide[14,15] and fenoldopam,[16] have failed in humans. A possible reason for the failure of these interven tional trials in AKI is the late intervention in the course of AKI due to the dependence on serum creatinine and BUN to diagnose AKI.

In this chapter, animal and clinical studies to diagnose established AKI and clinical studies to determine whether the biomarker detects early AKI of native kidneys will be reviewed for each biomarker. Biomarkers of AKI in a transplanted kidney will be discussed separately in Chapter 6.

2. INTERLEUKIN-18

Interleukin 18 (IL 18) is a pro inflammatory cytokine that plays a role in both the innate and acquired immune response. IL 18 plays an important role in host defenses against tumors and infections. Activated macrophages express high levels of IL 18. A wide variety of cells express IL 18 including mononuclear cells, keratinocytes, osteoblasts, intestine and renal epithelial cells and dendritic cells. Neutralization of IL 18 has potential therapeutic effects. Blockade of IL 18 using neutralizing antibodies,[17] exogenous IL 18 binding protein (IL 18BP)[18] or caspase 1 inhibition[19] protects mice from liver necrosis. Exogenously administered recombinant human IL 18BP is therapeutically effective in a mouse model of collagen induced arthritis.[20] IL 18BP reduces ischemic dysfunction in a suprafused human atrial myocardium model.[21] Strategies to block IL 18 using IL 18BP are

underway in clinical trials of rheumatoid arthritis.[22] Thus on the basis of IL 18 inhibition studies, IL 18 is a mediator of various diseases including ischemic AKI, intestinal ischemia, cardiac ischemia, brain ischemia and arthritis. Thus, on the basis of studies that IL 18 is a mediator of inflam mation in other organs, it was determined whether caspase 1 deficient mice that have a deficiency in the activation of IL 18 were protected against ischemic AKI.

Caspase 1 (previously known as interleukin 1β converting enzyme or ICE) activates the proinflammatory cytokines IL 1β and IL 18.[23] Caspase 1 deficient mice developed less ischemic AKI as determined by serum creatinine and acute tubular necrosis scores than wild type mice.[24] As IL 1β does not play an injurious role in ischemic AKI in mice,[25] a lack of the active form of IL 18 was investigated as the mechanism of the protection against AKI in caspase 1 deficient mice. In an electrochemiluminescence assay of whole kidney, IL 18 was more than 100% increased in wild type AKI as compared to sham operated controls. On immunoblot analysis, there was a conversion of the precursor to the active form of IL 18 in AKI wild type mice, but not in the caspase 1 deficient AKI mice and sham operated controls. To determine whether IL 18 plays an injurious role in ischemic AKI, wild type mice were injected with IL 18 neutralizing antiserum prior to the ischemic insult. These mice were protected against AKI to a similar degree as caspase 1 deficient mice. The conclusion of this study was that IL 18 is a mediator of ischemic AKI in mice.[24,26] Two subsequent studies using IL 18 binding protein transgenic mice, administration of IL 18 binding protein or IL 18 deficient mice have confirmed that IL 18 is a mediator of ischemic AKI in mice.[27,28]

Immunohistochemistry of mouse kidneys demonstrated an increase in IL 18 protein in injured tubular epithelial cells in AKI kidneys compared to normal controls. In a separate study using freshly isolated proximal tubules from mice, it was determined that hypoxic proximal tubules had high levels of IL 18.[29] On the basis of the demonstration of IL 18 in injured proximal tubules, IL 18 was measured in the urine. Urine IL 18 was increased in mice with ischemic AKI compared to sham operated mice.[24] Thus we developed the hypothesis that IL 18 could be released from the injured tubular epithelial cells into the urine and serve as a urinary biomarker of AKI in humans.

Subsequent studies in humans demonstrated that urine IL 18 is an early predictive biomarker of AKI[30] (Table 5.2). Urine IL 18 was measured in 72 patients and was significantly increased in patients with AKI vs normal

Table 5.2 Urine IL-18 as a biomarker of AKI

Situation	*N*	Study	Area under ROC curve	Reference
AKI	50	Urine IL 18 increased in ATN vs normals, prerenal azotemia, urinary tract infection, chronic kidney disease and nephrotic syndrome	0.95	31
ARDS patients in ICU	138	On multivariable analysis, urine IL 18 predicted development of AKI 24 and 48 h later. Urine IL 18 on the day of initiation of mechanical ventilation was a strong predictor of mortality	0.73	121
Children post CPB	55	Urine IL 18 increased at 4 6 h, peaked at 12 h, and remained elevated up to 48 h after CPB. SCr increased 48 72 h after CPB	0.73	33
Adults post CPB	33	Urine IL 18 and NGAL predict AKI		34
Adults post CPB	100	Urine IL 18 on arrival in the ICU did not predict AKI	0.53	35
Children in the ICU	137	Urine IL 18 rises prior to SCr in non septic critically ill children, predicts severity of AKI and is an independent predictor of mortality	0.31 0.77	32
Contrast nephropathy	51	Urine IL 18 was not different between cases and controls before as well as 24 and 72 h after cardiac catheterization		36
Contrast nephropathy	40	Urine IL 18 and NGAL at 24 h were increased in contrast nephropathy. IL 18 increased 24 h earlier than SCr. IL 18, but not SCr, is an independent predictive marker for later major cardiac events	0.75	37

ATN, acute tubular necrosis; ICU, intensive care unit; SCr, serum creatinine.

controls, prerenal azotemia, urinary tract infection, chronic kidney disease and nephrotic syndrome. This study in humans demonstrated the association of urine IL 18 with established tubular injury and formed the basis for examining urine IL 18 in more detail.

The ARDS network had collected urine samples in patients after the initiation of mechanical ventilation. These urine samples were obtained and a nested case control study within the ARDS network trial was performed to determine whether urinary IL 18 is an early diagnostic biomarker for AKI in critically ill patients in the ICU. On multivariable analysis, urine IL 18 values predicted development of AKI (defined as a 50% increase in serum creatinine) 24 and 48 h later. On diagnostic performance testing, urine IL 18 demonstrated an area under the receiver operated characteristic (ROC) curve of 73% to predict AKI in the next 24 h. The presence of sepsis in both control and AKI patients did not have a significant effect on urinary IL 18. On multivariable analysis, the urine IL 18 value on the day of initiation of mechanical ventilation for ARDS was a strong predictor of mortality.[31]

Next it was determined whether the finding that urine IL 18 is an early biomarker of AKI in critically ill adults could be reproduced in children. One hundred and thirty seven children with an average age of 6.5 years (53% male) were studied. The peak levels of IL 18 correlated with the severity of AKI by the pediatric RIFLE (pRIFLE) classification. In non septic AKI patients, urinary IL 18 rose to higher levels than control levels 2 days prior to a significant rise in serum creatinine. Urinary IL 18 was associated with increased mortality. Urinary IL 18 was also increased in patients with sepsis. In conclusion, urinary IL 18 rises prior to serum creatinine in non septic critically ill children predicts severity of AKI and is an independent predictor of mortality.[32]

AKI, as defined by a 50% increase in serum creatinine, occurs in about 25% of patients after cardiopulmonary bypass (CPB). In this high risk group, we tested whether urine IL 18 is a predictive biomarker for AKI in children following CPB. Urine IL 18 increased at 4–6 h after CPB, peaked at over 25 fold at 12 h, and remained markedly elevated up to 48 h after CPB. In contrast, using serum creatinine, AKI was detected only 48–72 h after CPB. The results indicate that IL 18 is an early, predictive biomarker of AKI after CPB.[33] On multivariate analysis, urine IL 18 was independently associated with number of days in AKI among cases suggesting that it may be a marker of AKI severity.[33]

In another study it was determined that urine IL 18 and neutrophil gelatinase associated lipocalin (NGAL) predict AKI after cardiac surgery.[34]

Thirty three patients undergoing CPB were classified as AKI (50% increase in serum creatinine within 48 h after CPB) and no AKI. Urine NGAL and IL 18 were increased in the AKI group at 2–4 h postoperatively. The concentrations of IL 18 and NGAL at 2 h postoperatively correlated with increased serum creatinine at 12 h postoperatively.

However, in another prospective observational cohort study in adults, it was determined that urine IL 18 does not predict AKI after cardiac surgery. One hundred patients undergoing CPB at a single center were studied. Twenty patients developed AKI. On arrival in the ICU and at 24 h post operatively, urine IL 18 was not different in patients who developed AKI compared to non AKI patients.[35]

In a nested case control study of 15 patients with contrast induced nephropathy and 36 matched controls, urinary IL 18 was measured before as well as 24 and 72 h after cardiac catheterization. No statistically significant differences in urine IL 18 were detected between cases and controls or between the patient samples obtained before and after the cardiac cathe terization.[36] However, in another much larger study it was determined that urinary IL 18 and NGAL are early predictive biomarkers of contrast nephropathy (CIN) after coronary angiography.[37] CIN was diagnosed in 13 of 150 patients. At 24 h after the procedure, urinary IL 18 and NGAL levels were significantly increased in the CIN group compared to non CIN patients. The time of AKI as predicted by urine IL 18 was 24 h earlier than the time of AKI as determined by the rise in serum creatinine. IL 18, but not serum creatinine, was also found to be an independent predictor of later major cardiac events up to 17 months after CPB. The area under the ROC curve for the early diagnosis of AKI was 74.9% for urine IL 18 and 73.4% for urinary NGAL.

In summary, the pro inflammatory cytokine IL 18 is both a mediator and a biomarker of ischemic AKI. IL 18 is a mediator of ischemic AKI in mice as evidenced by the studies that IL 18 expression increases in the kidney in AKI and inhibition of IL 18 is protective against AKI in mice.[24,26 28] The majority of published studies demonstrate that urine IL 18 is an early biomarker of AKI in humans and that urine IL 18 increases before serum creatinine in critically ill adults and children in the ICU, in adults and children after CPB and in adults after contrast administration.

Larger studies are needed to confirm that IL 18 is an early biomarker of AKI and to obtain FDA approval for IL 18 as an early biomarker of AKI. In this regard, the TRIBE AKI (Translational Research Investigating Biomarkers in Early Acute Kidney Injury) Clinical Consortium has been

established to accelerate the development of biomarkers. The consortium is a National Institutes of Health (NIH) funded multidisciplinary group and includes investigators from nine major academic centers who have expertise in preclinical, translational, epidemiologic and health services research. In the TRIBE AKI study, urine IL 18, NGAL and cystatin C are being studied as early biomarkers of AKI in a prospective multicenter observa tional cohort study of 1600 patients receiving cardiac surgery. In addition, the hypothesis will be tested that compared with serum creatinine, cystatin C will improve preoperative risk stratification, and that urine IL 18 and NGAL levels will be better markers of postoperative AKI than serum creatinine and predict the severity of AKI and short term mortality. The ultimate findings of this study will pave the way for interventional clinical trials to prevent or to treat AKI and for studies of biomarkers as predictors of long term outcomes, like development of chronic kidney disease and mortality, after AKI.

3. NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN

Neutrophil gelatinase associated lipocalin (NGAL) is a 21 kD protein of the lipocalin superfamily. NGAL is a critical component of innate immunity to bacterial infection and is expressed by immune cells, hepatocytes and renal tubular cells in various disease states.[38] NGAL is a small secreted polypeptide that is protease resistant and thus may be easily detected in the urine.

NGAL protein increases massively in the renal tubules and in the first urine output after ischemic AKI in rats and mice.[39] The appearance of NGAL in the urine preceded the appearance of other urinary markers such as the tubular proteins *N* acetyl beta D glucosaminidase and $beta_2$ microglobulin. Studies in cultured human proximal tubule cells subjected to in vitro hypoxic injury confirmed the origin of NGAL from tubule cells. NGAL was also detected in the urine of mice in the early stage of cisplatin induced nephrotoxicity.[40] These animal studies demonstrated that NGAL may represent an early, sensitive, non invasive urinary biomarker for ischemic and nephrotoxic kidney injury.

NGAL is the most extensively studied biomarker in AKI (Table 5.3). Urinary and serum NGAL were demonstrated to be sensitive, specific and highly predictive early biomarkers of AKI in children after cardiac surgery.[41] Seventy one children undergoing cardiopulmonary bypass were studied. Serial urine and blood samples were analyzed by Western blots and ELISA for NGAL expression. Diagnosis of AKI, defined as a 50% increase in serum

Table 5.3 NGAL as a biomarker of AKI

Situation	*N*	Study	Area under ROC curve	Reference
Children post CPB	71	By multivariate analysis, urinary NGAL at 2 h after CPB was an independent predictor of AKI	0.998	41
Children post CPB	71	2 h postoperative plasma NGAL strongly correlated with change in creatinine, duration of AKI, length of hospital stay and mortality	0.96	42
Adults post CPB	81	Urine NGAL was high at 3 and 18 h after cardiac surgery in patients who later developed AKI	0.8	43
Adults post CPB	100	Plasma NGAL and cystatin C predict AKI and are independent predictors of duration and severity of AKI and duration of ICU stay. Predictive value of plasma NGAL increases with grade of AKI	0.77	44 46
Adults post CPB	60	Plasma NGAL higher in standard CPB system compared to miniaturized CPB system		48
Adults post CPB	50	Plasma and urine NGAL at 2 h post CPB predicts AKI	0.8 0.96	47
Adults post CPB	60	Urine NGAL was not different in off pump vs on pump CPB		49
Adults post CPB	426	Urine NGAL peaked immediately after cardiac surgery and remained significantly higher 3, 18 and 24 h after surgery	0.506 0.611	122
Adults post CPB	90	Areas under the curve for NGAL to predict AKI immediately and 3 h post surgery were determined	0.59 and 0.65	70
Contrast nephropathy	35	Rise in serum NGAL 2 and 4 h after contrast. Rise in urinary NGAL 4 and 12 h after contrast. Serum NGAL correlated with SCr		50

(*Continued*)

Table 5.3 NGAL as a biomarker of AKI—cont'd

Situation	*N*	Study	Area under ROC curve	Reference
Contrast nephropathy in children	91	Urine and plasma NGAL increased within 2 h after cardiac catheterization. SCr increased at 6 24 h after cardiac catheterization	0.92	54
Children in the ICU	140	Urine NGAL rose in AKI, 2 days before a 50% or greater rise in SCr	0.78	51
Children in the ICU	168	Serum NGAL was increased in AKI compared with no AKI		123
Critically ill adults	88	Serum NGAL predicted the development of AKI	0.96	52
Critically ill children with sepsis	143	There was a significant difference in serum NGAL between healthy children, critically ill children with SIRS and critically ill children with septic shock		53
Emergency department	635	Patients with AKI had an elevated urine NGAL compared to patients with prerenal azotemia, chronic kidney disease or normal kidney function. Urine NGAL highly predictive of clinical outcomes	0.948	55
Critically ill/Trauma	31	Urinary NGAL is a predictor of AKI	0.98	56

CPB, cardiopulmonary bypass; SCr, serum creatinine.

creatinine from baseline, developed 1–3 days after cardiopulmonary bypass. In contrast, urinary NGAL rose significantly at 2 h after cardiopulmonary bypass. By multivariate analysis, the urinary NGAL at 2 h after cardiopulmonary bypass was a powerful independent predictor of AKI. In addition, 2 h postoperative plasma NGAL levels strongly correlated with change in creatinine, duration of AKI, length of hospital stay and mortality after cardiopulmonary bypass.[42]

Urinary NGAL is also an early biomarker of AKI in adults post cardiac surgery.[43] In 81 cardiac surgery patients, urine samples were collected immediately preoperatively and at various time intervals after surgery for NGAL. Mean urinary NGAL concentrations in patients who developed AKI were significantly higher early after surgery and remained significantly higher at 3 and 18 h after cardiac surgery compared with patients who did not develop AKI.

There are other studies confirming the value of NGAL as a biomarker of AKI in cardiac surgery patients. Serum NGAL and cystatin C were measured in 100 adult patients after cardiac surgery.[44] On arrival in the ICU, serum NGAL and cystatin C were independent predictors of AKI and were superior to BUN and serum creatinine for the prediction of AKI. In the same group of patients, it was also determined that NGAL and cystatin C correlated with and were independent predictors of duration and severity of AKI and duration of intensive care stay after cardiac surgery.[45] The combination of NGAL and cystatin C did not add to the predictive value. Also in the same group of patients, the predictive value of NGAL increases with the grade of AKI.[46] For example, plasma NGAL was higher for more severe AKI (greater than 50% increase in serum creatinine) compared to less severe AKI (greater than 25% increase in serum creatinine). NGAL also increased with increasing RIFLE classes of AKI. In 50 adult patients undergoing CPB, urinary and serum NGAL were predictive biomarkers of AKI as early as 2 h post operation.[47]

The next step in biomarker measurement in cardiac surgery patients has been to determine whether interventions prevent AKI and also lower plasma or urine biomarkers. In this regard, in a prospective study of 60 patients undergoing either standard CPB or miniaturized CPB, kidney function was better protected during miniaturized CPB as determined by urinary NGAL.[48] However, in 60 patients undergoing either off pump or on pump coronary artery bypass graft (CABG) surgery, urine NGAL was not different between the groups.[49] Serum creatinine and the incidence of AKI was also not different between the groups.

NGAL may also represent an early sensitive biomarker of AKI after contrast administration for coronary angiography.[50] NGAL was measured in the serum and urine before and at 2, 4, 12, 24 and 48 h after contrast administration. There was a significant rise in serum NGAL 2 and 4 h after contrast administration, and a rise in urinary NGAL 4 and 12 h after percutaneous coronary intervention (PCI).

Urine NGAL is an early biomarker of AKI in critically ill children aged between 1 month and 21 years who were on mechanical ventilation.[51] In 140 patients, mean and peak urine NGAL concentrations increased with worsening pediatric RIFLE maximum status. Urine NGAL concentrations rose in AKI, 2 days before and after a 50% or greater rise in serum creatinine. Urine NGAL was a good diagnostic marker for AKI development with an area under the receiver operating characteristic (ROC) curve of 0.78. Urine NGAL was a marker of persistence of AKI for 48 h or longer with an area under the ROC curve of 0.79. Urine NGAL was not a good marker for AKI severity when it was recorded after a rise in serum creatinine had occurred (area under the ROC curve of 0.63). In 88 critically ill adults, serum NGAL had an area under the ROC curve of 0.956, sensitivity of 85%, specificity of 97% to predict the development of AKI.[52] Median urinary pi glutathione *S* transferase (pi GST) was higher in critically ill patients compared to normal controls. However, the area under the curve for urinary pi GST indicated that it was not a good predictor of AKI.

A multicenter study of serum NGAL was performed in 143 critically ill children with systemic inflammatory response syndrome (SIRS) or septic shock during the first 24 h of admission to the pediatric ICU.[53] There was a significant difference in serum NGAL between healthy children, critically ill children with SIRS, and critically ill children with septic shock. Serum NGAL was significantly increased in critically ill children with AKI compared with those without AKI. The study concludes that serum NGAL is a highly sensitive but non specific predictor of AKI in critically ill children with septic shock.

NGAL is an early predictive biomarker of contrast induced nephropathy (CIN) in children.[54] Ninety one children (age 0–18 years) with congenital heart disease undergoing elective cardiac catheterization and angiography with contrast administration were studied. CIN, defined as a 50% increase in serum creatinine from baseline, was found in 11 subjects (12%). A signi ficant elevation of NGAL concentrations in urine and plasma was noted within 2 h after cardiac catheterization. In contrast, detection of CIN by an increase in serum creatinine was only possible 6–24 h after cardiac

catheterization. By multivariate analysis, the 2 h NGAL concentrations in the urine and plasma, but not patient demographics or contrast volume, were found to be powerful independent predictors of CIN.

The sensitivity and specificity of a single urine NGAL measurement for diagnosing AKI was determined in 635 patients in the emergency department (ED).[55] Patients with AKI had a significantly higher urine NGAL level than patients with prerenal azotemia, chronic kidney disease or normal kidney function. At a NGAL cutoff value of 130 μg/g creatinine, the sensitivity and specificity of NGAL for detecting acute injury were 0.900 (95% CI, 0.73 to 0.98) and 0.995 (CI, 0.990 to 1.00), respectively, and these values were superior to those for NAG, alpha$_1$ microglobulin, alpha$_1$ acid glycoprotein, fractional excretion of sodium and serum creatinine. In multiple logistic regression, urinary NGAL level was highly predictive of clinical outcomes, including nephrology consultation, dialysis and admission to the intensive care unit. In 31 multiple trauma patients, urinary NGAL concentration on admission was significantly higher in patients who subsequently developed AKI.[56]

The diagnosis of AKI is problematical in premature infants. Urinary NGAL was measured in 20 premature infants.[57] Neonates born at an earlier gestational age or low birth weight infants had higher urine NGAL concentrations. The study concludes that the use of NGAL as a biomarker of AKI in premature infants merits further investigation.

NGAL is also increased in other conditions besides ischemic AKI. Serum and urine NGAL levels were increased in 26 patients with autosomal dominant polycystic kidney disease (ADPKD) and a significant correlation was found between urine and plasma NGAL levels and residual renal function.[58] In a study of 34 children with diarrhea associated hemolytic uremic syndrome (HUS), the majority of patients with HUS had renal tubular epithelial injury as evidenced by elevated urinary NGAL which was associated with higher BUN and serum creatinine concentrations, and more frequent need for dialysis.[59] In a study of HIV infected children, elevated levels of NGAL were found in HIV associated nephropathy and HIV associated HUS.[60] In a study of 85 patients, urinary NGAL, but not plasma NGAL, was found to be a biomarker of activity in lupus nephritis.[61]

In summary, urine NGAL is an early biomarker of AKI in children and adults in the following situations: post cardiopulmonary bypass, after contrast administration, in critically ill ICU patients, in patients presenting to the emergency department and trauma patients (Table 5.3).

4. KIDNEY INJURY MOLECULE-1

Kidney injury molecule 1 (KIM 1) is a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain. KIM 1 mRNA and protein are expressed at a low level in normal kidney but are increased dramatically in post ischemic kidney[62] (Table 5.4). KIM 1 has recently been identified as the first non myeloid phosphatidylderine receptor that confers a phagocytic phenotype on injured epithelial cells both in vivo and in vitro.[63]

Urinary KIM 1 is a non invasive, rapid, sensitive and reproducible biomarker for the early detection of both cisplatin induced AKI and ischemic AKI in rats.[64] In this study, a sandwich KIM 1 ELISA test was used. At 1 day after cisplatin administration, there was a three to fivefold increase in the urinary KIM 1 compared to plasma creatinine, BUN, urinary *N* acetyl beta glucosaminidase (NAG), glycosuria and proteinuria that were not increased in the urine. At 24 h of post ischemic reperfusion after 10 min of bilateral renal pedicle clamping, urine KIM 1 levels were 10 fold higher than control rats and plasma creatinine and BUN, glycosuria, proteinuria and urinary NAG levels were not yet increased.

KIM 1 is also a tissue and urinary biomarker for nephrotoxicant induced kidney injury. Tissue and urinary expression were measured with three different nephrotoxins in the rat: *S* (1,1,2,2 tetrafluoroethyl) l cysteine (TFEC), folic acid and cisplatin. Marked increases in KIM 1 expression localized to proximal tubule epithelial cells were detected. In addition, KIM 1 protein was detected in urine of nephrotoxin treated rats.[65] KIM 1 is a sensitive and tissue specific biomarker of early AKI compared to BUN, serum creatinine and NAG in rats injected with gentamicin, mercury or chromium.[66] A rapid dipstix test for the detection of urinary KIM 1 (rat) or KIM 1 (human) has been developed.[67] On dipstix, the urinary KIM 1 band intensity significantly correlated with levels of KIM 1 in a dose and time dependent manner as measured by histo pathological damage and immunohistochemical assessment of renal KIM 1. KIM 1 was detected in rats with cadmium, gentamicin or ischemic AKI. In humans, the urinary KIM 1 band intensity was significantly greater in patients with AKI compared to healthy volunteers. KIM 1 was measured in rats with adriamycin induced nephropathy before and after angiotensin converting enzyme (ACE) inhibition.[68] Renal and urinary KIM 1 corre lated with proteinuria and interstitial damage. Reduction of proteinuria correlated with a decrease in renal and urinary KIM 1. KIM 1 has been

Table 5.4 KIM-1 as a biomarker of AKI

Situation	*N*	Study	Area under ROC curve	Reference
ATN	40	Extensive expression of KIM 1 in proximal tubules in ATN kidney biopsies. Urine KIM 1 was significantly higher in patients with ischemic AKI compared to other renal diseases. Urine brush border enzymes did not correlate with clinical diagnostic groupings		69
Kidney transplant	25	KIM 1 staining on kidney biopsy identified proximal tubular injury and correlated with the degree of renal dysfunction		124
Kidney transplant	145	Urine KIM 1 was a predictor of graft loss independent of creatinine clearance, proteinuria and donor age		125
Hospitalized patients	201	Patients with the highest levels in urinary NAG and KIM 1 had the higher odds for dialysis requirement or hospital death		71
Adults post CPB	90	Areas under the curve for KIM 1 to predict AKI immediately and 3 h post surgery were determined	0.68 and 0.65	70

ATN, acute tubular necrosis; NAG, *N*-acetyl-beta-(D)-glucosaminidase.

accepted by the Food and Drug Administration and European Medicines Agency as a highly sensitive and specific urinary biomarker to monitor drug induced kidney injury in preclinical studies and on a case by case basis in clinical trials.

KIM 1 is also a biomarker of AKI in humans. Urine samples were collected from 32 patients with various acute and chronic kidney diseases, as well as from eight normal controls. There was extensive expression of KIM 1 in proximal tubule cells in kidney biopsies from all six patients with biopsy confirmed acute tubular necrosis (ATN). Urinary KIM 1 levels were significantly higher in patients with ischemic ATN compared to patients with other forms of acute renal failure or chronic kidney disease. Concentrations of other urinary brush border enzymes, like gamma glutamyltransferase and alkaline phosphatase, did not correlate with clinical diagnostic groupings.[69] KIM 1 was also measured in 90 patients undergoing cardiac surgery.[70] Thirty six patients developed AKI within 72 h after surgery. The area under the curve to predict AKI immediately and 3 h post operatively was 0.68 and 0.65 for KIM 1, 0.61 and 0.63 for NAG and 0.59 and 0.65 for NGAL. Combining the three biomarkers, KIM 1, NAG and NGAL, increased the sensitivity for early detection of AKI to 0.75 and 0.78.

The relationship between urinary *N* acetyl beta (D) glucosaminidase activity (NAG) and KIM 1 level and adverse clinical outcomes was deter mined prospectively in 201 hospitalized patients with AKI. Patients with the highest levels in urinary NAG and KIM 1 had the higher odds for dialysis requirement or hospital death. This study demonstrates that urinary biomarkers of AKI such as NAG and KIM 1 can predict adverse clinical outcomes in patients with AKI.[71]

KIM 1 is also a sensitive biomarker of tubular injury in other renal diseases besides AKI. Renal KIM 1 expression was significantly increased in human kidney tissue in patients with focal glomerulosclerosis IgA nephropathy, membranoproliferative glomerulonephritis, membranous glomerulonephritis, acute rejection, chronic allograft nephropathy, systemic lupus erythematosus, diabetic nephropathy, hypertension and Wegener's granulomatosis compared to normal kidney tissue.[72] KIM 1 was primarily expressed at the luminal side of de differentiated proximal tubules, in areas with fibrosis and in areas of inflammation in macrophages. Renal KIM 1 positively correlated with renal damage, negatively with renal function, but not with proteinuria. Urinary KIM 1 was increased in the same group of patients and correlated positively with tissue KIM 1 and macrophages, negatively with renal function, but not with proteinuria. This study

demonstrates that KIM 1 is upregulated in renal disease and is associated with renal fibrosis and inflammation and that urinary KIM 1 can be used as a non invasive biomarker in multiple renal diseases.

5. TUBULAR ENZYMES

The apical membrane of proximal tubular epithelial cells contains numerous microvilli that form the brush border. The brush border contains enzymes that carry out the specialized functions of the proximal tubule. Intracellular enzymes can be released into the urine with injury either by exocytosis or leakage. The detection of proteins, especially enzymes, released from damaged proximal and/or distal tubular cells has also been used as a biomarker of AKI. Glutathione *S* transferase (GST) isomers are cyto plasmic enzymes found in proximal and distal tubular cells. *N* acetyl glucosaminidase (NAG) is a lysosomal enzyme found mostly in proximal tubules. Alkaline phosphatase (AP) and γ glutamyl transpeptidase (γGT) are brush border enzymes (Table 5.5).

Nearly 30 years ago, tubular enzymes in the urine were measured as a biomarker of AKI.[73] In acute tubular disorders, e.g. renal failure from acute pancreatitis, the concentrations of $alpha_1$ microglobulin were high in patients with acute tubular injury compared to normal controls.

Tubular enzymuria may be very sensitive to tubular injury from multiple causes. Dipeptidyl aminopeptidase was increased in the urine in patients with tubulointerstitial nephritis and chronic glomerulonephritis.[74] Of five brush border enzymes investigated, alkaline phosphatase was the most sensitive to detect contrast nephropathy.[75] In 73 consecutive patients with non oliguric AKI, urinary excretion of $alpha_1$ and $beta_2$ microglobulin, cystatin C, retinol binding protein, alpha glutathione *S* transferase, gamma glutamyl transferase, lactate dehydrogenase and *N* acetyl beta D glucosaminidase was measured early in the course of the AKI.[76] Urinary excretion of cystatin C and $alpha_1$ microglobulin had the highest diagnostic accuracies as indicated by the largest areas under the ROC curves in identifying patients requiring dialysis. This study concluded that in non oliguric AKI, increased urinary excretion of cystatin C and $alpha_1$ microglobulin may predict an unfavorable outcome, as indicated by the requirement for dialysis.[76] Neutral endopeptidase (NEP) and retinol binding protein (RBP) were increased in the urine of patients after open heart surgery independent of kidney failure.[77] It has also been demonstrated that hemodialysis exacerbates tubular enzymuria in patients with AKI.[78]

Table 5.5 Tubular enzymuria as a biomarker of AKI

Situation	*N*	Study	Area under ROC curve	Reference
Non oliguric AKI	73	Urine excretion of cystatin C and alpha$_1$ microglobulin had the highest diagnostic accuracy in identifying patients requiring renal replacement therapy	0.92 and 0.86	76
Adults post CPB	34	Urine NEP and RBP were increased in patients after open heart surgery independent of kidney failure		77
Adults post CPB	90	Areas under the curve for NAG to predict AKI immediately and 3 h post surgery were determined	0.61 and 0.63	70
Critically ill patients in the ICU	26	GGT, AP, NAG and GST but not LDH were higher in the AKI group on admission and were useful in predicting AKI	0.845 0.950	80
Sepsis patients in the ICU	40	Urinary alpha GST and pi GST are elevated early in all patients with sepsis syndrome, but are not predictive of AKI as defined by AKIN		81

AP, alkaline phosphatase; GGT, gamma-glutamyl transferase; GST, glutathione *S*-transferase; LAP, leucine aminopeptidase; NAG, *N*-acetyl-glucosaminidase; NEP, neutral endopeptidase; RBP, retinol-binding protein.

Alkaline phosphatase (AP), gamma glutamyl transferase (GGT), leucine aminopeptidase (LAP) and dipeptidyl peptidase IV (DPP) were measured in kidney transplant patients with normal graft function, ATN, acute rejection and healthy controls. Enzymuria was increased with both acute rejection and ATN. Successful treatment of rejection resulted in a decrease in the enzymuria.[79] In a prospective pilot study of 26 consecutive critically ill adult patients admitted to the intensive care unit, urinary levels of gamma glutamyl transpeptidase (gamma GT), alkaline phosphatase (AP), *N* acetyl glucosaminidase (NAG), and alpha and pi glutathione S transferase

(alpha and pi GST) but not lactate dehydrogenase (LDH), were higher in the AKI group on admission and were useful in predicting AKI.[80]

Urinary alpha GST and pi GST were measured during the 48 h after ICU admission in 40 consecutive patients who were admitted with a diagnosis of sepsis.[81] AKI was diagnosed according to the AKIN criteria. Nineteen patients developed AKI, all within 24 h of ICU admission. Urinary alpha GST level was not increased in patients who developed AKI vs non AKI patients. Median urinary pi GST level was significantly higher in those who developed Stage 1 AKI, and in those who developed Stage 3 AKI compared to the non AKI group. Median urinary pi GST level at ICU admission was higher in all groups than in healthy control subjects. The area under the receiver operating characteristics curve for urinary pi GST level indicated that it was not a good predictor of AKI. The conclusion of this study was that urinary pi GST is elevated early in all patients with sepsis syndrome, but is not predictive of AKI as defined by AKIN.

Five urinary biomarkers, retinol binding protein, alpha$_1$ microglobulin, microalbumin, *N* acetyl beta D glucosaminidase and intestinal alkaline phosphatase, were measured in 172 patients randomized to receive cold blood or cold crystalloid for renal perfusion during thoracoabdominal aortic aneurysm repair.[82] Twenty seven patients in the cold blood group and 21 patients in the cold crystalloid group developed AKI ($P = 0.4$). Changes in renal biomarkers were similar in the groups.

In summary, measurement of tubular enzymuria is inexpensive and easy to measure. However, tubular enzymuria may be increased in multiple causes of tubular injury including ATN, acute rejection and acute tubulointerstitial nephritis.

6. CYSTATIN C

Butler at al in 1961 studied the urine proteins of 223 individuals by starch gel electrophoresis and found a new urine protein fraction in the post gamma globulin fraction.[83] This protein was named cystatin C. Cystatin C is a 13 kD protein produced by all nucleated cells. It is a polypeptide chain with 120 amino acid residues. It is freely filtered by the glomerulus, completely reabsorbed by the proximal tubules and is not secreted by the renal tubules.[84] Thus some of the limitations of serum creatinine, e.g. effect of muscle mass, diet, gender and tubular secretion, may not be a problem with cystatin C (Table 5.6). Cystatin C is best measured by an immunonephelometric assay.

Table 5.6 Cystatin C in AKI

Situation	*N*	Study	Area under ROC curve	Reference
Contrast nephropathy	127	Serum cystatin C of greater than 1.3 mg/L had an 88% sensitivity and 96% specificity for the detection of kidney failure (an iopromide clearance of less than 80 mL/min/m^2)		87
Uninephrectomy for living kidney donation	10	Serum cystatin C increased 1 day after uninephrectomy compared to SCr that increased at 2 days after uninephrectomy		9
Decompensated liver cirrhosis	36	Serum cystatin C was an accurate GFR marker in cirrhotic patients. SCr and calculated creatinine clearance were of no practical value		89
Liver cirrhosis	97	Serum cystatin C was a better marker of renal function than SCr in patients with moderately impaired liver function and Child Pugh Class C patients	0.69	126
Hospitalized patients	85	Serum cystatin C increased earlier than the increase in SCr	0.82 0.97	90
Critically ill patients	50	Serum cystatin C correlated better with GFR (creatinine clearance) than did SCr	0.927	91
Adults post CPB	72	Urine cystatin C at 6 h predicted AKI (a 25% or greater increase in SCr or renal replacement therapy)	0.734	94
Critically ill children	25	Serum cystatin C and B2M were better than SCr to identify a creatinine clearance of under 80 mL/min	0.792 0.851	93

B2M, beta$_2$ microglobulin; GFR, glomerular filtration rate; SCr, serum creatinine.

In studies using Cr–EDTA clearance as the reference standard, the blood concentration of this post gamma globulin fraction, known as cystatin C, was identified as a measure of GFR.[85] Cystatin C is a better marker of GFR than serum creatinine as demonstrated in the following studies: serum cystatin C and cystatin C based formulae were as good in estimating GFR as the Modification of Diet in Renal Disease (MDRD) formula.[86] Uzun et al studied the diagnostic significance of cystatin C using non creatinine measures of GFR.[74] Serum $^{(99m)}$Tc DTPA clearance was compared with serum cystatin C, creatinine, beta$_2$ microglobulin levels and creatinine clearance in a group of patients with GFRs of 10–60 mL/min/1.73 m^2 and healthy controls. Reference clearance, determined by serum $^{(99m)}$Tc DTPA, was best correlated with creatinine clearance ($r = 0.957$) and cystatin C ($r = 0.828$), compared to beta$_2$ microglobulin ($r = 0.767$) and creatinine ($r = 0.682$). As these patients had a GFR of less than 60, it was concluded that serum cystatin C level can be used as a marker of GFR in patients with kidney failure.[74] Artunc et al compared serum creatinine, serum cystatin C and the clearance of the iodinated contrast dye iopromide (reference standard) in 127 patients undergoing cardiac catheterization. Serum cystatin C showed a higher non parametric correlation ($r = 0.805$) to the iopromide clearance compared to serum creatinine ($r = 0.652$) and compared to GFR estimated by the Cockcroft–Gault formula ($r = 0.690$). A serum cystatin C value of greater than 1.3 mg/L demonstrated an 88% sensitivity and 96% specificity for the detection of kidney failure (an iopromide clearance of less than 80 mL/min/m^2).[87] At a multinational meeting held in 2002 in Germany[88] it was decided that: (1) cystatin C is at least equal if not superior to serum creatinine as a marker of GFR; (2) the independence from height, gender, age and muscle mass is advantageous; and (3) select patient groups such as children, the elderly and patients with reduced muscle mass may benefit from its use as a marker of GFR.

The following studies have determined the use of cystatin C as a marker of low GFR in patients with AKI. Changes in cystatin C occur sooner after changes in kidney function than serum creatinine. Herget Rosenthal studied patients after uninephrectomy for living kidney donation. Serum cystatin C increased 1 day after uninephrectomy compared to serum creatinine that increased at 2 days after uninephrectomy.[9] Serum creatinine concentration and calculated creatinine clearance are thought to be of limited value as GFR markers in patients with decompensated liver cirrhosis. Thirty six patients with decompensated liver cirrhosis and 56 non cirrhotic controls were studied. Inulin clearance, serum cystatin C

and creatinine clearances were studied. Plasma cystatin C concentration was found to be an accurate GFR marker in cirrhotic patients. Plasma creatinine concentration and calculated creatinine clearance were of no practical value, as their reference values varied with the severity of the liver disease.[89]

In patients with AKI, cystatin C rises prior to serum creatinine. In 85 patients at high risk to develop AKI, it was determined whether cystatin C detected AKI earlier than serum creatinine. AKI was defined according to the RIFLE classification. Serum cystatin C increased by more than 50% at 0.6 days earlier than the increase in serum creatinine. Serum cystatin C also demonstrated a high diagnostic value to detect AKI as indicated by area under the ROC curve on the two days before the R or 'risk of renal dysfunction' criteria was fulfilled by creatinine. This study concluded that serum cystatin C is useful for the detection of AKI and may detect AKI one to two days earlier than creatinine.[90] In another study in critically ill patients, serum creatinine, serum cystatin C and 24 h creatinine clearance were determined. Serum cystatin C correlated better with GFR than did creatinine and was diagnostically superior to creatinine.[91] During contin uous veno venous hemofiltration (CVVH), the quantity of cystatin C removed is less than 30% of its production and no rapid changes in its serum concentration are observed.[92] This study suggests that CVVH is unlikely to significantly influence serum concentrations of cystatin C and that cystatin C can be used to monitor residual kidney function during CVVH.

Serum cystatin C and beta$_2$ microglobulin (B2M) were measured in 25 children in the ICU in a prospective, observational study set.[93] The ability of serum cystatin C and B2M to identify a creatinine clearance rate and a Schwartz creatinine clearance rate under 80 mL/min/1.73 m^2 was better than that of creatinine (areas under the ROC curve: 0.851 and 0.792 for cystatin C, 0.802 and 0.799 for B2M, and 0.633 and 0.625 for creati nine). This study concluded that serum cystatin C and B2M were better than serum creatinine to detect AKI in critically ill children.

A study evaluated the use of urinary cystatin C for the early diagnosis of AKI. Plasma and urine were prospectively collected from 72 adults under going elective cardiac surgery.[94] Acute kidney injury was defined as a 25% or greater increase in plasma creatinine or renal replacement therapy within the first 72 h following surgery. Plasma cystatin C and NGAL did not predict the development of AKI within the first 6 h following surgery. However, both urinary cystatin C and NGAL were increased in the 34 patients who later developed AKI, compared to patients with no AKI. The urinary cystatin C at 6 h after ICU admission was the most useful for predicting AKI.

The reason why cystatin C rises before serum creatinine is not clear. A possible explanation is that cystatin C represents the ideal endogenous marker of GFR: it is produced by all nucleated cells at a constant rate, is not affected by changes in body mass, nutrition or gender and is not degraded or secreted by the renal tubules. In contrast, serum creatinine is affected by many non renal factors that affect generation of creatinine and tubular secretion.

There are limitations to the use of cystatin C as a marker of GFR. Abnormalities of thyroid function[95] and glucocorticoid therapy[96,97] may affect cystatin C independently of kidney function. Levels of C reactive protein may increase cystatin C levels and it has been suggested that cystatin C is a marker of inflammation.[98]

7. OTHER BIOMARKERS OF ACUTE KIDNEY INJURY

Other biomarkers of AKI are given in Table 5.7. These biomarkers are now discussed in more detail.

7.1. IL-6 and IL-8

IL 6 is a pro inflammatory cytokine. IL 6 also plays a role in the immune response and hematopoiesis. IL 6 is present in macrophages, fibroblasts and endothelium. IL 8 is also a pro inflammatory cytokine and the prototype of the 'chemokine superfamily'. IL 8 recruits and activates neutrophils.

Eighteen cytokines and chemokines were measured in a mouse model of AKI.[99] IL 8 levels in ischemic kidney tissues were significantly increased early after induction of AKI. There were also significant increases in IL 6 and IL 12 (p40) early after the induction of ischemia. TNF α showed a trend to increase with time after ischemia. IL 1α, IL 2, IL 4 and IFN γ were significantly decreased in AKI. MIP 1α was significantly higher at 24 h of AKI. No differences in AKI versus no AKI were found for kidney levels of IL 1β, IL 3, IL 5, IL 10, IL 12 (p70), IL 17, GM CSF and RANTES. As there was a significant rise in IL 8 in the kidney in AKI, IL 8 was measured in serum and urine. IL 8 levels in serum and urine were highest 3 h after induction of ischemia and before a significant rise in serum creatinine. IL 8 was markedly elevated in urine from humans who received deceased donor kidney transplants and developed DGF compared with deceased donor kidney recipients with prompt graft function.

Table 5.7 Biomarkers of AKI

Biomarker	Situation	*N*	Study	Area under ROC curve	Reference
Na/H exchanger isoform 3 (NHE3)	ICU patients	68	Urine NHE3 was increased in AKI and correlated positively with SCr		127
Adenosine deaminase binding protein (ABP)	Neonates treated with tobramycin	33	Urinary ABP and its excretion rate was significantly increased from the first day of tobramycin treatment		128
Platelet activating factor (PAF), IL 1, IL 6, IL 8	Septic shock	12	Blood and urine PAF, IL 1, IL 6 and IL 8 were higher in AKI than controls		129
IL 8	Renal allograft recipients deceased donors	17	Urine IL 8 was markedly elevated in urine from humans who developed delayed graft function compared with patients with prompt graft function		99
IL 6	Severe sepsis patients	547	Increased log plasma IL 6 and APACHE II score were significant risk factors of AKI. The increase in IL 6 did not correlate with measures of hypotension, e.g. mean arterial pressure		100
Endothelin (ET)	Contrast nephropathy	12	Urine ET increased after contrast in the patients with AKI. NAG and B2M showed a similar pattern		130
Hepatocyte growth factor (HGF)	ATN	73	Urine HGF markedly increased in patients with ATN compared to normal, CKD, PKD		131

Type I and II receptors for tumor necrosis factor α (TNF α)	Septic shock	537	Elevated serum TNF RI and RII were associated with development of ARF. TNF R was an independent predictor of mortality in patients developing ARF	132
Cytodiagnostic indices in urine	ATN	51	Tubular cells, casts higher in ATN patients. Granular, waxy, leukocytic, broad casts, renal cells higher in patients requiring dialysis. Positive correlation between cytodiagnostic indices and rise of SCr	133
Liver fatty acid binding protein (L FABP)	Kidney transplant patients	12	A significant direct correlation was found between urinary L FABP level and both peritubular capillary blood flow and the ischemic time of the transplanted kidney as well as hospital stay	103
Liver fatty acid binding protein (L FABP)	Children after CPB	40	Increased L FABP levels at 4 and 12 h after CPB in patients that developed AKI. Both bypass time and urinary L FABP were significant independent risk indicators for AKI	104
Liver fatty acid binding protein (L FABP)	ICU patients	80	Urinary L FABP levels in patients with septic shock were significantly higher than those in patients with severe sepsis without shock, patients with ARF and healthy subjects	105

(*Continued*)

Table 5.7 Biomarkers of AKI—cont'd

Biomarker	Situation	*N*	Study	Area under ROC curve	Reference
L1 cell adhesion molecule (CD171)	ATN	24	Urine L1 was higher in patients with ATN compared to patients with prerenal azotemia or other causes of ARF		106
Netrin 1	ARF	20	Urinary netrin 1 was increased in 13 acute renal failure patients compared to 6 healthy volunteer urine samples		109
Exosomal ATF3	AKI		Increased in rats with AKI before the SCr. Increased in humans with AKI compared to no AKI		110
Exosomal fetuin A	AKI		Increased in rats with AKI before the SCr. Increased in humans with AKI compared to no AKI		11
Urinary aprotinin	AKI	106	The 2 h level correlated with SCr, duration of AKI and length of hospital stay	0.98	112

ATF3, activating transcription factor-3; ATN, acute tubular necrosis; B2M, beta-2 microglobulin; GGT, gamma-glutamyl transferase; NAG, *N*-acetyl-glucosaminidase; PKD, polycystic kidney disease; SCr, serum creatinine.

Patients from the placebo group of the Prospective Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis (PROWESS) dataset were studied.[100] Of the 547 patients studied, 127 (23.2%) developed AKI. In a multivariable Cox regression, the predictors of AKI were log IL 6 and APACHE II score. Increased log IL 6 and APACHE II score were significant risk factors of AKI in patients with severe sepsis. The increase in IL 6 did not correlate with measures of hypotension, e.g. mean arterial pressure.

7.2. Liver fatty acid binding protein (L-FABP)

FABPs are a family of carrier proteins for fatty acids and other lipophilic substances such as eicosanoids and retinoids. FABPs facilitate the transfer of fatty acids between extra and intracellular membranes. Some FABPs transport lipophilic molecules from outer cell membrane to certain intra cellular receptors such as PPAR.

L FABP, was correlated with the degree of tubulointerstitial damage in a model of folic acid (FA) induced nephropathy in mice.[101] The protein expression levels of human L FABP in both the kidney and urine signifi cantly correlated with the degree of tubulointerstitial damage, the infiltra tion of macrophages and the deposition of type I collagen.

Urinary L type fatty acid binding protein (L FABP) was measured in mice with ischemic AKI and cisplatin induced AKI.[102] In both ischemic AKI and cisplatin induced AKI, urinary L FABP was increased in the urine before the increase in BUN. Renal histology scores worsened with longer ischemic time or increased dose of cisplatin. In both AKI models, urinary L FABP showed a better correlation with histology injury scores and GFR, as measured by fluorescein isothiocyanate labeled inulin injection, than with BUN and urinary *N* acetyl D glucosaminidase.

L FABP was evaluated as a biomarker of renal ischemia in both human kidney transplant patients and a mouse model of AKI.[103] In 12 living related kidney transplant patients, intravital video analysis of peritubular capillary blood flow was performed immediately after reperfusion of the transplanted organs. A significant direct correlation was found between urinary L FABP level and both peritubular capillary blood flow and the ischemic time of the transplanted kidney as well as hospital stay. Human L FABP transgenic mice demonstrated lower blood urea nitrogen levels and less histological injury than injured wild type mice. In addition, human L FABP transgenic mice subjected to AKI demonstrated the transition of L FABP from the

cytoplasm of proximal tubular cells to the tubular lumen on immunohistochemistry. These data show that increased urinary L FABP after ischemic reperfusion injury is a biomarker of AKI.

Urine L FABP was measured in 40 pediatric patients prior to and following cardiopulmonary bypass surgery.[104] Enzyme linked immunosorbent assay analysis demonstrated increased L FABP levels of about 94 and 45 fold at 4 and 12 h, respectively, following surgery in the 21 patients who developed AKI. Western blot analysis confirmed the presence of L FABP in the urine. Both bypass time and urinary L FABP were significant independent risk factors for AKI. This study demonstrates that urinary L FABP levels represent a sensitive and predictive early biomarker of AKI after cardiac surgery.

Urinary and serum L FABP was measured in 80 critically ill patients.[105] Urinary L FABP levels in patients with septic shock were significantly higher than those in patients with severe sepsis without shock, patients with ARF and healthy subjects ($P < 0.001$). Serum L FABP levels showed no significant differences between patients with septic shock, patients with severe sepsis, patients with ARF and healthy subjects.

In summary, urinary L FABP is increased in rodents with AKI before the increase in serum creatinine. Urinary L FABP is also increased in humans with AKI. Urinary L FABP, but not serum L FABP, is increased in patients with severe sepsis. In view of studies that L FABP is also a biomarker of progression of chronic kidney disease, larger and multicenter studies of L FABP as an early biomarker of AKI in patients are warranted.

7.3. L1 cell adhesion molecule

The L1 cell adhesion molecule (CD171) is a multidomain membrane glycoprotein of the immunoglobulin superfamily. The L1 cell adhesion molecule was examined in 24 kidney biopsies from patients with acute tubular necrosis.[106] In acute tubular necrosis biopsies, L1 lost its polarized distribution being found in both the basolateral and apical domains of the collecting duct. It was also induced in thick ascending limb and distal tubule cells in ATN biopsies. Urinary L1 was significantly higher in all 24 patients with acute tubular necrosis compared to five patients with prerenal azotemia and to six patients with other causes of AKI.

7.4. Netrin

Netrins are laminin like molecules with a distinctive domain organization. Netrins belong to the laminin related family of axon guidance molecules.

Netrins 1, 3 and 4 are encoded by distinct genes. Mouse netrin 1 shares 52% amino acid identity with mouse netrin 3. Netrins act via two receptors, 'deleted in colon cancer' (DCC) and UNC5. Netrins play a role in axonal guidance including development of mammary gland, lung, pancreas and blood vessels, inhibition of leukocyte migration and chemoattraction of endothelial cells. Netrin 1 is a potent inhibitor of leukocyte chemoattraction. The kidney has high levels of netrin expression.

The role of netrin in ischemic AKI in mice was determined.[107] In ischemic AKI, netrin 1 and 4 mRNA expression was downregulated while expression of netrin 3 was upregulated. Netrin 1 protein levels were increased between 3 and 24 h of reperfusion. Immunolocalization showed that netrin 1 increased in tubular epithelial cells early in AKI. Administration of recombinant netrin 1 significantly improved kidney function and histology suggesting that the downregulation of netrin 1 in vascular endothelial cells may promote endothelial cell activation and infiltration of leukocytes into the kidney resulting in tubular injury. In another study, it was demonstrated that netrin 1 overexpression protects against ischemic AKI in mice by inhibition of apoptosis.[108]

As netrin 1 expression is increased early in the tubules during ischemic AKI, netrin 1 was investigated as an early biomarker of AKI.[109] Urinary netrin 1 excretion was determined in ischemic , cisplatin , folic acid and endotoxin induced AKI in mice. Urinary netrin 1 levels increased markedly within 3 h of ischemia reperfusion, reached a peak level at 6 h, and returned to near baseline by 72 h. Serum creatinine significantly increased only after 24 h of reperfusion. Netrin 1 was also measured in patients with AKI. Urinary netrin 1 was increased in 13 patients with AKI and no urinary netrin 1 was found in six healthy volunteer urine samples. Thus netrin 1 is a promising biomarker of AKI that merits further study in humans.

7.5. Exosomes

Urinary exosomes can be released from every segment of the nephron, including podocytes. Exosomes are 50–90 nm vesicles. An exosome is created inside the cell when a segment of the cell membrane invaginates and is endocytosed. The internalized segment is broken into smaller vesicles that can be expelled from the cell. The released vesicles are called exosomes. Exosomes consist of a lipid raft. Exosomes are secreted by cells under normal and pathological conditions under control of RNA called 'exosomal shuttle RNA'. The detection of urinary exosomal transcription factors may

provide understanding of cellular regulatory pathways as well as being biomarkers of disease.

Exosomes were isolated by differential centrifugation in rats and humans with AKI.[110] The exosomes were found to contain activating transcription factor 3 (ATF3) detected by Western blot. ATF3 was found in the concentrated exosomal fraction, but not in whole urine. ATF3 was present in urine exosomes in rat models of AKI before the increase in serum creatinine. ATF3 was found in exosomes isolated from patients with AKI but not from patients with chronic kidney disease or controls.

Exosomal Fetuin A was increased in rats with cisplatin induced AKI compared to control rats.[111] By immunoelectron microscopy and elution studies, Fetuin A was localized to inside urinary exosomes. Urinary exosomal Fetuin A was increased in three ICU patients with AKI compared to patients without AKI. The study concluded that proteomic analysis of urinary exo somes can provide candidate biomarkers for the diagnosis of AKI.

7.6. Urinary aprotinin

Aprotinin, also known as bovine pancreatic trypsin inhibitor or BPTI (Trasylol, Bayer), is a drug used to reduce bleeding during cardiopulmonary bypass (CPB). Aprotinin slows down fibrinolysis, the process that leads to the breakdown of blood clots. Use of aprotinin is associated with AKI and this drug was withdrawn from the market. Aprotinin does decrease the need for blood transfusions and decrease blood loss during CPB.

Proteomic analysis of urine of children undergoing CPB identified a prominent protein with a mass to charge ratio of 6.4 kDa.[112] Tandem mass spectrometry of urine identified the protein as aprotinin. Urinary aprotinin levels were measured in 106 pediatric patients undergoing CPB and receiving aprotinin therapy. Urinary aprotinin levels 2 h after initiation of CPB were predictive of AKI (area under the curve of 0.98). By multi variate analysis, the urinary aprotinin level at 2 h after CPB was an inde pendent predictor of AKI. The 2 h urinary aprotinin level correlated with serum creatinine, duration of AKI and length of hospital stay.

Urine NGAL was measured as a biomarker of aprotinin induced AKI in 369 patients undergoing CPB.[113] In this group, 205 patients received aprotinin and 164 patients did not receive aprotinin. The association of aprotinin use with urine NGAL and the incidence of AKI was determined. Postoperative urinary NGAL was increased in cardiac surgical patients receiving aprotinin compared to patients that did not receive aprotinin.

In summary, urinary aprotinin is a biomarker of AKI in patients undergoing CPB and receiving aprotinin. In addition, AKI caused by aprotinin can be detected by measuring urinary NGAL.

7.7. Nephronectin

Nephronectin (NPNT), a ligand for $alpha_8beta_1$ integrin, that is expressed in the ureteric bud epithelium during kidney morphogenesis, was examined in a mouse model of nephrotoxic AKI.[114] NPNT expression was greatly increased in regenerating tubular cells during the maintenance and recovery phases of ATN. On day 1 following onset of ATN, NPNT was present in the urine. NPNT expression preceded proliferating cell nuclear antigen protein expression in regenerating renal tubular epithelial cells.

7.8. Angiotensin converting enzyme insertion/deletion (I/D) genetic polymorphisms

Angiotensin converting enzyme genetic polymorphism was screened for genotype (I/D polymorphism analysis by polymerase chain reaction amplification) and phenotype (measurement of the circulating angiotensin converting enzyme by spectrophotometry) in 180 consecutive patients admitted to the ICU.[115] AKI was defined in terms of the RIFLE classifi cation. II and ID genotypes were associated with lower baseline circulating rates of angiotensin converting enzyme. There was a significantly greater II genotype proportion in AKI patients (42%) compared to patients without AKI. After adjustment for the identified prognostic factors, II genotype was independently associated with increased risk of AKI and death among patients with AKI. This study suggests that genetic factors may affect the susceptibility to and prognosis of AKI.

8. COMBINATIONS OF AKI BIOMARKERS

The classical biomarker paradigm is that one test detects one disease, e.g. troponin for acute myocardial infarction, prostate specific antigen (PSA) for prostate cancer. However, AKI is a complex disease with multiple causes and it is possible that one biomarker will not be sufficient to make an early diagnosis. Thus, a panel of biomarkers may be necessary in AKI.[116]

Both urinary NGAL and IL 18 were measured in children that devel oped AKI after CPB.[33] NGAL increased 25 fold within 2 h and declined within 6 h after surgery. In contrast, urine IL 18 increased at 4—6 h after

CPB, peaked at over 25 fold at 12 h, and remained markedly elevated up to 48 h after CPB. Also, on multivariate analysis, both IL 18 and NGAL were independently associated with number of days in AKI among cases. These results indicate that NGAL and IL 18 are increased in tandem after CPB. The combination of these two biomarkers may allow for the reliable early diagnosis and prognosis of AKI at all times after CPB, much before the rise in serum creatinine.[33] A panel of biomarkers of AKI may improve the early diagnosis of AKI in different populations of patients with AKI.

Urinary levels of matrix metalloproteinase 9 (MMP 9), *N* acetyl beta D glucosaminidase (NAG) and kidney injury molecule 1 (KIM 1) were examined in 44 patients with various acute and chronic kidney diseases, and 30 normal subjects in a cross sectional study.[117] In addition, a case control study of children undergoing CPB surgery was performed. AKI was defined as a greater than 50% increase in the serum creatinine within the first 48 h after surgery. In the cross sectional study, combining all three biomarkers achieved a perfect score, as determined by area under the ROC curve, for diagnosing AKI. In the case control study, KIM 1 was better than NAG at all time points for early diagnosis of AKI after CPB, but combining both was no better than KIM 1 alone. Urinary MMP 9 was not a sensitive marker in the case control study.

The diagnostic performance of nine urinary biomarkers of AKI was evaluated in 204 patients with or without AKI: healthy volunteers, patients undergoing cardiac catheterization and patients admitted to the intensive care unit.[118] The biomarkers studied were: KIM 1, NGAL, IL 18, hepatocyte growth factor (HGF), cystatin C, NAG, vascular endothelial growth factor (VEGF), chemokine interferon inducible protein 10 (IP 10; CXCL10) and total protein. Using a logic regression model, the area under the curve (0.94) was greater for the combination of biomarkers than for the individual biomarkers. Age adjusted levels of urinary KIM 1, NAG, HGF, VEGF and total protein were significantly higher in patients who died or required renal replacement therapy compared to those who survived or did not need renal replacement therapy.

Urinary KIM 1, NGAL and NAG were measured at five time points for the first 24 h after surgery in 90 adults undergoing cardiac surgery.[70] Thirty six patients developed AKI as defined by an increase of serum creatinine of 0.3 within 72 h after surgery. The areas under the curve to predict AKI immediately and 3 h postoperatively were 0.68 and 0.65 for KIM 1, 0.61 and 0.63 for NAG and 0.59 and 0.65 for NGAL. Combining the three biomarkers improved the areas under the curve to 0.75 and 0.78. This study

demonstrates that a combination of biomarkers may be better than individual biomarkers for the early detection of AKI before a rise in serum creatinine.

In a systematic review of biomarkers of AKI, Parikh et al determined methodological quality of biomarker studies reported on MEDLINE and EMBASE databases between 2000 and 2006.[119] In total, 31 studies evaluated 21 novel urine and serum biomarkers of AKI. Urine IL 18, KIM 1 and NAG performed best in some studies for the diagnosis of established AKI. Serum cystatin C, urine NGAL, IL 18 and brush border enzymes (glutathione *S* transferase) performed best for the early diagnosis of AKI. KIM 1 and IL 18 performed best for the prediction of mortality risk in patients with AKI.

In summary, more than one biomarker may be necessary to obtain sufficient sensitivity and specificity for AKI screening. In combination, a panel of AKI biomarkers and serum markers of GFR like cystatin C may result in a greater potential to identify AKI earlier than we currently can, with resultant clinical implications. Also, clinicians who are aware of the limitations of different biomarkers in different diseases and at different time points after the AKI insult, may request a specific test or a panel of tests.

9. SUMMARY

There are multiple promising serum and urinary biomarkers, e.g. IL 18, NGAL, KIM 1, cystatin C and L FABP, that detect AKI before the rise in serum creatinine and predict outcome in patients with AKI. However, the testing of panels of different biomarkers in AKI is necessary. Determination of biomarkers of AKI and GFR in patients with AKI due to different causes, e.g. sepsis, ischemia, nephrotoxins and contrast, is important. Establishing the optimal biomarker or biomarkers for a given clinical scenario will require prospective validation in large numbers of patients with a variety of causes of AKI, preferably with measurement of numerous candidate biomarkers. Prospective screening studies to determine the use of these biomarkers in larger populations are necessary. In this regard, an NIH funded clinical consortium consisting of investigators from nine academic centers called TRIBE AKI (Translational Research Investigating Biomarkers in Early Acute Kidney Injury) has been established. Currently, the consortium is performing a prospective multicenter observational cohort study of 1800 patients receiving cardiac surgery to determine whether urine IL 18, urine NGAL and serum cystatin C are biomarkers for

the early diagnosis and long term outcomes of AKI. Ultimately, disease control studies to determine the impact of biomarker screening on reducing the burden of disease are desirable. In this regard, a prospective study is testing whether erythropoietin therapy decreases the incidence of AKI as determined by serum creatinine and serum cystatin C and lowers levels of urinary IL 18, NGAL and KIM 1 in over 500 ICU patients in New Zealand.[120]

10. BIOMARKERS OF EXTRA-RENAL COMPLICATIONS OF ACUTE KIDNEY INJURY

Hospital acquired acute kidney injury (AKI) is a common complication that is associated with significant morbidity and mortality.[134] Hospital stay is longer in patients with AKI[135] and patients are more likely to be discharged to short or long term care facilities.[135,136] A wealth of epidemiological data has accumulated that AKI is independently associated with increased mortality. Increased in hospital mortality occurs in patients with both mild[3,137–139] and severe (requiring renal replacement therapy) AKI.[140,141] Long term mortality is also increased in patients with AKI. For example, patients with normal renal function who developed AKI after cardiothoracic surgery had increased risk of death at 10 years when controlling for other variables; increased long term mortality risk occurred even in patients who had complete recovery of kidney function.[142]

Although AKI is clearly associated with increased risk of in hospital and long term mortality, the mechanisms by which AKI contributes to death are unclear. It is possible that the development of AKI is a marker of susceptibility to injury or of the severity of underlying illness. However, emerging clinical and experiment data are accumulating that AKI contributes to distant organ injury. Thus, the high mortality of AKI may be due to deleterious short and long term systemic effects. In experimental models of AKI, it has been demonstrated to adversely affect pulmonary, cardiac, immune, GI, brain and hepatic function.[143,144] Thus, an important approach to reducing the significant mortality of AKI will be to identify and target its systemic complications.[145–147]

Although the focus of biomarker development in AKI has been to detect AKI early in its course with the goal of initiating therapy to improve kidney function, early identification of AKI may also facilitate treatments to target extra renal complications. Since AKI is diagnosed so late in its course,[39] the inflammatory and other systemic consequences of AKI may be greatly

under appreciated. In fact, lung and other organ failures appear to precede AKI and are thus assumed to cause AKI. Thus, AKI causing extra renal organ dysfunction is likely a clinically under recognized phenomenon, especially since the systemic effects of AKI may occur early in AKI and AKI is typically diagnosed late. The development of biomarkers that diagnose AKI earlier in its course will not only assist in the treatment of AKI, but may be used to better establish the course of extra renal complications and the role of kidney failure in the development of other organ dysfunction. Furthermore, an additional area of potential development is the identification of biomarkers that might predict specific extra renal complications. Although numerous extra renal complications may occur in patients with AKI, the best studied complications that have associated biomarkers related to AKI in patients are the effects of AKI on inflammation and lung injury. Therefore, in this section, the inflammatory and pulmonary complications of AKI as well as their potential biomarkers will be discussed. Data from animal as well as clinical studies will be reviewed.

10.1. AKI and inflammation

10.1.1. Proinflammatory cytokines mediate organ dysfunction

It is well understood that an exuberant inflammatory response is an underlying mechanism behind the development of organ failure in patients with multiple organ dysfunction syndrome (MODS). The proinflammatory cytokines TNF α and IL 1β initiate the cascade of events resulting in the systemic inflammatory response syndrome (SIRS) that can lead to MODS. In animals, injection of either TNF α or IL 1β results in a shock like state characterized by fever, hypotension, cardiac dysfunction and lung injury with pulmonary edema and inflammation.[148] Increased production of both TNF α and IL 1β occurs after both infectious and non infectious assaults such as sepsis, pancreatitis and trauma. These proinflammatory cytokines mediate organ dysfunction and trigger increased production of a cascade of other, downstream cytokines such as IL 6 and IL 8.

10.1.2. Proinflammatory cytokines are increased in the serum in animal models of AKI

Data in animal models of AKI suggest that the inflammatory response in AKI is dysregulated. The effect of AKI on the production and elimination of proinflammatory cytokines may be a key mechanism by which patients with AKI have increased distant organ dysfunction and increased mortality. In animals with AKI, TNF α,[147,149] IL 1β,[147,149,150]

IL 6,[150 152] KC[99,150,152] and GCSF[153] increase in the serum after AKI. Since cytokine production also increases in the kidney,[99,151,154 156] renal cytokine production may contribute to renal injury and cause the increase in serum cytokines. Circulating cytokines may then contribute to extra renal organ injury (discussed further below). Although the kidney is likely an important source of cytokine production in AKI, data suggest that extra renal cytokine production may also occur. For example, serum cytokines such as IL 6 are increased after bilateral nephrectomy;[150,152] because both kidneys are removed in this model of acute renal failure, the kidney cannot be the source of increased serum cytokines in this model. The increase in serum cytokines after bilateral nephrectomy is notable as it demonstrates that the systemic milieu of acute renal failure results in a proinflammatory state possibly due to increased extra renal cytokine production.[157 159]

10.1.3. Clearance of proinflammatory cytokines may be impaired in acute kidney injury

The metabolism and clearance of IL 1β, IL 6, IL 10, GCSF and TNF α have been examined in animals and data suggest that the kidney plays a role in the elimination of these cytokines.[160 165] In patients with chronic renal failure not on dialysis, serum IL 6 and TNF α are increased,[166] suggesting that impaired kidney function results in increased cytokine levels. Additionally, increasing levels of serum IL 6 are significantly correlated with decreasing levels of glomerular filtration rate,[167] further suggesting that impaired kidney function may affect cytokine clearance. Although filtration and excretion cytokines may occur,[150] current evidence suggests that cytokines are not primarily cleared via filtration and excretion, but may be filtered, reabsorbed and metabolized by the proximal tubule.[162]

10.1.4. Excess production and impaired clearance of proinflammatory cytokines may occur in AKI

Although many insults affect cytokine production, AKI may be a unique scenario where *both* production and clearance of cytokines are affected. Because numerous insults which lead to cytokine production may occur in patients already with AKI (e.g. hemorrhage, infection), impaired elimination and accumulation of cytokines would have significant clinical consequences. The excess cytokine burden due to increased production and impaired cytokine clearance may explain the development of distant organ dysfunction in patients with AKI.

10.1.5. Serum cytokines are increased in patients with AKI

A number of studies have demonstrated that the inflammatory response of patients with AKI may be dysregulated and that the effect of AKI on cytokines may predict adverse outcomes. As discussed below, serum IL 6, IL 8 and IL 10 may be particularly relevant biomarkers of AKI that are associated with adverse outcomes and thus have the potential to be biomarkers of extra renal complications of AKI as well.

10.1.6. Serum IL-6, IL-8 and IL-10 are increased in patients with established AKI and predict mortality

One of the first studies to examine serum cytokine levels and outcomes in patients with AKI was an analysis of a subset of patients in the Program to Improve Care in Acute Renal Disease (PICARD) study. PICARD was a prospective multicenter cohort study designed to examine the natural history and outcomes of critically ill ICU patients with established AKI. In order to examine the effect of AKI on inflammation, serum IL 1β, TNF α, IL 6, IL 8, C reactive protein, and IL 10 were determined in a subset of 98 patients from the PICARD study at the time of enrollment and then weekly for the duration of the hospital stay. Briefly, patients were enrolled into the PICARD at the time of nephrology consultation, indicating that the patients studied were those with established AKI. For patients with a base line serum creatinine less than 1.5 mg/dL, AKI was defined as an increase in serum creatinine of at least 0.5 mg/dL within 48 h; for those with a baseline creatinine of greater than 1.5, AKI was defined as an increase in serum creatinine of at least 1.0 mg/dL within 48 h. IL 1β, TNF α, IL 6 and IL 8 are proinflammatory cytokines, CRP is an acute phase reactant that is typically increased in inflammatory conditions, and IL 10 is an anti inflammatory cytokine. Compared to healthy controls ($n = 48$), patients with AKI had significantly elevated levels of all serum markers as deter mined at baseline (i.e. at the time of entry into the study). Compared to stable end stage renal disease patients, patients with AKI had significantly increased IL 6, IL 10 and CRP (IL 1β, TNF α and IL 8 were not deter mined in the end stage renal disease (ESRD) patients).

To determine if increases in cytokines or CRP might portend worse outcomes, a multivariate analysis of cytokines for predictors of in hospital mortality and adjusted for demographics and sepsis status was performed. After adjustment, increased serum levels of IL 6, IL 8 and IL 10 at baseline were significantly correlated with increased in hospital mortality in patients with AKI. Specifically, increasing quartiles of cytokine values were

associated with increasing risk of mortality. For example, the IL 6 values (pg/mL) of 65.4 (quartile 1), 110.8 (quartile 2), 227.4 (quartile 3) and 641.7 (quartile 4) were associated with an increased odds ratio of death of 1.0, 1.3, 1.8 and 3.0, respectively. The fact that increases in proinflammatory cyto kines (IL 6 and IL 8) and an anti inflammatory cytokine (IL 10) predicted increased mortality, suggests that the cytokine response in patients with AKI is significantly dysregulated. Given that increased proinflammatory cyto kines are associated with organ dysfunction and that increased anti inflammatory cytokines might be associated with increased risk of infection, it is plausible that the increase in these cytokines are not just biomarkers of poor outcome, but may play a role in mediating extra renal complications (e.g. lung injury, cardiac dysfunction, infections). In the PICARD study, it is important to note that when cytokines values were further adjusted for severity of illness (APACHE III scores), only IL 6 remained an independent predictor of mortality. Thus, IL 6 may have particular clinical relevance regarding outcomes in patients with AKI.

Another study has also examined the relationship between increases in plasma IL 6, IL 8 and IL 10 on outcomes in critically ill ICU patients with AKI.[168] In this study, HLA DR expression and plasma IL 6, IL 8 and IL 10 were determined in 103 consecutive critically ill ICU patients with the systemic inflammatory response syndrome, with and without AKI.[168] HLA DR and plasma cytokines were determined prospectively on the day of admission and 2 days after. Patients with AKI had signif icantly lower HLA DR expression and higher plasma levels of IL 6, IL 8 and IL 10 than patients without AKI. Thus, even in patients with SIRS, the cytokines IL 8, IL 6 and IL 10 emerged as notably elevated with patients with AKI. On day 2, serum levels of IL 6 and IL 10 demon strated moderate significant power to predict survival (AUCs of 0.703 and 0.749, respectively).

In summary, two studies of critically ill patients with AKI found that serum IL 6, IL 8 and IL 10 were increased and that the increases in serum IL 6 and IL 10 were associated with increased mortality.

10.1.7. Serum IL-6 is an early biomarker of AKI

In the studies discussed above, serum IL 6 and other inflammatory markers were found to be increased in patients with *established* AKI. A number of subsequent studies have found that serum IL 6 and other proinflammatory factors are also increased *early* after AKI and may be used to identify patients with AKI prior to a rise in serum creatinine.

In an analysis of patients from the Prospective Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis (PROWESS) dataset, predictors of AKI in critically ill patients with severe sepsis were examined. PROWESS was a prospective randomized controlled study of the use of drotrecogin α to treat severe sepsis. For their analysis, the charac teristics of the 547 patients who developed AKI in the placebo arm of the study were examined (there were 840 total patients in the placebo arm of this study). AKI was defined as an increase in serum creatinine of 25% or 0.3 mg/dL during the first week. Data analysis included biochemical, clinical and demographic data, platelet count, protein C concentration, APACHE II scores and plasma IL 6 concentration. Interestingly, increasing quartiles of plasma IL 6 were significantly correlated with the development of AKI as judged by an increase in serum creatinine.

IL 6 and other inflammatory markers as predictors of the development of AKI were also studied in an analysis of 879 patients involved in the low tidal volume versus high tidal volume mechanical ventilation study database of the first National Heart, Lung, and Blood Institute Acute Respiratory Distress Syndrome Clinical Network (ARDS net) trial. In this trial, 209 (24%) patients developed AKI as defined by an increase in serum creatinine of at least 50% from baseline. Baseline values of IL 6, IL 8, IL 10, von Willebrand factor, TNF α, types I and II soluble TNF receptors (sTNF I and II), protein C, plasminogen activator inhibitor 1 (PAI 1), surfactant protein A, surfactant protein D, and intercellular adhesion molecule 1 were correlated with the development of AKI. After adjustments for demo graphics, interventions, and severity of illness, increased levels of IL 6, sTNFR I, sTNFR II and PAI 1 levels were independently associated with the development of AKI.

In summary, analysis of the PROWESS study and ARDS net trial demonstrated that increased serum IL 6 was independently associated with the development of AKI in patients with sepsis and ARDS, respectively.

10.2. Pulmonary complications of AKI

Pulmonary complications are the most common and well recognized extra renal complication of AKI.[169] In fact, respiratory failure requiring mechanical ventilation occurs twice as often in patients with AKI than in similarly ill patients without AKI.[140,170] The requirement for mechanical ventilation is even higher for AKI patients that require renal replacement therapy (74% vs 30%).[140] The development of respiratory failure in patients

with AKI is a particularly ominous occurrence and is associated with a marked increased mortality.[140,170 178] The need for mechanical ventilation is an independent predictor of mortality in patients with AKI, even when adjusted for severity of illness.[140,170 178] In one study, the mortality rate for AKI with mechanical ventilation was 81% vs 29% for those not requiring mechanical ventilation;[171] and respiratory failure was associated with the worst prognosis of all associated organ failures with an odds ratio of death of 10.3 for associated respiratory failure vs 1.7 for associated non respiratory organ failure.[171] For those who do survive, the development of respiratory failure is also associated with increased morbidity. Patients with AKI and respiratory failure also have an increased likelihood of being discharged to an extended care facility.[136]

Data suggest that AKI can both cause and exacerbate pulmonary function that may ultimately lead to respiratory failure requiring mechanical ventilation. For example, a recent study demonstrated that patients with AKI require mechanical ventilation longer and have an impaired ability to wean from mechanical ventilation.[179] In this observational, retrospective analysis, the outcomes of critically ill cancer patients with respiratory failure were compared between those with and without AKI (defined as in increase in serum creatinine to at least 1.5). The median duration of mechanical ventilation was 10 days in AKI patients vs 7 in patients without AKI; the duration of weaning was 41 days in AKI patients vs 21 days in patients without AKI. In another study, prevention of AKI with *N* acetylcysteine administration prior to intravenous contrast administration significantly reduced the need for mechanical ventilation.[180]

Pulmonary complications due to renal failure have been recognized for over 100 years;[181] however, the pathogenesis of AKI associated respiratory failure remains to be clearly explained. Fluid retention and overload leading to hydrostatic (cardiogenic) pulmonary edema is well known to cause respiratory failure in patients with both AKI and end stage kidney disease.[146] This form of pulmonary edema is typically characterized by signs of fluid overload, including lower extremity edema, increased pulmonary capillary occlusion (wedge) pressure and increased central venous pressure (CVP). The presence of this form of respiratory compromise is typically confirmed by resolution of symptoms with fluid removal via diuretics or ultrafiltration by dialysis. Although fluid overload is one mechanism of pulmonary edema that occurs in patients with AKI, data suggest that other mechanisms of lung injury may also occur. Pulmonary edema and shortness of breath in the presence of normal or low pulmonary capillary wedge pressure has been

demonstrated,[182,183] suggesting that AKI may also cause non cardiogenic pulmonary edema.

Non cardiogenic pulmonary edema is the hallmark of acute lung injury (ALI), and its more severe form, acute respiratory distress syndrome (ARDS). Acute lung injury is defined by the following: (1) chest X ray evidence of bilateral infiltrates; (2) no evidence of heart failure (pulmonary capillary wedge pressure less than 18); and (3) Pa_{O_2}/Fi_{O_2} between 201 and 300 mmHg.[184] ARDS is the more severe form which is defined by the same parameters, except a Pa_{O_2}/Fi_{O_2} of 200 or less. Pa_{O_2} (arterial oxygen pres sure) is in mmHg and the fraction of inspired oxygen (Fi_{O_2}) ranges from 0.21 (room air) to 1 (100% oxygen). Thus, a patient with a Pa_{O_2} of 60 mmHg on 100% oxygen would have a Pa_{O_2}:Fi_{O_2} ratio of 60 and be considered to have ARDS. Although caused by a wide variety of initial insults (e.g. trauma, sepsis, pneumonia), central to the pathogenesis of ALI is an exuberant proinflammatory response that results in the upregulation of adhesion molecules and chemokines in the lung which facilitates neutrophil infiltration. Lung neutrophil infiltration and activation directly injures the capillary endothelial barrier leading to the influx of proteinaceous edema fluid accumulation in the interstitial and alveolar space. In patients with AKI and pulmonary edema, lung neutrophil infiltration has been documented in autopsy studies suggesting that neutrophil mediated capillary injury may occur in AKI.[185,186] Thus, clinical data suggest that AKI causes inflam mation, endothelial damage and non cardiogenic pulmonary edema.

10.2.1. Lung inflammation in experimental AKI

Animal data support the notion that AKI may cause non cardiogenic pulmonary edema via neutrophil infiltration. Lung injury has been exam ined after ischemic AKI[150,152,187 193] as well as bilateral nephrec tomy[150,152,189,190,193,194] and is characterized by pulmonary edema and neutrophil infiltration. Ischemic AKI is a common cause of AKI in hospi talized patients, and as such, it is a clinically relevant model. It is well known, however, that ischemia reperfusion injury of other organs (e.g. hind limb, gut, liver) is also associated with lung injury. Bilateral nephrectomy is a useful model to study the systemic effects of acute renal failure because renal failure occurs in the absence of renal ischemia. Remarkably, lung injury is similar after ischemic AKI and bilateral nephrectomy and is char acterized by neutrophil infiltration and pulmonary edema within 4 h.[150,152] Unilateral renal ischemia, a model of renal ischemia without renal failure (serum creatinine and BUN are normal), is not associated with lung

injury.[150] Thus, renal ischemia in the absence of renal failure is insufficient to cause lung injury.

Further supporting the role of inflammatory mediators in the patho genesis of AKI mediated lung injury is the demonstration that cytokines (e.g. TNF α[187]), adhesion molecules (e.g. ICAM 1[187]), chemokines (e.g. KC[150,152], MIP 2[150,152], CINC2[190], CXCR2[190]), heat shock proteins (e. g. HSP70,[190] HSP47[190]) and NFκB[187] are all increased in the lung after AKI. Genomic responses in the lung after ischemic AKI or bilateral nephrectomy are associated with increased inflammatory genes.[189] In addition, anti inflammatory treatment with IL 10,[150] alpha MSH[187] or a p38 MAPkinase inhibitor[191] each protect against AKI mediated lung injury.

Recently, two studies have utilized gene chip technology to characterize the pulmonary effects of AKI.[188,189] In this first study, 22,626 genes were analyzed in the lung 6 and 36 h after ischemic AKI and bilateral nephrec tomy. At 6 h, 266 lung genes were upregulated and 615 lung genes were downregulated after ischemia reperfusion injury, while no genes were changed 6 h after bilateral nephrectomy. At 36 h, 600 lung genes were unregulated and 327 were downregulated after ischemic AKI; 519 lung genes were upregulated and 226 were downregulated after bilateral nephrectomy. In the follow up study, the 'inflammatory transcriptome' in the lung after ischemic AKI was examined. One hundred amd nine inflammatory genes were examined in the kidney and lung after ischemic AKI. Interestingly, the changes in genes in the kidney and lung were similar. It was noted that the innate immunity genes Cd14, Socs3, Saa3, Lcn2 and IL1r2 were changed. Upon functional analysis, it was demonstrated that IL 10 and IL 6 signaling was particularly involved in the effects of ischemic AKI on the lungs.

10.2.2. Potential role of IL-6 in AKI-mediated lung injury

To determine if IL 6 mediates lung injury after AKI, IL 6 deficient mice and IL 6 antibody treated mice have been studied. Both IL 6 deficient mice and IL 6 antibody treated mice had improved lung injury after ischemic AKI and bilateral nephrectomy. The improvement in lung injury with IL 6 inhibition was associated with reduced lung neutrophil accumulation, reduced pulmonary edema and reduced lung CXCL1 (also known as KC). CXCL1 is the murine analog of human IL 8. CXCL1 and IL 8 are neutrophil chemokines. Alveolar macrophage production of IL 8 is thought to be a key mediator of acute lung injury in patients.[195 197]

10.2.3. Serum IL-6 and IL-8 increase 2 h after cardiopulmonary bypass-associated AKI and predict prolonged mechanical ventilation

To determine whether serum cytokines might be early biomarkers of AKI and predict the adverse outcome of prolonged mechanical ventilation, a case control study of serum cytokines in pediatric patients undergoing cardiac surgery was performed.[198] Levels of serum interleukin (IL) 1α, IL 5, IL 6, IL 8, IL 10, IL 17, IL 18, interferon (IFN) γ, tumor necrosis factor α (TNF α), granulocyte colony stimulating factor (G CSF) and granulocyte macrophage colony stimulating factor (GM CSF) were deter mined in 18 cases (with AKI) and 21 controls (without AKI) at 2, 12 and 24 h following cardiopulmonary bypass (CPB). AKI was defined as a 50% increase in serum creatinine within 3 days of CPB. Serum IL 6 levels at 2 and 12 h and serum IL 8 levels at 2, 12 and 24 h were significantly associated with the development of AKI. Of note, none of the other cytokines were significantly changed in cases versus controls at these time points.

To determine if the increases in serum IL 6 and IL 8 might be associated with complications in patients with AKI, the relationship of levels of these cytokines with prolonged mechanical ventilation (greater than 24 h) then was determined. In patients with AKI, serum IL 6 levels were significantly associated with prolonged mechanical ventilation with an area under the receiver operator characteristic (ROC) curve of 0.95. IL 8 levels at 2 h predicted prolonged mechanical ventilation in all patients. Although several previous studies had determined that certain serum cytokines were increased in patients with AKI, this is the first study to document that serum cytokines increase very early (within 2 h of AKI) and predict an adverse outcome (prolonged mechanical ventilation). It is remarkable that the pattern of cytokine increase and decline noted in patients with AKI is similar to the rise and fall of serum cytokines in animal models of AKI.[150] Specifically, serum IL 6 and KC (CXCL1, the murine analog of human IL 8) are also increased by 2 h after AKI, where the levels are the highest, and then begin to decline at 12 and 24 h.

Another recent study has examined whether increases in plasma IL 8, specifically, might be a biomarker of AKI.[199] In this study, plasma IL 8 was determined before and at 2, 24 and 48 h in 143 adult patients following cardiopulmonary bypass. AKI was defined by two criteria: (1) an increase in serum creatinine by at least 0.3 mg/dL or 50% (AKI network [AKIN] stage 1) or (2) an increase in serum creatinine by at least 50% alone. Increased serum IL 8 at 2 h predicted the identification of AKI by both criteria.

In summary, in both patient and animal models, early AKI is associated with a proinflammatory burst that is characterized by the early increase in serum IL 6 and KC/IL 8. A proinflammatory burst such as this is the common link and accepted mechanism by which disparate inciting events (e.g. hemorrhage, trauma and pancreatitis) mediate respiratory com plications and acute lung injury in other settings.[200,201] Other pro inflammatory cytokines have not been shown to be increased early in patients with AKI. Thus, the increase in serum IL 6 and IL 8 in patients with AKI who develop prolonged mechanical ventilation is particularly relevant as these cytokines may be both biomarkers of AKI and prolonged mechanical ventilation as well as therapeutic targets of pulmonary complications of AKI.

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CHAPTER 6

Biomarkers in Kidney Transplantation

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Contents

Biomarkers in Kidney Disease
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1. BIOMARKERS: AN OVERVIEW

The use of biomarkers in the diagnosis and prognostication of specific diseases is an area of intense research. Simple methods to non invasively diagnose and monitor diseases hold obvious clinical appeal. In trans plantation, the ability to non invasively diagnose and monitor the various causes of allograft dysfunction might result in improved graft and patient outcomes.

A number of authors have attempted to define the characteristics of a biomarker. Parikh and Deverajan[1] suggested the following characteristics were desirable for biomarkers of acute kidney injury (AKI): (1) the biomarker should be easily detectable, using simple bedside or standard clinical laboratory techniques, in readily available clinical samples (such as urine and blood); (2) the biomarker should be easy, rapid and reliable; (3) the biomarker should possess a high sensitivity to detect the relevant disease early; (4) the biomarker should have a range of cutoff points that would allow for risk stratification; and finally (5) the biomarker should demonstrate strong performance on statistical analysis.

In discussing potential biomarkers of brain injury, Bakay and Ward[2] suggested a biomarker should be: (1) specific and sensitive for the brain; (2) appear in serum rapidly; and (3) demonstrate a reliable temporal rela tionship with injury. These cogent definitions could be more broadly applied to all biomarkers.

Sandler et al[3] suggested that biomarkers should also allow a clinician to determine the prognosis of a particular disease and enable physicians to plan diagnostic and treatment interventions.

Kidney transplantation is distinct from AKI of native kidneys and from brain injury in that the clinician is able to obtain tissue more easily. Many investigators have therefore suggested the use of specific biomarkers obtained from the allograft (as opposed to serum or urine) to identify disease states early, prognosticate outcome and modify treatment.

2. BIOMARKERS OF ACUTE KIDNEY INJURY POST-TRANSPLANTATION (TABLE 6.1)

2.1. Pre-transplant biomarkers

2.1.1. Tissue markers

Oberbauer et al[4] assessed whether apoptosis of tubular epithelial cells in donor kidney biopsies was associated with early renal allograft function.

Table 6.1 Biomarkers of AKI post-transplantation

Biomarker	*N*	Result	Time of collection	Source	Reference
Apoptosis of RTE cells in donor kidney biopsies	89	Biopsies with subsequent ATN had significantly greater apoptotic RTE cells vs immediate trans plant function or early AR A significantly greater percentage of apoptotic cells were found in the distal vs the proximal tubule in all groups	Donor biopsy	Tissue	4
ICAM 1, VCAM 1 and E selectin	73	LRT donor biopsies had significantly lower expres sion of ICAM 1 and VCAM 1 vs CRTs Less expression of RTE cell ICAM 1 in CRTs with subsequent prompt function vs DGF	Donor biopsy	Tissue	5
Donor sCr	51	DGF more frequent in the group with falling donor sCr Mean recipient serum creatinine and allograft survival not different in donors with falling vs rising sCr More donor hypertension and more chronic lesions in the biopsies of donors with a rising sCr	Expanded criteria donor sCr	Serum	6
KIM 1	62	KIM 1 seen in 100% of cases with obvious tubular injury. KIM 1 seen in 92% of biopsies with AR Highest intensity for KIM 1 seen in tubular injury group followed by AR, and lowest in protocol biopsies KIM 1 correlated with BUN and sCr	Allograft biopsy	Tissue	10
Soluble glycoprotein 130 (sgp130)	105	Pre transplant sgp130 plasma levels significantly reduced in patients who went on to have ATN vs those who had immediate graft function or AR	Pre transplant plasma	Plasma	7

(*Continued*)

Table 6.1 Biomarkers of AKI post-transplantation—cont'd

Biomarker	*N*	Result	Time of collection	Source	Reference
Serum calcium	585	Serum calcium levels correlated independently with DGF. The use of calcium channel blockers prior to transplantation protected against DGF. Nephrocalcinosis was found in 17% of biopsies but was not associated with DGF or serum calcium levels	Post transplant serum	Serum	11
NGAL	25	NGAL expression significantly increased in CRT biopsies vs LRTs. NGAL staining intensity correlated with cold ischemia time and with peak post transplant sCr. Most intense staining for NGAL seen in DGF	Allograft biopsy	Tissue	12
uIL 18	72	ATN had greater uIL 18 vs all other conditions including: controls; prerenal azotemia, UTI, CRI, and nephrotic syndrome. CRTs with DGF had a higher median uIL 18 than CRTs with PGF and LRTs with PGF. Lower uIL 18 associated with steeper decline in sCr POD 0 4	Allograft urine	Urine	13
uIL 18 and NGAL	53	Urine NGAL and IL 18 were significantly elevated in DGF. ROC analysis for the prediction of DGF based on urinary NGAL or IL 18 on POD 0 showed an AUC of 0.9. Urine NGAL and IL 18 on POD 0 predicted the postoperative trend in sCr by multivariate analysis	Allograft urine	Urine	14

uIL 18, NGAL and KIM 1	91	Median levels of NGAL and IL 18, but not KIM 1, had significant separation at all time points in DGF, SGF and IGF. ROC curve analysis suggested urine NGAL or IL 18 on POD 1 were moderately accurate to predict dialysis within 1 week. Multivariate analysis confirmed elevated levels of uIL 18 or NGAL predicted the need for dialysis. NGAL and IL 18 predicted graft recovery up to 3 months post transplant	Allograft urine	Urine	118
$Beta_2$ microglobulin (i/cβ2m), RBP, NGAL and α1m	100	None of the biomarkers allowed for clear differentiation between stable transplants with normal tubular histology and stable transplants with subclinical tubulitis	Allograft urine	Urine	16
Urinary actin, GGTP, LDH, IL 6, TNF α and IL 8	40	ROC curve analysis showed elevated urinary actin, IL 6, and IL 8 on POD 0 were predictors of sustained ARF	Allograft urine	Urine	17
SNPs of genes for CD28, CTLA4, ICOS and PPCD1	678	DGF associated with 2 SNPs on the ICOS gene, rs10183087 and rs4404254 ICOS SNP rs10932037 associated with decreased graft survival None of the SNPs were associated with AR	Deceased donor recipients	SNPs (genes)	19
SNPs in donor genes for TNF α, TGFβ1, IL 10, p53, TP53 and HMOX1	965	DGF significantly associated with the G allele of TNF α SNP rs3093662	Donors	SNPs (genes)	21

α1m, α_1-microglobulin; HMOX1, heme oxygenase 1; POD, postoperative day; BBP, retinol-binding protein; RTE, renal tubular epithelial; uIL-18, urinary IL-18.

Donor biopsies of patients with biopsy proven acute tubular damage after engraftment but no signs of rejection ($n = 23$) had significantly greater apoptotic tubular epithelial cells when compared to patients with immediate transplant function ($n = 44$) or early rejection ($n = 22$). A significantly greater percentage of apoptotic cells were found in the distal vs the proximal tubule in all groups. The authors suggested that the number of apoptotic renal tubular epithelial cells in donor biopsies prior to engraftment was predictive of subsequent acute tubular injury in the early postoperative course of patients undergoing kidney transplantation.

Schwarz et al[5] examined the contribution of adhesion molecule expression in donor kidney biopsies to early allograft dysfunction. Biopsies were obtained from living ($n = 20$) and deceased ($n = 53$) donor kidneys before engraftment and examined for the expression of the cell adhesion molecules intercellular adhesion molecule 1 (ICAM 1), vascular cell adhesion molecule 1 (VCAM 1) and endothelial leukocyte adhesion molecule (E selectin). Living donor biopsies uniformly demonstrated significantly lower expression of ICAM 1 and VCAM 1 vs deceased donor biopsies. There was no difference in tubular epithelial cell expression of adhesion molecules between transplants with primary function vs allografts with early rejection in either living or deceased donor kidneys. Signifi cantly less expression of tubular epithelial cell ICAM 1 was seen in deceased donor kidney that subsequently had prompt function ($38 \pm 29\%$) vs delayed graft function in cadaveric kidneys (65 ± 24, $P < 0.05$). The authors suggested that tubular epithelial cell adhesion molecule expression was not a predictor of acute rejection but could predict post transplant AKI due to ischemia.

2.1.2. Plasma markers

Morgan et al[6] examined whether progressively rising serum creatinine in expanded criteria donors predicted outcome. Allografts from donors with peak serum creatinine levels of >2.0 mg/dL were divided into two groups defined by the terminal donor serum creatinine: Group 1 had decreasing creatinine ($n = 27$) with a terminal creatinine ≤ 0.2 mg/dL than the peak serum creatinine, and group 2 had increasing serum creatinine ($n = 24$) with a terminal creatinine equal to the peak creatinine. Donor peak serum creatinine was not significantly different between the two groups (Group $1 = 3.1 \pm 1.3$; Group $2 = 3.2 \pm 1.3$; $P = 0.6521$). As expected, the mean terminal creatinine was significantly higher in group 2 (3.2 ± 1.3 mg/dL) vs group 1(1.9 ± 0.9 mg/dL; $P < 0.0001$). Surprisingly, the

outcomes were not statistically different between recipients of allografts from either group. DGF occurred more frequently in the group with falling donor serum creatinine (Group 1 = 32%; Group 2 = 24%; $P = 0.7881$) although the difference did not achieve statistical significance. Mean recipient serum creatinine (Group 1 = 1.6 ± 0.6; Group 2 = 1.6 ± 0.4; $P = 0.3533$) and allograft survival were also not significantly different at follow up (Group 1 = 89%; Group 2 = 92%; P = NS). The findings of proportionately more donor hypertension (Group 1 = 30%; Group 2 = 13%; $P = 0.1331$) and more chronic lesions in the biopsies of donors with a rising serum creatinine (Group 1 = 41%; Group 2 = 0%; $P = 0.0023$) may explain the poorer outcomes in the allografts from Group 1.

Sadeghi et al[7] examined the association between pre transplant plasma levels of the anti inflammatory molecule, soluble glycoprotein 130 (sgp130) and post transplant ATN in 105 first time, deceased donor kidney trans plant recipients. ATN was diagnosed in 29% (30/105) of patients and acute rejection was diagnosed in 18/130 patients. Pre transplant sgp130 plasma levels were significantly reduced in patients who went on to have acute tubular necrosis (ATN) as compared with patients who had immediate graft function ($P = 0.004$) or acute rejection ($P = 0.009$). The odds ratio of ATN was 4.3 on multivariable logistic regression analysis with a pre transplant sgp130 of ≤ 250 pg/mL.

2.2. Post-transplant biomarkers

2.2.1. Tissue markers

Kidney injury molecule 1 (KIM 1) is a transmembrane type 1 epithelial cell protein that belongs to the immunoglobulin gene superfamily. The extra cellular component contains a novel six cysteine immunoglobulin like domain and a mucin domain. Normal rat kidneys express low levels of KIM 1 mRNA and protein. In contrast, postischemic rat kidneys express significantly increased levels of KIM 1 mRNA and protein in regenerating proximal tubule epithelial cells at 48 h.[8] The extracellular component of KIM 1can be cleaved by metalloproteinases, resulting in its appearance in urine.[9]

Zhang et al[10]investigated the expression of KIM 1 as a biomarker for diagnosing early tubular injury in randomly selected kidney transplant renal biopsies by immunohistochemistry. Expression of KIM 1 was compared with morphological findings of tubular injury and acute cellular rejection. The authors also determined whether KIM 1 staining intensity correlated with renal function. Three groups were examined: Group 1 ($n = 25$) − a

control group of protocol renal transplant biopsies without any obvious injury obtained within the first year post transplant; Group 2 ($n = 25$) − biopsies demonstrating obvious tubular injury without acute cellular rejection; and Group 3 ($n = 12$) − biopsies demonstrating Banff criteria IA and IB mild acute cellular rejection (ACR). KIM 1 was absent in 72% of the protocol biopsies (Group 1). In the remaining 28%, KIM 1 expression was focal, low grade and localized to proximal tubules. No morphological difference was detected between KIM 1 positive and negative cases, leading the authors to suggest that KIM 1 expression was more sensitive than routine histology examination for detection of low grade proximal tubule injury. Group 2 biopsies demonstrated obvious tubular injury and KIM 1 expression was seen in 100% of cases. Expression localized to the plasmalemmal surface of proximal luminal epithelium but extended to the lateral cellular membranes if epithelial junctions were disrupted. The basal aspect of the epithelium was negative for KIM 1 staining even when there was marked tubular injury morphologically. In the acute rejection group (Group 3), KIM 1 expression was seen in 11/12 biopsies (92%). KIM 1 expression was not seen on infiltrating inflammatory cells, including lymphocytes, monocytes, and plasma cells in all groups. The highest intensity for KIM 1 staining was seen in the tubular injury group followed by the acute rejection group, and was lowest in the protocol biopsies. In the protocol biopsy group, greater levels of KIM 1 staining portended better recovery of function over 18 months. KIM 1 immunoreactivity correlated with BUN and creatinine when all three groups were combined as well as in each individual group.

2.2.2. Plasma markers

Boom et al[11] investigated whether serum calcium levels were a risk factor for the development of delayed graft function in a cohort of 585 cadaveric transplants. Serum calcium metabolism and the presence of nephrocalci nosis, ATN or acute rejection in biopsies obtained in the first post transplant week were related to the occurrence of delayed graft function (DGF). The incidence of DGF was 31%. Serum calcium levels correlated independently with DGF (odds ratio = 1.14 (95% confidence interval = 1.04−1.26) per 0.1 mmol/L). The use of calcium channel blockers prior to transplantation protected against DGF (OR 0.5 (95% CI 0.29−0.87)). Nephrocalcinosis was found in 17% (12/71) of biopsies but was not associated with DGF or serum calcium levels.

2.2.3. Urine markers

Mishra et al[12] hypothesized that the expression of neutrophil gelatinase associated lipocalin (NGAL) could serve as an early biomarker of AKI following transplantation. NGAL expression was assessed in specimens obtained from 13 deceased and 12 living donor kidneys approximately 1 h after engraftment. Staining intensity of NGAL was correlated with the need for dialysis, peak serum creatinine post transplant and cold ischemia time. NGAL expression was significantly increased in deceased donor biopsies compared with living donor kidneys (2.3 ± 0.8 vs 0.8 ± 0.7 respectively, $P < 0.001$). NGAL staining intensity correlated with cold ischemia time ($R = 0.87$, $P < 0.001$) and with peak post transplant serum creatinine that occurred days later ($R = 0.86$, $P < 0.001$). The most intense staining for NGAL was seen in four patients who developed delayed graft function and required dialysis in the first post transplant week. The authors concluded that NGAL staining intensity in early protocol biopsies was a novel predictive biomarker of early AKI in renal allografts.

Parikh et al[13] examined whether urinary IL 18 might serve as a biomarker of ATN in a study of 72 subjects, including healthy controls, patients with different forms of AKI and patients with other renal diseases. Patients with ATN had significantly greater median urinary IL 18 concentrations (644 pg/mg creatinine; $P < 0.0001$) compared with all other subjects, including healthy controls (16 pg/mg creatinine), patients with prerenal azotemia (63 pg/mg creatinine), patients with urinary tract infection (63 pg/mg creatinine), patients with chronic renal insufficiency (12 pg/mg creatinine) and patients with nephrotic syndrome (34 pg/mg creatinine). Median urinary IL 18 concentrations measured in the first 24 h after kidney transplantation were significantly greater in recipients of deceased donor kidneys with DGF vs recipients with prompt graft function. Recipients of deceased donor kidneys with DGF had a median urinary IL 18 of 924 pg/mg creatinine vs 171 pg/mg creatinine in patients who received a deceased donor kidney with prompt graft function and 73 pg/mg creatinine in patients who received a living donor kidney with prompt graft function ($P < 0.002$). Lower urinary IL 18 levels were associated with a steeper decline in serum creatinine concentrations on postoperative days 0–4 following kidney transplantation ($P = 0.009$).

In a follow up study the same authors[14] assessed whether urine NGAL and IL 18 were predictive biomarkers for delayed graft function (defined as dialysis requirement within the first post transplant week). Urinary NGAL

and IL 18 from recipients of living donor kidneys ($n = 23$), deceased donor kidneys with prompt graft function ($n = 20$) and deceased donor kidneys with DGF ($n = 10$) were assessed on postoperative day 0. Peak postoperative serum creatinine requiring dialysis was found to occur 2–4 days after transplant in recipients with DGF. Urine NGAL and IL 18 values were significantly elevated levels in the DGF group ($P < 0.0001$). Receiver operating characteristic (ROC) analysis for the prediction of DGF based on urinary NGAL or IL 18 on postoperative day 0 showed an area under the curve of 0.9. Both urine NGAL and IL 18 on day 0 predicted the postoperative trend in serum creatinine by multivariate analysis, after adjusting for effects of age, gender, race, urine output and cold ischemia time ($P < 0.01$).

Hall et al[15] performed a prospective, multicenter, observational cohort study of deceased donor kidney transplant recipients to assess IL 18, NGAL and KIM 1 as biomarkers for predicting graft recovery and the need for dialysis within 1 week of transplant. Serial urine samples were collected on the first 3 post transplant days and analyzed for the putative biomarkers. Graft recovery was defined as delayed graft function (DGF), slow graft function (SGF) or immediate graft function (IGF). Of the 91 recipients studied, 34 had DGF, 33 had SGF and 24 had IGF. Median levels of urine NGAL and IL 18 levels showed significant separation at all time points in all the three groups. Median urine KIM 1 levels on the other hand were not statistically different between groups. ROC curve analysis suggested that the urine NGAL or IL 18 measured on the first postoperative day were moderately accurate when used to predict dialysis within 1 week. Multivariate analysis confirmed that elevated levels of urine IL 18 or NGAL predicted the need for dialysis even after adjustment for serum creatinine, cold ischemia time, urine output, and recipient and donor age. Furthermore, NGAL and IL 18 quantiles also predicted graft recovery up to 3 months after transplantation.

Schaub et al[16] investigated whether non invasive screening of urinary biomarkers of tubular injury correlated with subclinical tubulitis found in protocol biopsies. Recipients were divided into four groups: (1) recipients with stable graft function and normal tubular histology ($n = 24$); (2) recipients with stable graft function and subclinical tubulitis on protocol biopsy ($n = 38$); (3) recipients with clinical tubulitis Ia/Ib ($n = 18$); and (4) recipients with other clinical tubular pathologies ($n = 20$). Urine was examined for intact/cleaved beta$_2$ microglobulin (i/cβ2m), retinol binding protein (RBP), neutrophil gelatinase associated lipocalin (NGAL) and

alpha$_1$ microglobulin (α1m). Tubular proteinuria was found in 38% (RBP) − 79% (α1m) of Group 1. Group 2 had slightly higher but non significant levels of i/cβ2m ($P = 0.11$), RBP ($P = 0.17$), α1m ($P = 0.09$) and NGAL ($P = 0.06$) than Group 1with substantial overlap. Groups 3 and 4 had significantly greater levels of RBP, NGAL and α1m than stable trans plants with normal tubular histology or stable transplants with subclinical tubulitis ($P < 0.002$). The authors concluded that none of the biomarkers allowed for clear differentiation between stable transplants with normal tubular histology and stable transplants with subclinical tubulitis.

Kwon et al[17] found that urinary actin, interleukin 6 (IL 6) and IL 8 were associated with sustained ischemic AKI in renal allografts. Urine specimens were collected in the first post transplant week from 30 recipients of deceased donor (including 9 with 'sustained ARF' and 21 patients deemed 'recovery') and 10 recipients of living donor kidneys. Urine was analyzed for actin, gamma glutamyl transpeptidase (GGTP), lactate dehy drogenase (LDH), IL 6, tumor necrosis factor alpha (TNF α) and IL 8. Post transplant day 0 urinary actin, GGTP, IL 6 and IL 8 were elevated in recipients who subsequently had sustained acute renal failure (ARF) vs recipients who subsequently recovered, although these did not always reach statistical significance. In contrast, recipients with recovering function had increased urinary TNF α and LDH compared to recipients with sustained ARF. ROC curve analysis demonstrated that elevated urinary actin, IL 6 and IL 8 on day 0 were predictors of sustained ARF. Using a cutoff value for actin of 24.8 μg/g urine creatinine, the sensitivity and specificity were 0.67 and 0.86 respectively. The AUC was 0.75, whereas the AUC for predicting recovery was 0.25. Using a cutoff value for IL 6 of 60.2 ng/g urine creatinine, the sensitivity and specificity were 0.83 for both parameters, and the AUC was 0.91. Using a cutoff value for IL 8 of 78.3 ng/g urine creatinine, the sensitivity and specificity were 1.00 and 0.61 respectively, while the AUC was 0.82. The authors concluded that increased urinary actin, IL 6 and IL 8 on postoperative day 0 could be biomarkers for the prediction of sustained ischemic AKI post transplant. The study also reported the urinary levels of the putative biomarkers in patients excluded from the analysis due to minimal urine flow. Unfortunately the results were generally reported per mL of urine output and could therefore not be compared directly with the cutoff values. Nevertheless, such patients are often not reported in biomarker studies, which are often conducted on selected patients. This obviously indicates a limitation of all urinary biomarker studies.

2.2.4. Genetic biomarkers of DGF

The molecule, Cluster of Differentiation 28 or CD28, is a costimulatory molecule found in T cells. Interaction with B7−1 and B7−2 on antigen presenting cells results in T cell activation, whereas interaction with cyto toxic T lymphocyte antigen 4 (CTLA 4) causes T cell inhibition.[18] The genes for CD28, CTLA4, programmed cell death and inducible costim ulator (ICOS) are all found on chromosome 2q.[19]

Haimila et al[19] examined the association of genetic variations in inducible costimulator genes with kidney transplant outcomes. Single nucleotide polymorphisms (SNPs) of the genes for CD28, CTLA 4, ICOS and PPCD1 were investigated in 678 deceased donor recipients and correlated with kidney transplant outcome. Delayed graft function (DGF) was defined as a serum creatinine of > 500 μmol/L (> 5.65 mg/dL) in the first post transplant week, the need for more than one dialysis session or the presence of oliguria (defined as < 1 L/day for more than 2 days). The occurrence of DGF was associated with two SNPs on the ICOS gene, rs10183087 and rs4404254 (odds ratio $= 5.8$; $P = 0.020$ and odds ratio $= 5.8$; $P = 0.019$, respectively). However, the associations reported were no longer significant after a Bonferroni correction for multiple tests. ICOS expression has been found to be decreased in rs4404254 TT homozygotes compared with CT or CC genotypes.[20] The authors suggested therefore that rs4404254 TT homozygotes perhaps expressed lower levels of ICOS and thus endured more DGF. The ICOS SNP rs10932037 was associated with decreased graft survival ($P = 0.026$). None of the SNPs examined were associated with acute rejection.

Israni et al[21] performed a cross sectional study of DGF in 965 recipients of deceased donor kidneys from 512 donors. DGF was defined as the need for dialysis therapy in the first week after transplantation. Single nucleotide polymorphisms (SNPs) in the donor genes for TNF α, transforming growth factor beta$_1$ (TGF β_1), interleukin 10 (IL 10), p53 (TP53) and heme oxygenase 1 (HMOX1) were correlated with the occurrence of DGF, as well as secondary outcomes including acute rejection and estimated glomerular filtration rate. DGF was significantly associated with the G allele of TNF SNP rs3093662 (odds ratio $= 1.85$ compared with A allele; 95% CI $= 1.16$−2.94; $P = 0.009$; $n = 965$) after adjustment for cold ischemia time, recipient race, extended criteria donor, donor cause of death, donor race, donor age and source of DGF information. This association however became non significant after adjustment for multiple comparisons.

The authors suggested that the study had inadequate sample size for the study of infrequent genotypes and multiple comparisons.

3. BIOMARKERS OF ACUTE REJECTION (TABLE 6.2)

A number of biomarkers derived from a variety of sources have been used to determine the possible risk of acute rejection both in the donor and in the recipient.

3.1. Genetic biomarkers of acute rejection (Table 6.3)

Dmitrienko et al[22] used a case control design to examine the polymorphic frequencies of the T cell signaling genes CD45, CD40L and CTLA 4, and the cytokine genes TNF α, IFN γ, IL 10 and TGF β in 100 deceased and living donor recipients of first kidney transplants. Fifty recipients with biopsy proven acute rejection (BPAR) were compared with 50 recipients who did not have acute rejection (AR). Fifty normal subjects were included as an indicator of local polymorphic gene frequency. Multivariate analysis showed no significant association between BPAR and single nucleotide polymorphisms in CTLA 4, TGF β, IL 10 or TNF α genes or dinucleo tide repeat polymorphisms in IFN γ and CD40L genes. Allele TGF β 25pro was significantly associated with increased graft failure ($P = 0.0007$) while CD40L 147 was associated with reduced graft failure ($P = 0.004$). No subject had a CD45 G (guanosine instead of cytosine) allele detected, likely due to the inclusion of only Caucasian patients. Thus immune response gene polymorphisms examined in this study showed no significant association with BPAR in subjects receiving triple immunosuppression.

The Fc gamma receptor IIA (FcγRIIA) is a member of the Fc receptor family. Unlike the Fc gamma receptors FcγRI and FcγRIIIa which are common to both mice and humans, the FcγRIIA is unique to humans.[23] These receptors activate cells via src family kinases and are thought to play a central role in leukocyte activation and cytotoxicity, and the initiation of the complement cascade.[24]

Yuan et al[24] examined whether FcγRIIA genotypes were associated with renal allograft rejection. The distribution of the genotypes in the study patient group differed from the control groups. The study included 53 recipients who had suffered graft loss within 1 year of transplant (including 42 recipients who had lost their graft within 3 months) due to histologically confirmed acute rejection and 46 renal allograft recipients with well functioning grafts for at least 1 year. A group of 58 normal, random blood donors were also

Table 6.2 Biomarkers of acute rejection

Biomarker	*N*	Result	Time of collection	Source	Reference
CXCL9 and CXCL10	69	Urine MIG significantly increased in AR patients In AR, urinary MIG increased 5 days prior to biopsy	Urine collected for a median 29 days post transplant	Urine	34
Pre transplant serum CXCL10	316	Increased CXCL10 associated with significantly greater AR More severe and steroid resistant AR with significantly higher CXCL10 levels Multivariate analysis showed CXCL10 and DGF had highest predictive power of graft loss	Pre transplant sera	Sera Tissue	35
Fractalkine, monokine induced by IFN γ, IFN γ inducible protein 10 (IP 10), MIP3α, granzyme B, and perforin	215	AUC for AR; fractalkine 0.834, Mig 0.901, IP 10 0.810, MIP3α 0.734, granzyme B 0.765 and perforin 0.779 Fractalkine, IP 10 and granzyme B together were best able to distinguish AR from no AR Only changes in urinary fractalkine distinguished recipients with AR from ATN	Protocol urines every 2 weeks × first 2 months, on biopsy day, and at end of anti AR therapy	Post transplant urine	39

sCD30	3899	Five year graft survival lower with high serum sCD30 vs graft survival with low sCD30 Less need for anti rejection therapy in year 2, but not year 1, with low sCD30	Pre transplant	Sera	52
sCD30	120	During 47.5 months of follow up, pre transplant sCD30 was not associated with differences in graft survival rate Higher incidence of AR in the low sCD30 High sCD30 was associated with significantly elevated serum creati nine 3 years post transplant	Pre transplant	Sera	6
sCD30	56	ROC analysis on postoperative days 3 5 showed sCD30 identified recipients who subsequently developed AR or those with ATN in the absence of rejection	Within the first 20 days post transplant	Plasma	55
TGF β_1	115	Plasma TGF β_1 greater in allograft recipients vs normal controls; did not distinguish AR from chronic vascular rejection or ATN Urine TGF β_1 was similar in normals and allograft recipients	At time of biopsy	Plasma Urine	62

(*Continued*)

Table 6.2 Biomarkers of acute rejection—cont'd

Biomarker	*N*	Result	Time of collection	Source	Reference
CD103/18S ribosomal (r)RNA	49	CD103 mRNA increased in AR vs other findings on allograft biopsy, CAN and stable graft function. 18S rRNA did not vary significantly among the groups	Within 24 h of biopsy	Urine	61
IL 4, IL 5 and IL 6, IFN γ, perforin and granzyme B mRNA	61	IL 4, IL 5 and IL 6, IFN γ, perforin and granzyme B mRNA were significantly associated with AR Patients with infections, ATN, CsA nephrotoxicity and 'uncertain rejec tion episodes' were excluded. Not all AR was confirmed by biopsy	For 3 months post transplant	Sera	45
Perforin, granzyme B and cyclophin B mRNA	122	Levels of perforin and granzyme B mRNA, but not levels of constitutively expressed cyclophin B mRNA, were greater in the urinary cells from patients with AR versus no AR and were significantly higher in patients who developed AR within the first 10 days post transplant	First 10 days post transplant	Urine	46

Perforin and granzyme B	67	Recipients with AR had increased levels of granzyme B and perforin transcripts on days 5 7, 8 10, 11 13, 17 19, 20 22 and 26 29. Best diagnostic result achieved with samples taken on postoperation days 8 10 Diagnosis of AR could be made at a median of 11 days before the diagnosis by standard criteria Both perforin and granzyme B gene expression decreased after initiation of anti rejection therapy	First month post transplantation	Blood	48
MCP 1	20 (tissue studies) 38 (urine studies)	Urine and tissue MCP 1 significantly higher than that seen with ATN or in normal tissue	1 60 months post transplant	Tissue Urine	30
PS, ES, platelets, leukocyte common antigen, macrophages, T cells and neutrophils	77	Significantly more recipients with AR if the donor biopsy was positive for PS, contained $\geq$ 5 leukocytes/ glomerulus, contained $>$ 9.3 leukocytes/hpf or were both PS positive and contained $>$ 9.3 leukocytes/hpf	Pre transplant	Tissue	71

(*Continued*)

Table 6.2 Biomarkers of acute rejection—cont'd

Biomarker	*N*	Result	Time of collection	Source	Reference
Serum CRP	441	Serial CRP measurements provide economical and reproducible evidence of immune activation, help discriminate renal dysfunction due to CsA nephrotoxicity or rejection, and allow appropriate modification of immunosuppressive therapy	Daily	Serum	65
Serum CRP	97	Pre transplant CRP levels greater in patients who subsequently developed AR Recipients within the lowest CRP quartile had longer times to rejection 3 month incidence of AR lower in the lowest CRP quartile group Only pre transplant CRP level was an independent risk factor for AR	Pre transplant	Serum	66
CD20	27	AR with CD20+ interstitial infiltrates was significantly more likely to be steroid resistant allograft loss	First year post transplant	Tissue	78

CD20+ lymphoid clusters (LC)	120	LC most frequent in patients who had not received lymphoid depletion or were treated with thymoglobulin vs patients treated with Campath Banff 1a/1b AR more frequent in LC positive vs LC negative group No difference in LC positive and negative with respect to time to ACR, sCr, steroid resistance and graft loss	Biopsy at mean of 8 10 post transplant months	Tissue	56
ELISPOT	55	Frequency of pre transplant IFN ELISPOTS was significantly greater in patients with AR Pre transplant IFN ELISPOT did not correlate with sCr at 6 or 12 months post transplant	First year post transplant	Serum	64
E selectin HLA class II antigens	94	High levels of intertubular capillary E selectin expression in deceased donor kidneys vs living donor kidneys Increased expression of tubular antigens seen prior to transplantation in biopsy proven AR. No significant association between tubular antigen expression and 3 and 6 month sCr levels, DGF and the number of rejection episodes	Pre transplant	Tissue	70

(*Continued*)

Table 6.2 Biomarkers of acute rejection—cont'd

Biomarker	*N*	Result	Time of collection	Source	Reference
Anti HLA DR, anti CD3, anti CD14, anti CD54, ICAM 1 and anti CD25 IL 2R	30	AR was associated with the presence of HLA DR positive cells and ICAM 1 positive cells ICAM 1 or CD3 positive cells and IL 2R receptor or HLA DR positive cells were highly specific for the diagnosis of AR CAN associated with CD14 positive cells HLA DR positive cells most accurate predictor of AR	On admission for graft dysfunction 10 days 3.5 years post transplant (median of 28 days)	Urine cells	83

ES, E-selectin; PS, P-selectin; sCr, serum creatinine.

Table 6.3 Genetic biomarkers of acute rejection

Allele/SNP	*N*	Result	Reference
T cell signaling genes: CD45, CD40L and CTLA 4	150	No significant association between BPAR and SNPs in CTLA 4, TGF β, IL 10 or TNF α genes or DNPs in IFN γ, and CD40L genes TGF β 25pro significantly associated with increased graft failure	22
Cytokine genes: TNF α, IFN γ, IL 10 and TGF β		CD40L 147 associated with reduced graft failure	
FcγRIIA genotypes	157	Homozygosity for FcγRIIA R/R131 significantly more frequent in AR vs no AR and blood donors AR associated with a distinct distribution of FcγRIIA genotypes Frequency of the R/R131 genotype significantly greater in recipients with graft loss vs both control groups	24
CTLA 4 gene polymorphisms: dinucleotide (AT)n repeat in exon 3; single nucleotide polymorphism A/G at position 49 in exon 1	374	(AT)n repeat polymorphism: increased incidence of AR with alleles 3 and 4 in both liver and kidney A/G single nucleotide polymorphism was not associated with AR	18
Chemokines: CCR2 V64I and CCR5 59029 A	163	Less AR in human renal transplantation with these alleles	28
Toll like receptors: TLR4/CD14 and TLR3	216	Higher rejection free survival rates associated with TLR4 genotype rs10759932 in human allografts. SNPs of TLR3 or CD14 not associated with AR	116

included in the analysis. Homozygosity for FcγRIIA R/R131 was significantly more frequent in recipients with acute rejection than in non rejectors with well functioning grafts and blood donors ($P < 0.05$). Renal allograft recipients with well functioning grafts followed the predicted distribution of FcγRIIA genotypes and allele frequencies when compared with normal, random blood donors ($P = 0.989$). Recipients with acute rejection were found to have a distinct distribution of FcγRIIA genotypes: the distribution of FcγRIIA R/R131, FcγRIIA R/H131 and FcγRIIA H/H131 was 45%, 42% and 13% in patients with acute rejection vs 20%, 52% and 28%, respectively, in the 46 recipients with well functioning grafts ($P < 0.05$), and 21%, 52% and 27%, respectively in the normal blood donors ($P < 0.05$). The frequency of the R/R131 genotype was significantly greater in recipients with graft loss compared to both control groups (45% vs 20% and 21%, respectively, $P < 0.05$). The frequencies of FcγRIIA R131 and FcγRIIA H131 were 0.66 and 0.34, respectively, in patients with acute rejection, and were significantly different from recipients with well functioning grafts ($P < 0.05$). The authors suggested that FcγRIIA polymorphisms could be useful markers for potential risk of rejection.

Slavcheva et al[18] retrospectively examined the association between acute rejection and two polymorphisms in the CTLA4 gene, the dinucleotide (AT)n repeat polymorphism in exon 3 and the single nucleotide polymorphism A/G at position 49 in exon 1. The study included 207 liver and 167 renal transplant recipients. Both populations had a higher than expected rate of acute rejection (53.7% and 34% for liver and renal grafts respectively). The authors acknowledged this and suggested it was due to the use of azathioprine based triple drug therapy. With respect to the (AT)n repeat polymorphism, there was an increased incidence of acute rejection in association with alleles 3 and 4 in both liver and kidney ($P = 0.002$ and 0.05, respectively). Allele 1 was less frequently observed in African American recipients vs Caucasian liver and kidney transplant recipients (frequency of 33.8% and 69%, respectively ($P < 0.0001$)). Patients with allele 1 had a tendency towards a lower rate of acute rejection (42% vs 57.8%, $P = 0.058$), suggesting that allele 1 was potentially protective. The A/G single nucleotide polymorphism was not associated with acute rejection in the patients studied.

3.2. Chemokines and acute rejection

Chemokines are small proteins characterized by four conserved cysteine residues.[25] They are cytokines that activate G protein coupled receptors and

cause cells to migrate along a concentration gradient. Chemokines therefore allow homing of cells to a specific tissue or tissue compartment where chemokine production is maximal. For example, chemokines direct T or B cells to antigen presented by antigen presenting cells in the lymphatic system.[26] Other chemokines have pro inflammatory properties and are produced by cells during infection or pro inflammatory stimuli. These 'inflammatory' chemokines then direct leukocytes to areas of tissue damage or inflammation, and may cause the white blood cells (WBCs) to become activated.[25] Chemokines have been implicated in the development of allograft rejection.[27]

Abdi et al examined the association of human chemokine receptor genetic variants and outcome in 163 recipients of deceased and living donor kidney transplants.[28] The percentage of recipients who had a rejection episode was more than twofold lower in individuals possessing a CCR2−V64I allele compared to recipients who lacked this allele (19% vs 44%; odds ratio (OR), 0.30; 95% CI, 0.12−0.78; $P = 0.014$). In addition, fewer recipients homozygous for the CCR5−59029 A allele experienced at least one episode of rejection vs those possessing a CCR5−59029 G allele (23% for 59029 A/A vs 44% for 59029 A/G or 59029 G/G; OR, 0.37; 95% CI, 0.16−0.85; $P = 0.016$). Recipients with a CCR5−59029 G allele also had a significantly higher number of rejection episodes compared to recipients with the CCR5−59029 A allele. These differences persisted after correction for other known risk factors. The authors acknowledged that association of the CCR5−59029 A allele with a lower risk of acute rejection is 'counterintuitive' since CCR5−59029 A/A homozygotes demonstrate higher CD4+ T cell CCR5 cell surface expression.[29] The association of the CCR2−V64I allele with less rejection was more biologically plausible. Monocyte chemotactic peptide 1 (MCP 1), the ligand of CCR2, is an important monocyte chemoattractant. Both CCR2 and MCP 1 are upregulated in renal transplant rejection.[30] Additionally, CCR2 knockout mice had a doubling of allograft survival in a fully mismatched MHC murine cardiac transplant model.[31] Abdi et al postulated that the polymorphism observed in their study could reduce graft allogenicity, possibly by reducing monocyte migration into the graft.[28]

Two families of chemokine receptors have been identified: the CC receptors 1−10 (CCR1−CCR10) which bind CC chemokines, and the CXC receptors 1−5 (CXCR1−CXCR5) which bind CXC chemokines.[32] Two important ligands of chemokine receptor CXCR3 are IFN inducible protein 10 (IP 10 or CXCL10) and monokine induced by IFN γ (MIG or CXCL9), which are both upregulated in rejecting murine heart allografts.

Mice deficient in CXCR3 as well as mice deficient in IP 10 have prolonged delay in the development of cardiac allograft rejection.[32,33]

Hauser et al[34] examined whether the monokine induced by IFN γ (MIG, otherwise known as CXCL9) and IFN γ inducible protein 10 (IP 10, otherwise known as CXCL10) were early markers of AR. Urine was prospectively collected from 69 de novo renal transplant recipients for a median of 29 days. Acute rejection was diagnosed clinically in 15 of 69 recipients and confirmed by biopsy in 14. Urine MIG was significantly elevated in 14 of 15 AR patients with a median of 2809 pg/mL (quartiles 25% and 75% = 870 and 13,000; $n = 15$) vs both non rejecting allograft recipients (median, 25% and 75%: 96, 1.0 and 161, $n = 54$) and healthy controls (median, 25% and 75%: 144, 19 and 208, $n = 13$) ($P < 0.0001$). Urinary MIG predicted AR with a sensitivity of 93% and a specificity of 89%. In patients with acute rejection, urinary MIG was elevated (greater than the cutoff level) 5 days prior to biopsy on average ($P < 0.0001$), and corresponded well with increased urinary IP 10. The authors also suggested that urinary MIG and IP 10 indicated therapeutic success ($P < 0.0001$ and $P < 0.05$ respectively), while neither granzyme B nor serum creatinine were as useful as indicators of adequate antirejection therapy. MIG was also dissociated from infections or other causes of graft dysfunction.

Lazzeri et al[35] examined the expression and distribution of CXCL10 in tissue specimens obtained from 22 patients with acute rejection or chronic allograft nephropathy (CAN). The authors also retrospectively assayed pre transplant sera from 316 deceased donor kidney recipients for serum CXCL10 levels. Widespread CXCL10 expression was seen in biopsy specimens obtained from patients with CAN, both in infiltrating inflam matory cells, and also in vessels, tubules and glomeruli. Recipients with very low pre transplant serum CXCL10 levels (< 65 pg/mL; $n = 80$) had significantly better 5 year graft survival rate than recipients with very high (> 157 pg/mL; $n = 78$) or intermediate serum CXCL10 levels > 97 and < 157 pg/mL ($n = 80$) ($P = 0.0002$ and $P = 0.03$, respectively). In addition, pre transplant serum CXCL10 levels > the 75th percentile (> 157 pg/mL) were associated with significantly greater acute rejection vs serum CXCL10 levels < the 75th percentile (34.8% vs 21.4%; $P = 0.01$). More severe and steroid resistant acute rejection ($n = 14$) was associated with significantly higher pre transplant median serum CXCL10 levels compared to 60 recipients with less severe rejection episodes (216.1 vs 112.4 pg/mL; $P = 0.04$). Multivariate analysis showed that CXCL10 (RR 2.8) and delayed graft function (RR 3.7) had the highest predictive power of graft loss.

A common histopathological feature of acute renal transplant rejection is mononuclear cell infiltration.[36] Monocyte chemotactic peptide 1 (MCP 1) is a chemotactic and activating chemokine specific for monocytes that is encoded by the early response gene.[37,38] Grandaliano et al[30] examined monocyte infiltration, MCP 1 gene and protein expression, and urine MCP 1 levels in kidney transplant recipients biopsied for acute graft dysfunction. Tissues from 13 patients with AR were compared to seven with acute tubular injury, as well as normal kidney tissue. MCP 1 gene expression was undetectable in normal human kidneys, but increased significantly in tubular injury and AR. MCP 1 in situ hybridization demonstrated MCP 1 interstitial infiltrating mononuclear cells and prox imal tubular cells. MCP 1 expression was greater in tissue demonstrating tubular injury than normal tissue, but significantly less than in AR. There was good correlation between expression of the chemokine and the number of infiltrating monocytes ($r = 0.87$, $P < 0.05$). Urinary MCP 1 was measured by ELISA in eight normal subjects (36 ± 16 pg/mg urine creatinine), 13 clinically stable recipients (33 ± 9 pg/mg, NS vs normals), 12 recipients with BPAR (250 ± 46 pg/mg, $P < 0.01$ vs normals) and five transplant recipients with acute tubular injury (97 ± 33 pg/mg, $P < 0.05$ vs normals and patients with BPAR). Successful treatment of BPAR led to a significant decrease in urinary MCP 1 levels.

Peng et al[39] examined whether the urinary excretion of several chemokines, including fractalkine, chemokine monokine induced by IFN γ, IFN γ inducible protein 10 (IP 10), macrophage inflammatory protein 3 alpha, granzyme B and perforin could predict the occurrence of acute rejection. Urine was collected every 2 weeks during the first 2 months post transplant, on the day of biopsy, at the end of anti rejection therapy (average of 4 days; range = 1−15 days) from 215 allograft recipients and 80 healthy control subjects. Sixty seven patients developed acute rejection. Areas under the ROC curve to distinguish acute rejection from patients without rejection for fractalkine, Mig, IP 10, MIP3α, granzyme B and perforin were 0.834, 0.901, 0.810, 0.734, 0.765 and 0.779, respectively. A cutoff point for fractalkine of 102.88 ng/mmol creatinine yielded a sensitivity and specificity of 82.1% and 76.5% respectively ($P < 0.001$). The best set of markers to distinguish acute rejection from the absence of acute rejection was the combination of fractalkine, IP 10 and granzyme B (sensitivity and specificity of 83.6% and 95.0% respectively). Of all the markers, only changes in urinary fractalkine distinguished recipients with acute rejection from those with acute tubular necrosis. The area under the ROC curve for

fractalkine was 0.734 (95% CI: 0.604–0.865), whereas the area under the ROC curve for the other chemokines and cytotoxic effector molecules was not significant. In addition, among all markers, the area under the ROC curve for fractalkine could best differentiate steroid resistant ($n = 39$) from steroid sensitive acute rejection ($n = 28$). When a cutoff point for fractal kine of 233.76 ng/mmol creatinine was to diagnose steroid resistant rejection, the specificity and sensitivity was 75.0% and 74.4% respectively ($P < 0.001$).

3.3. Toll-like receptors (TLRs) and acute rejection

Toll like receptors (TLRs) are innate immune system receptors encoded by the germ line, that are considered important in host defense.[40] They are expressed on many cell types including antigen presenting cells (APCs), epithelial and endothelial cells. Activation of TLRs contributes to the upregulation of selectins and chemokines on endothelial cells.[41] TLRs on the surface of APCs are important in the priming of naïve T cells.[40]

Hwang et al[42] examined the impact of TLR4/CD14 and TLR3 poly morphisms on acute rejection in 216 donor–recipient pairs undergoing living donor kidney transplantation. TLR4 genotype rs10759932 was associated with higher rejection free survival rates (log rank test, $P = 0.0053$) and no episode of acute rejection occurred when the genotype was present in either the donor or the recipient. Single nucleotide poly morphisms of TLR3 or CD14 were not associated with acute rejection.

3.4. Gene transcripts and acute rejection

The granules of cytotoxic T lymphocytes contain perforin, a pore forming protein which, upon release, forms pores in target cell membranes,[43] and granzyme B is a serine peptidase[44] that causes apoptotic cell death via activation of caspase 3.[45]

Dugre et al[46] investigated whether cytokine gene transcripts and the mRNA expression of cytotoxic molecules from mitogen induced periph eral blood mononuclear cells (PBMCs) of renal transplant recipients could predict acute rejection prior to biopsy. PBMCs were collected twice weekly during the peri transplant period and weekly thereafter for 3 consecutive months. Interleukins 4, 5, and 6, IFN γ, perforin, and granzyme B mRNA levels were significantly associated with acute rejection. Upregu lation of ≥ 2 of these cytokines in a given patient identified 75% of rejecting recipients compared with 15% of non rejecting patients. A limitation of the

study was the exclusion of 40/61 enrolled patients for infections, acute tubular necrosis, CsA nephrotoxicity and 'uncertain rejection episodes'. In addition some AR episodes were diagnosed clinically, without biopsy confirmation.

Li et al[47] also examined gene transcript analysis of perforin and granzyme B as a non invasive diagnostic test for acute rejection, but focused instead on urinary cells. The granules of cytotoxic T lymphocytes contain perforin, a pore forming protein which, upon release, forms pores in target cell membranes,[43] and granzyme B is a serine peptidase[44] that causes apoptotic cell death via activation of caspase 3.[45] Urine specimens ($n = 24$) were collected from 22 renal allograft recipients with biopsy proven acute rejection, as well as 127 samples from 63 recipients without evidence of acute rejection. Log transformed mean (± SE) levels of perforin and granzyme B mRNA, but not levels of constitutively expressed cyclophin B mRNA, were greater in the urinary cells from the patients with acute rejection vs recipients without acute rejection (perforin, 1.4 ± 0.3 vs 0.6 ± 0.2 fg/μg of total RNA; $P < 0.001$; and granzyme B, 1.2 ± 0.3 vs 0.9 ± 0.2 fg/μg of total RNA; $P < 0.001$). ROC analysis showed that acute rejection could be predicted with a sensitivity and specificity of 83% (for both parameters; using a cutoff value of 0.9 fg of perforin mRNA/μg of total RNA), and with a sensitivity and specificity of 79% and 77% respec tively (using a cutoff value of 0.4 fg of granzyme B mRNA/μg of total RNA). The authors subsequently analyzed sequential urine samples from 37 allograft recipients during the first 9 days post transplantation. The levels of perforin and granzyme B mRNA, but not that of cyclophilin B, were significantly higher in patients who developed acute rejection ($n = 8$) within the first 10 days post transplant vs 29 recipients in whom acute rejection did not develop (granzyme B, $P = 0.02$ on days 4–6 and $P = 0.009$ on days 7–9; perforin, $P = 0.003$ on days 4–6, and $P = 0.01$ on days 7–9). The authors suggested that mRNA encoding cytotoxic proteins in urinary cells could represent a non invasive method of diagnosing acute renal allograft rejection.

Graziotto et al[48] analyzed the expression of perforin (P), granzyme B (GB) and fas ligand (FL) in 68 renal biopsies and 64 samples of peripheral blood lymphocytes (PBL) in three groups of patients: (1) pre reperfusion biopsies and PBL from recipient prior to transplantation; (2) biopsies and PBLs collected 5–10 days post transplant for graft dysfunction; and (3) protocol biopsies and PBLs obtained at 2 months post transplant in patients with stable renal function. Perforin and granzyme B expression increased

significantly over the first 2 months post transplant in non rejecting grafts (perforin, $P < 0.05$; granzyme B, $P < 0.01$). Perforin overexpression in pre reperfusion biopsies was associated with biopsy proven acute and subclinical rejection in the subsequent 2 months ($chi^2 = 3.93$; $P < 0.05$). No significant increase in CTL transcription was found in PBL samples taken during episodes of AR. The authors noted considerable variability in each sample and suggested that the use of biomarkers may be hindered by time related variability in their expression, and the need for a sizable quantity of renal tissue to ensure an adequate sensitivity.

In a similar study, Simon et al[49] examined whether peripheral blood gene expression of perforin and granzyme B transcripts could predict renal allograft rejection. Peripheral blood was collected twice weekly during the first month post transplantation. Gene expression was measured using real time polymerase chain reaction (PCR) in 364 samples from 67 patients. Clinical rejection was either biopsy proven or based on clinical response to anti rejection therapy in one patient.

Recipients with acute rejection ($n = 17$) had increased levels of gran zyme B and perforin transcripts on days 5−7, 8−10, 11−13, 17−19, 20−22 and 26−29, vs patients without rejection ($n = 50$, $P < 0.05$ in all cases). The diagnosis of acute rejection, using gene expression criteria, determined by ROC curve analysis, could be made 2−30 days before the diagnosis by standard criteria (median 11 days). The best diagnostic result was achieved with samples taken on post operative days 8−10. These samples yielded a sensitivity and specificity of 82% and 90% respectively for perforin, and a sensitivity and specificity of 72% and 87% respectively for granzyme B. Both perforin ($P < 0.01$) and granzyme B gene expression ($P < 0.05$) decreased after initiation of anti rejection therapy.

A unique population of suppressor T cells, also known as regulatory T cells or Tregs, has been implicated in AR.[50] Tregs are CD4+CD25+ and are distinguished from other cells by constitutive expression of the forkhead winged helix transcription factor FOXP3.[51] Veronese et al[50] used immu nohistochemistry to examine 80 human donor and recipient kidney biopsies for Treg transcription factor FOXP3, as well as CD4 or CD8. FOXP3(+) cells were found in the interstitium of biopsies with type I and II acute cellular rejection. Ninety six percent of the FOXP3(+) cells were CD4(+) while a minority expressed CD8. The FOXP3(+)CD4(+) cells were localized primarily to the tubules.

Muthukumar et al[52] examined urinary cells for FOXP3, CD25, CD3epsilon, perforin and 18S ribosomal messenger RNA in 36 subjects

with acute rejection (AR), 18 subjects with chronic allograft nephropathy (CAN) and 29 subjects with normal biopsy results (NL). Seventy five urine specimens were collected prior to biopsy (presumably on the day of biopsy − the timing of collection was not further defined) and 8 samples were collected after the biopsy. Subjects with acute rejection demonstrated greater urinary log transformed mean FOXP3/18S ribosomal mRNA copies than subjects with CAN or normal histology (AR 3.8 ± 0.5; CAN 1.3 ± 0.7; NL 1.6 ± 0.4; $P < 0.001$). ROC analysis demonstrated that reversal of acute rejection could be predicted with 90% sensitivity and 73% specificity using a cutoff for FOXP3 mRNA of 3.46 ($P = 0.001$). In addition, the 18S normalized, log transformed mRNA levels of CD25 (6.9 ± 0.4, 4.0 ± 0.5 and 2.8 ± 0.6, respectively; $P < 0.001$), CD3 epsilon (8.2 ± 0.4, 4.3 ± 0.5 and 1.6 ± 0.5; $P < 0.001$), and perforin (7.6 ± 0.4, 4.5 ± 0.4 and 2.8 ± 0.4; $P < 0.001$) were also greater in subjects with AR compared to subjects with CAN or NL histology. However CD25, CD3epsilon and perforin did not predict reversal of AR or graft failure.

CD30 is a transmembrane glycoprotein belonging to the tumor necrosis factor (TNF)/nerve growth factor receptor family.[53] It is 120 kDa in size and is primarily expressed on CD4+ and CD8+ T cells of the Th2 phenotype with little or no expression Th1 type T cells.[54] Activated CD30+ T cells release a soluble form of CD30 (sCD30) into the bloodstream.[55]

Cinti et al[54] retrospectively examined the ability of panel of reactivity antibodies (PRA) and soluble CD30 (sCD30) in stored pre transplant sera to predict the occurrence of AR in the first 6 months following living donor or deceased donor kidney transplantation. PRA was measured using flow PRA beads and was considered positive when the percentage fluo rescence was > 5%. Biopsy proven acute rejection (BPAR) occurred in 58.3% of patients (14/24). sCD30 was found in the pre transplant sera of 37.5% of patients (9/24) and all of these patients subsequently developed AR. PRA was found in the pre transplant sera of 25% patients (6/24), four of whom developed later AR. Both sCD30 and PRA were very specific for AR (sCD30 100%; PRA 80%), while sCD30 demonstrated better speci ficity accuracy (79.1% vs 50%) and positive predictive value 100% vs 66.6%. However neither sCD30 nor PRA demonstrated good sensitivity (sCD30 = 64.2%; PRA = 28.5%) nor negative predictive value (sCD30 = 66.6%; PRA = 44.4%).

In a separate study, Susal et al[53] performed a multicenter study involving 29 transplant centers in 15 countries to determine whether pre transplant

serum sCD30 could predict kidney allograft outcome. Pre transplant sera from 3899 cadaver kidney recipients was examined for serum sCD30 concentration by ELISA and correlated with subsequent allograft survival. Five year graft survival was significantly lower (64 ± 2%) in 901 recipients with high pre transplant serum sCD30 (≥ 100 U/mL) than the allograft survival of 75 ± 1% rate in 2998 recipients with low sCD30 (< 100 U/mL) ($P < 0.0001$). After the first post transplant year, recipients with a high pre transplant serum sCD30 had a death censored half life of 20.5 years vs 29.4 years for patients with a low sCD30. High pre transplant sCD30 was associated with the need for significantly more rejection treatment (10%) in the second post transplant year vs patients with a low sCD30 (5%, $P = 0.0003$), although this difference did not exist in the first year post trans plant. High pre transplant serum sCD30 appeared to also predict a worse rate of graft loss during the 5 year follow up period.

Pelzl et al[56] examined whether soluble CD30 (sCD30) was a useful biomarker of acute rejection in 56 kidney allograft recipients during the early post transplant period. The recipients were divided into three groups: (1) recipients with primary graft function, an uncomplicated course and no acute rejection ($n = 20$); (2) recipients with primary non function due to acute tubular necrosis (ATN) without evidence of acute rejection ($n = 11$); and (3) recipients who experienced an episode of biopsy proven acute rejection within the first 20 post transplant days ($n = 25$). Plasma sCD30 levels were measured on postoperative days 3−5, 7−9, 12−14 and 17−19. ROC analysis revealed that on postoperative days 3−5, plasma sCD30 allowed recipients who subsequently developed acute allograft rejection ($n = 25$) to be distinguished from recipients with an uncomplicated course ($P < 0.0001$, AUC 0.96, specificity 100%, sensitivity 88%) or those with ATN in the absence of rejection ($P = 0.001$, AUC 0.85, specificity 91%, sensitivity 72%).

Kim et al[57] retrospectively correlated pre transplant sCD30 levels (high vs low) with post transplant graft survival, incidence of acute rejection and graft function in 120 allograft recipients. During 47.5 ± 11.4 months of follow up, pre transplant sCD30 was not significantly associated with differences in graft survival rate ($P = 0.5901$). High sCD30 (≥ 115 U/mL) was associated with a higher incidence of acute rejection (33.9% vs 22.4% in the low sCD30) but this difference did not reach statistical significance ($P = 0.164$), suggesting the study may have been under powered. A similar trend was seen in response rate to anti rejection therapy. Patients with high sCD30 had an inferior response compared to patients with a low sCD30

(33.3% vs 7.7% respectively) but this also failed to reach statistical significance ($P = 0.087$). In contrast, patients with a high sCD30 had significantly elevated serum creatinine 3 years post transplant vs the low sCD30 group ($P < 0.05$). By multiple regression analysis, pre transplant sCD30 levels, acute rejection episodes, donor age, and kidney weight/recipient body weight ratio, were all independent variables affecting the serum creatinine level 3 years post engraftment.

Kotsch et al[58] examined whether kinetic real time RT PCR based gene expression profiling of urinary cells from outpatients could predict the occurrence of acute rejection. Urine was collected during the first 3 months post transplant from 35 kidney transplant recipients, including nine patients who subsequently developed biopsy proven acute rejection. Increased granulysin transcription was found in 11 of 14 cases of acute rejection, but was never observed above the confidence interval in any of the 159 urine specimens collected from the non rejecting group (100% specificity and 80% sensitivity). Granzyme B, perforin, FasL, TNF α, RANTES, IL 2, IL 10, IFN γ, TGF β, CD3 and CCR1 all showed less specificity and sensitivity. The authors also suggested that increased urinary granulysin gene expression was predictive of acute rejection occurring more than 4 weeks post transplant. This was confirmed in only two patients however. A modification of an RNA extraction protocol was also reported that permitted a reporting of results within 4–5 hours.

CD103 (formerly known as alpha E integrin) is found on the surface of a major subset of CD8+ cytotoxic T lymphocytes (CTL), and functions as a receptor for E cadherin on epithelial cells.[59] It allows CD103+ CTLs to bind epithelial cells through E cadherin.[60] CD103+ T cells have been found exclusively restricted to the tubules during human renal allograft rejection.[61] CD103+ cells are absent in normal renal tissue.[62]

Ding et al[62] tested the hypothesis that CD103 mRNA would be present in high abundance in urinary cells obtained during an episode of AR. Eighty nine urine specimens were collected from 79 recipients of renal allografts. Real time quantitative polymerase chain reaction assay was used to measure CD103 mRNA levels as well as a constitutively expressed 18S ribosomal (r)RNA. CD103 mRNA levels were greater in urinary cells from 30 patients with AR as compared to levels in 12 patients with other findings on allograft biopsy, 12 patients with biopsy proven CAN and 25 patients with stable graft function ($P = 0.001$; one way analysis of variance). In contrast, levels of constitutively expressed 18S rRNA did not vary significantly among the four diagnostic categories ($P = 0.44$). However, CD103

mRNA levels predicted AR with a sensitivity of only 59% and a specificity of 75% when a natural log transformed value of 8.16 CD103 copies/μg was used as the cutoff value ($P = 0.001$). The calculated area under the curve was 0.73 (95% CI, 0.62−0.82) for CD103 mRNA levels and 0.59 for 18S rRNA levels.

Not all biomarkers are able to distinguish between different disease states. Coupes et al[63] used ELISA to examine whether circulating active TGF β_1 was detectable in renal allograft recipients, and whether plasma levels correlated with episodes of AR. Several groups of patients were included in the study: 43 healthy controls, 11 patients with membranous nephropathy (MN) and impaired renal function, 17 transplant recipients with stable renal function, 27 patients with biopsy proven acute cellular rejection, 7 patients with biopsy proven chronic vascular rejection and 10 patients with biopsy proven acute tubular necrosis and/or cyclosporine toxicity. Plasma samples were collected at the time of biopsy in the latter three groups. Urine TGF β_1 was also measured in all groups. Plasma TGF β_1 was not detected in any of the healthy controls or MN patients (detection limit of assay 0.1 ng/mL). In contrast, TGF β_1 was significantly increased in all transplant recipients but could distinguish the different diagnoses. TGF β_1 was found in most of the urine samples including those from healthy controls. The transplant urines had values comparable with normal controls.

3.5. ELISPOT as a biomarker of acute rejection

Gebauer et al[64] developed an enzyme linked immunoabsorbent spot (ELISPOT) assay for the detection of cytokine secretion by individual, antigen reactive T cells. The latter were found to be antigen specific IFN γ producing T cells expressing a cell surface phenotype of memory T cells (CD45RO+, CD45RA−).[64]

Hricik et al[65] used the ELISPOT approach to serially measure the frequency of peripheral blood lymphocytes (PBLs) producing IFN γ in response to stimulator cells from donors or third parties in 55 primary kidney transplant recipients. Of this cohort 37 had donor stimulated IFN ELISPOTS measured before transplantation, including five recipients who subsequently developed acute rejection. The mean frequency of pre transplant IFN ELISPOTS was significantly higher in patients who expe rienced acute rejection (79 ± 69 vs. 30 ± 44 spots per 300,000 cells; $P = 0.039$ vs recipients without clinically evident rejection). Pre transplant IFN

ELISPOT did not correlate with serum creatinine at 6 ($R = 0.014$, $P =$ NS) or 12 months post transplant ($R = 0.114$, $P =$ NS).

3.6. Platelet activation and acute rejection

Zhang et al[66] examined the association of pre transplant platelet activation with post transplant AR and ATN. ELISA was used to determine the expression of the following glycoproteins in the peripheral blood of 203 first kidney transplant recipients of non heart beating donor kidneys: CD62p (a platelet activation dependent granule membrane protein; CD63 (a lyso somal enzyme glycoprotein); CD42a (a macula densa granule membrane glycoprotein); and PAC 1 (a fibrinogen receptor monoclonal antibody). Pre transplant expression of CD63 was 15.45 ± 6.55 in recipients who subsequently developed acute rejection vs 1.74 ± 0.71 and 1.72 ± 1.36 in patients who had subsequent immediate graft function or ATN ($P < 0.01$).

3.7. Serum markers of inflammation and acute rejection

Harris et al[67] studied whether serial daily measurements of serum C reactive protein (sCRP) could help differentiate renal dysfunction due to rejection from cyclosporine (CsA) nephrotoxicity. The total study population of 441 included 187 transplant recipients within 90 days of engraftment, 104 normal controls (healthy blood donors) and 150 patients on renal replace ment therapy awaiting transplants (95 on hemodialysis (HD), 55 patients on continuous ambulatory peritoneal dialysis (CAPD)). Median sCRP concentration in normal controls was 0.5 βμg/mL (range, < 0.03−10 μg/mL), while HD patients had a median concentration of 3.1 μg/mL (range, < 0.03 to ≥ 15 μg/mL) and CAPD patients had a median value of 2.9 μg/mL (range, < 0.03 to ≥ 15 μg/mL). CRP was noted to increase in some patients with inflammatory diseases such as Crohn's disease or CAPD peritonitis. Following transplant sCRP peaked in recipients with excellent primary graft function on post operative day 2 (median, 29 μg/mL; range, 4 to > 200 μg/mL) followed by decline to < 20 μg/mL in all patients by day 5 (median, 7 μg/mL; range, 2−19 μg/mL). Median sCRP of recipients with stable graft function (defined as a mean creatinine of 155 μg/mL or 1.7 mg/dL) was 4 μg/mL (range, 1−19 μg/mL). In 30 episodes of steroid sensitive acute rejection, sCRP was initially significantly increased to a median of 49 μg/mL ($P < 0.001$ vs uncomplicated controls) but fell rapidly with treatment to a median of 11 μg/mL, with further subsequent decreases. In 19 episodes of steroid resistant acute rejection, median initial sCRP levels

were significantly higher (119 μg/mL, $P < 0.001$ vs uncomplicated controls) but remained elevated (median = 77 μg/mL) at the end of the treatment. Twenty four patients with graft dysfunction attributed to CsA nephrotoxicity showed no increase in sCRP concentrations (median sCRP < 5 μg/mL throughout the episodes). Serum CRP levels were not signif icantly different from uncomplicated controls in six biopsy proven cases of ATN (median sCRP concentrations for the start, middle and end of the episode were 7 μg/mL (range, 1−9 μg/mL), 5 μg/mL (range, 1−6 μg/mL) and 2 μg/mL (range, 1−3 μg/mL), respectively). Other casues of increased sCRP were wound infections, pyelonephritis and sepsis. It was not clear from the study whether the increases in sCRP preceded the rise in sCr.

Perez et al[68] examined whether pre transplant serum levels of CRP would predict the development of acute rejection episodes after kidney transplant. Pre transplant serum CRP was measured in 97 consecutive renal transplant recipients. The mean length of follow up was 564 days (SD = 274 days) with a range of 6−1059 days. Acute rejection occurred in 39 (40%) recipients, with the majority occurring within the first 100 days post transplant (median = 85 days). Serum CRP were found to range from 0 to 60 μg/mL, with a median of 9.0 and a mean 14.5 ± 1.6 μg/mL. The lower and upper quartiles for CRP were < 2 μg/mL and > 21 μg/mL respec tively. Pre transplant CRP levels were greater in patients who subsequently developed acute rejection vs those who did not (22.2 ± 2.9 vs 11.7 ± 1.8 μg/mL, respectively, $P = 0.003$). Recipients whose pre transplant CRP was less than the median had a significantly longer time to rejection vs recipients with higher CRP levels ($P = 0.002$). Recipients within the lowest CRP quartile had longer times to rejection vs those in the highest quartile ($P = 0.006$). Similarly, the 3 month incidence of rejection was 13% (3/23) in the lowest CRP quartile group vs 44% (11/25) in the upper quartile group ($P = 0.027$, Fisher exact test). The difference remained significant at 6 months. Of all covariates analzsed by Cox proportional hazards regression multi variate analysis, only pre transplant CRP level was an independent risk factor for rejection ($P = 0.044$).

Myeloid related protein 8 (MRP8) and MRP14 (MRP14) are S100 family calcium binding proteins abundant in neutrophils and monocytes.[69] Upon interaction with activated endothelium these proteins form a heterodimer known as calprotectin (MRP8/14) that becomes associated with endothelium at sites of monocyte and neutrophil adhesion. Cal protectin subsequently increases the endothelial transcription of pro inflammatory chemokines and adhesion molecules.[70]

Burkhardt et al[71] used ELISA to measure MRP8/14 serum levels for 28 days in a pilot group of 20 renal allograft recipients and subsequently in a validation cohort of 36 renal allograft recipients. Serum MRP8/14, C reactive protein and creatinine levels were correlated with biopsy proven acute rejection. There were seven episodes of acute rejection (five of which were biopsy proven) in the pilot group that occurred a median of 7 days post transplant (IQR = 21 days). Of the 36 patients in the validation study, 18 experienced at least one acute rejection episode during the first 4 weeks after transplantation. Serum levels of MRP8/14 but not CRP were significantly increased for several days during the first 2 weeks in patients with the acute rejection groups in both studies ($P < 0.005$, on day 6 post transplant). Using ROC curves, an optimal cutoff of 4.2 μg/mL on post transplant day 6 for MRP8/14 yielded a specificity of 100% and a sensitivity of 67% for acute rejection in the pilot study. In the validation study, serum MRP8/14 levels were significantly increased on days 2−10 and on days 12−14 in recipients who later developed acute rejection. The best discrimination between the acute rejection and non rejection groups was found to be on postoperative day 6, as in the pilot study ($P < 0.001$). Plasma CRP did not differ significantly between patients with and without acute rejection ($P = 0.311$ on postoperative day 6). Serum creatinine was also not able to differentiate significantly between the rejection and non rejection groups ($P = 0.214$ on day 6 after transplantation). On post operative day 6 a cutoff of 4.2 μg/mL, a value derived from the pilot study, discriminated between the rejection and no rejection with a specificity of 100% and a sensitivity of 73%. Increased MRP8/14 serum levels preceded acute rejection episodes by a median of 5 days. Serum MRP8/14 was below the cutoff in patients with delayed graft function, urinary tract infections or cytomegalovirus infections, and these values did not differ significantly from control values.

3.8. Tissue biomarkers of acute rejection

Koo et al[72] compared the expression of adhesion molecules and HLA class II antigens in pre transplant biopsies from deceased and living donor kidneys ($n = 65$ and 29 respectively). High levels of intertubular capillary E selectin expression (grade 2) were detected in 35 out of 65 (54%) deceased donor kidneys compared with no expression in any of the living donor kidneys ($P < 0.00001$). Expression of HLA DR antigens, ICAM 1 and VCAM 1 was found in proximal tubules of deceased donor kidneys, whereas

living donor kidneys had markedly reduced expression. Increased expression of tubular antigens was seen before transplantation in all 11 cadaver renal allografts with biopsy proven acute rejection within 7 days of engraftment. Tubular antigens were absent in 15 out of 54 (28%) donor kidneys with no rejection in the first 7 days ($P < 0.05$). There was no significant association between tubular antigen expression and 3 and 6 month serum creatinine levels, delayed graft function and the number of rejection episodes.

Benson et al[73] performed a prospective immunohistochemical analysis of the correlation between the inflammatory markers in the pre transplant biopsies and subsequent acute rejection in the recipient. Pre transplant biopsies were taken in 77 adult renal transplant recipients, of whom 29 (38%) rejected. The biopsies were examined for P selectin (PS), E selectin (ES), platelets, leukocyte common antigen, macrophages, T cells and neutrophils. Significantly more recipients rejected if the donor biopsy was positive for PS (63 vs 24%, $P = 0.0007$), contained ≥ 5 or more leukocytes/glomerulus (48 vs 21%, $P = 0.03$), contained > 9.3 leukocytes/hpf (46.5 vs 10.5%, $P = 0.006$) or were both PS positive and contained > 9.3 leukocytes/hpf (61.9 vs 0.0%, $P = 0.0001$). The PS was found to be primarily of platelet origin and most of the leukocytes were macrophages. The authors suggested that immunohistochemical changes present prior to transplantation could identify those recipients with a greater risk of acute rejection, and allow for tailored immunosuppression.

TNF α is a cytokine synthesized by a number of cell types including monocytes macrophages[74] and T lymphocytes.[75] TNF α expression is increased during human acute allograft rejection[76] and it is thought to be involved in the induction of adhesion molecules on graft endothelium and the recruitment of cells into the allograft.[77] TNF α binds receptors known as TNFRI and TNFRII[78] while naturally occurring soluble TNFRI and TNFRII (sTNFRI and sTNFRII) released after proteolytic cleavage of cell surface TNF receptors may regulate its bioactivity.[79]

Oliveira et al[74] studied the expression of IL 1β, soluble IL 1 receptor II (IL 1RII), TNF α, soluble TNFRI (sTNFRI) and soluble TNFRII (sTNFRII), and leukemia inhibitory factor (LIF) analysis in fine needle aspiration biopsy (FNAB) culture supernatants following kidney transplantation. FNABs were performed on 66 kidney transplant recipients on days 7 and 14 after transplant, and again whenever there was acute rejection. The cohort was divided into four groups: Group 1 comprised stable patients studied on post operative day 7 ($n = 30$); Group 2 included patients studied on day 7 after transplantation, and 8 ± 4.5 days before acute rejection

diagnosis ($n = 12$); Group 3 comprised patients studied on the first day of acute rejection diagnosis ($n = 17$); and Group 4 included stable patients studied on postoperative day 14 ($n = 32$). Recipients of groups 1 and 4 did not experience any acute rejection during the first 6 months, and every patient in group 2 was studied again on the first day of acute rejection. Serum levels for sIL 1RII, sTNFRI and sTNFRII were also measured. Soluble TNFRI was found to be significantly higher in group 2 vs 1 ($P = 0.002$). When the acute rejection groups (Groups 2 and 3) were combined, sTNFRI was found to be significantly higher than in the groups repre senting stable patients (groups 1 and 4) ($P < 0.0001$). A similar pattern was seen with sTNFRII, which was significantly greater in group 2 vs group 1 ($P = 0.02$), and significantly lower in all stable patients ($P = 0.0001$). For sTNFRI, a cutoff value for acute rejection of > 480 pg/mL resulted in a sensitivity of 89.6%, specificity of 78.3%, positive predictive value of 76.4% and negative predictive value of 90.6%. For sTNFRII, a cutoff value of > 700 pg/mL resulted in a sensitivity of 91.6%, specificity of 80.6%, positive predictive value of 64.7% and negative predictive value of 96.1%. IL 1β and sIL 1RII did not differ significantly among the groups.

3.9. B-cell activation and acute rejection (tissue biomarkers as predictors of response to therapy)

Hippen et al[80] examined the relationship between the presence of CD20 positive B lymphocytes in kidney transplants undergoing acute cellular rejection and graft survival. Biopsies from 27 recipients with biopsy proven Banff 1 A or 1 B rejection in the first year post transplant were stained for CD20 and C4d. The staining patterns were correlated with follow up data of 4 years for each patient studied. Six patients were found to have interstitial CD20+ B cell clusters while 21 patients were negative for CD20 infiltrates. Patients in the former group had a significantly greater peak serum creatinine at the time of acute rejection, suggesting worse impairment of renal function in the CD20+ group (median 3.1 mg/dL vs 2.2 mg/dL in the CD20 group, $P = 0.047$). Recipients with CD20+ interstitial infiltrates were significantly more likely to have steroid resistant acute rejection ($P = 0.015$ vs CD20 recipients) and to experience immunological (death censored) allograft loss ($P = 0.024$). However, when death with graft function was included as a cause of graft loss, the trend toward poorer outcomes for the CD20+ group remained but failed to reach statistical significance ($P = 0.153$), suggesting the study may have been underpowered. The authors

suggested that identification of B cell infiltrates could distinguish a unique subset of patients for whom anti B cell therapy may be beneficial.

In a similar study, Kayler et al[81] determined the influence of lymphocyte depleting therapy on B cell clusters in 120 allograft biopsies obtained during the first episode of acute cellular rejection in 120 recipients. Lymphoid clusters were found in 59% of the biopsies (71/120). CD20+ B cells were found in all 71 biopsies and accounted for 5−90% of the cluster leukocyte content. Lymphoid clusters were most frequent in patients who had not received lymphoid depletion or had been treated with thymoglobulin (79% and 75%, respectively) compared to 37% in patients treated with Campath ($P = 0.0001$). Banff 1a/1b acute cellular rejection was more frequent in the lymphoid clusters positive vs negative group (96% vs 80%, respectively; $P = 0.0051$). However, over a follow up of 953 ± 430 days, lymphoid clusters positive and negative did not differ significantly with respect to time to acute cellular rejection, steroid resistance, serum creatinine and graft loss. In contrast to the study by Hippen, CD20+ lymphoid clusters did not predict glucocorticoid resistance or worse outcomes. The authors suggested that lymphoid clusters contain variable and heterogenous collections of B cells, and suggested a small subset of high risk patients could potentially exist.

3.10. Cytokines as biomarkers of acute rejection

Hu et al[82] screened the urine of healthy controls and kidney transplant recipients using antibody array and a multiplex beads assay. The kidney transplant recipients included 84 patients with renal allograft injury, 29 patients with stable graft function and 19 healthy individuals. A number of cytokines were elevated in both acute and chronic injuries including interferon γ induced protein of 10 kDa, monokine induced by interferon γ, macrophage inflammatory protein and osteoprotogerin. Unfortunately none of the four biomarkers were able to differentiate specific causes of graft dysfunction. The authors pointed out that since both alloimmune and non alloimmune causes of graft dysfunction increase cytokine levels, their discriminatory power in general may be limited.

Kutukculer et al[83] examined whether plasma levels of lymphokines IL 2, IL 3, IL 4, IL 6, IL 8 and soluble CD23 could predict acute rejection in 16 renal transplant recipients during the first 14 days after engraftment. Of the 16 patients, seven had clinical evidence of acute allograft rejection and five showed stable graft function. The remaining four patients had primary

non function. Plasma levels of IL 2, whenever detected, were predictive of impending graft rejection. Plasma levels of IL 4 and IL 6 were more reliable for diagnosis of rejection, while IL 3, IL 8 and soluble CD23 were not diagnostic or predictive of rejection. The authors pointed out that post transplant infections could affect the diagnostic performance of these biomarkers.

Crispim et al[84] evaluated tissue levels of the pro inflammatory cytokine IL 17 by enzyme linked immunosorbent assay method in 19 recipients of living and deceased donor transplants, and healthy controls. Tissue IL 17 was significantly increased in grafts undergoing rejection (125.7 ± 27.06 pg/mL) vs grafts without rejection group (30 ± 13.32 pg/mL) ($P < 0.05$). Biopsies from healthy controls had no IL 17.

3.11. Urine flow cytometry and the diagnosis of acute rejection

Roberti et al[85] examined the ability of urine flow cytometry (UFC) to diagnose acute rejection. UFC was performed in 30 patients (32 events) admitted for evaluation of graft dysfunction (defined as serum creatinine increment ≥ 0.6 mg/dL above baseline). The UFC analysis was compared with the subsequent discharge diagnosis. Acute rejection was confirmed by biopsy in all cases. The discharge diagnoses were as follows: acute rejection ($n = 15$); CAN ($n = 8$); drug toxicity ($n = 4$); urine leak ($n = 2$); recurrence of primary disease ($n = 1$); lymphocele ($n = 1$); and unknown ($n = 1$). Urine analysis was performed on a FACScan and 10,000 cells were counted in each sample. The cells were assessed for anti HLA DR, anti CD3, anti CD14, anti CD54 (ICAM 1) and anti CD25 (IL 2 receptor (IL 2R)). Acute rejection was associated with the presence of $\geq$ 5% HLA DR positive cells and ICAM 1 positive cells in 100% and 53% of samples respectively ($P < 0.01$ vs others). A number of markers were highly specific for the diagnosis of acute rejection including ICAM 1 or CD3+ cells (100% specificity) and IL 2R receptor or HLA DR+ cells (specificity = 88%). CAN was associated with CD14+ cells ($P = 0.03$ vs others; specificity = 87.3%). The most accurate finding associated with the diagnosis of acute rejection was the finding of HLA DR+ cells with only a 12% rate of false positive results (sensitivity = 100%, specificity = 88%, positive predictive value (PPV) = 88%, negative predictive value (NPV) = 100%). Samples from patients with drug toxicity, urological problems or recurrence of primary disease lacked expression of the antigens studied. The authors

suggested that UFC of urinary cells could differentiate acute rejection from other causes of acute allograft dysfunction. HLA DR was found to be the most sensitive, and ICAM 1 the most specific marker for acute rejection.

In a follow up study the same authors evaluated whether serial UFC correlated with clinical outcome.[86] A variety of cell surface antigens (anti CD3, anti CD14, anti HLA DR, anti CD54 and anti IL 2R) were examined by UFC during a 30 day period after the diagnosis and treatment of 24 AR episodes. The study included 59 urine specimens, from 17 patients meeting the diagnostic criteria for AR. The most common antigen seen during the first 2 days of AR was HLA DR (91.7% of the samples), followed by CD14 (50%) and CD54 (41.7%). Expression of HLA DR+, CD14+ and CD54+ cells after day 4 correlated with the need for anti lymphocytic drugs. The most accurate marker was CD54, with a sensitivity = 100% and specificity = 90.9% ($P = 0.001$). CD54+ and CD14+ urinary cells persisted in those patients who had permanent graft injury after treatment of AR.

3.12. Proteomic-based approaches to finding biomarkers of acute rejection

Schaub et al[87] examined whether proteins detected in urine using mass spectrometry could serve as biomarkers of acute rejection. Four patient groups were selected based on allograft function, clinical course and allograft biopsy result: (1) acute clinical rejection group ($n = 18$); (2) stable transplant group ($n = 22$); (3) acute tubular necrosis group ($n = 5$); and (4) recurrent (or de novo) glomerulopathy group ($n = 5$). Urine was collected on the day of the allograft biopsy, and the median time to biopsy ranged from 0 to 253 weeks post transplant. A control group of 28 urines from healthy individ uals, as well as five urines from non transplanted patients with lower urinary tract infection were also analyzed. The authors also performed sequential urine analysis in patients in the acute clinical rejection and stable transplant function (groups 1 and 2). Ninety four percent (17/18 patients) with acute rejection episodes were found to have three prominent peak clusters, whereas only 18% (4/22) of patients without clinical and histological evidence for rejection, and 0 of 28 normal controls ($P < 0.001$) had a similar finding. The presence or absence of these peak clusters correlated with the clinicopathological course in most patients. Urinary protein profiles in recipients with ATN and glomerulopathy groups were distinct from those with the pattern of rejection. In a follow up study[88] the protein peaks were

found to derive from non tryptic cleaved forms of β_2 microglobulin. Cleavage of intact β_2 microglobulin was found to require a urine pH < 6 and the presence of aspartic proteases. Accordingly, patients with acute tubulointerstitial rejection had a lower urine pH, and greater urine aspartic proteases and intact β_2 microglobulin. The authors proposed that these factors resulted in increased amounts of cleaved urine β_2 microglobulin.

4. BIOMARKERS OF CHRONIC ALLOGRAFT NEPHROPATHY (TABLE 6.4)

4.1. Tissue markers

Kirk et al[89] hypothesized that clinically stable human kidney transplants were subject to detectable ongoing immune activity which could be correlated with worsening of allograft function. Forty stable renal allografts were biopsied 2–3 years post transplantation. Biopsies were evaluated by RT PCR for CD3γ mRNA (a marker of T cell receptor turnover) as well as genes associated with acute rejection (TNF α, IFN γ, IL 1β, IL 2, IL 4, IL 6 and IL 8). Gene expression was then correlated with clinical findings at the time of biopsy and 2 years post biopsy. Cytokine gene transcription and histological evidence of injury were found in more than two thirds of clinically stable grafts. Increasing lymphocytic infiltration correlated with the proteinuria ($P = 0.034$) and worsening interstitial fibrosis ($P = 0.005$). The fibrosis demonstrated a significant positive correlation with baseline creatinine ($P = 0.006$) and negatively correlated with the GFR measured on the day of the biopsy ($P = 0.037$). Intragraft CD3γ signal also correlated with increasing proteinuria ($P = 0.043$), implicating increased T cell activity with deteriorating graft function. On the other hand, CD3γ did not correlate with fibrosis, serum creatinine or GFR. Both fibrosis ($P = 0.01$) and tubular atrophy ($P = 0.01$) on the original biopsy were correlated with declining renal function at follow up. CD3γ levels at the time of original biopsy correlated with the highest change in GFR over time ($P = 0.045$). The authors suggested that significant injury and immune activity could be found in patients with clinically stable allografts, and that this injury may be a cause of chronic allograft nephropathy.

Nickel et al[90] examined whether intragraft expression of perforin, granzyme B and Fas ligand correlated with long term clinical outcome following an episode of acute rejection. Gene transcript analysis was per formed on 22 human renal biopsies for the expression of perforin, granzyme B, Fas ligand and Fas. Expression levels were correlated with Banff rejection

Table 6.4 Biomarkers of chronic rejection

	N	Result	Time of collection	Source	Reference
CD3γ TNF α, IFN γ, IL 1β, IL 2, IL 4, IL 6, and IL 8		Cytokine gene transcription and histological evidence of injury found in clinically stable grafts Increasing lymphocytic infiltration correlated with the proteinuria and worsening interstitial fibrosis Fibrosis showed significant correlation with renal function Both fibrosis and tubular atrophy on the original biopsy were correlated with declining renal function at follow up Intragraft CD3γ correlated with increasing proteinuria and renal function	Biopsy 2 3 years post transplantation	Tissue	87
HLA antibodies DSA	1014 including 195 examined prospectively	DSA positive recipients had significantly lower graft survival Lower graft survival in DSA negative but HLA antibody positive patients Patients repeatedly negative for HLA antibodies had a higher survival vs patients who developed de novo HLA antibodies after the first testing	Median of 5 years post transplant	Sera	89

PAPP A CRP, IL 6, TNF α	178	PAPP A found to correlate with the other inflammatory markers like CRP, IL 6, TNF α Multiple regression analysis showed PAPP A and TNF α to be predictors of CAN PAPP A and CRP were predictors of cardiovascular events	Pre transplant	Sera	90
IL 1β, IL 1RII, TNF α, sTNFRI, sTNFRII and LIF	91	sTNFRI and sTNFRII significantly higher in patients with AR	POD 7 and 14	FNAB	72
ICAM 1, E selectin, and L selectin	306	A variant allele in exon 4 of ICAM 1 (R241) was significantly more frequent in recipients with chronic allograft failure More rapid time to graft failure was asso ciated with another ICAM 1 variant in the recipient (E469) in exon 6 No significant association was detected between the selectin polymorphisms studied and chronic allograft failure			94
HβD 1 and ACT	73	Patients with AR had significantly reduced HβD 1 and increased ACT Using both HβD 1 and ACT combined: AUC for the diagnosis of AR = 0.912	Pre biopsy Mostly in first 6 months after transplant	Urine	100

(*Continued*)

Table 6.4 Biomarkers of chronic rejection—cont'd

	N	Result	Time of collection	Source	Reference
CXCL10/IP 10	316	Patients with stable graft function had significantly lower serum CXCL10 than those with graft failure The frequency of AR in the first post transplant month was also increased based on pre transplant serum CXCL10 levels Pre transplant serum CXCL10 levels pre dicted increased frequency of AR CXCL10 was the most predictive of graft loss among the variables analyzed	Pre transplant sera Median post trans plant follow up of 39 months	Sera	93
Intact/cleaved i/cβ2m, RBP, NGAL and α1m	100	Recipients with clinical tubulitis (Ia/Ib) and recipients with other clinical tubular pathologies had significantly increased levels of RBP, NGAL and α1m than stable transplants with normal tubular histology or stable transplants with subclinical tubulitis	Variable	Tissue and urine	16

ACT, alpha-1-antichymotrypsin; α1m, alpha$_1$-microglobulin; i/cβ2m, beta$_2$-microglobulin; DSA, donor-specific antibodies.

grades allograft function in the course of acute rejection, and clinical outcome at 1 year. Fas ligand, but not perforin nor granzyme B, was significantly upregulated in therapy resistant acute rejections ($n = 7$) vs therapy sensitive acute rejection ($n = 8$). There was no relation between cytotoxic marker expression, Banff rejection grades or peak serum creatinine.

4.2. Plasma markers

Lachmann et al[91] examined 1014 deceased kidney transplant recipients for HLA antibodies using Luminex Single Antigen beads. Thirty percent of recipients were found to have HLA antibodies, and of these 31% were found to have donor specific antibodies (DSA). DSA positive recipients had significantly lower graft survival (49% vs 83% in the HLA antibody negative group; $P \leq 0.0001$). Lower graft survival was also seen in recipients who were DSA negative but HLA antibody positive (70% vs 83%; $P = 0.0001$). In a prospective analysis of 195 patients those who were repeatedly negative for HLA antibodies had a superior survival probability compared with patients who developed de novo HLA antibodies after the first testing (94% vs 79%; $P = 0.05$). The authors concluded that HLA antibodies were detrimental to graft survival, even late in the transplant course.

Lauzurica et al[92] postulated that cardiovascular disease and CAN are both manifestations of persistent, post transplant inflammation. The authors studied the role of pregnancy associated plasma protein A (PAPP A) in the development of post transplant cardiovascular events and CAN. PAPP A is a metalloproteinase linked to zinc that has been used in the diagnosis of fetal Down's syndrome.[93] PAPP A has also been found in atheromatous plaques, and circulating levels are increased in acute coronary syndromes.[94] It has also been associated with acute coronary syndrome and atheromatous plaque instability. Lauzurica et al[92] examined whether serum concentration of pre transplant PAPP A was associated with post transplant cardiovascular events and CAN. Pre transplant levels of ultrasensitive CRP, IL 6, TNF α and ultrasensitive PAPP A were measured in 178 renal transplant recipients. During the follow up period of 49.3 $\pm$ 33.6 months, 19 recipients devel oped biopsy proven CAN and 27 recipients had a cardiovascular event. PAPP A was found to correlate with the other inflammatory markers (PAPP A vs CRP, $r = 0.218$; $P = 0.004$; PAPP A vs IL 6, $r = 0.235$; $P < 0.001$; PAPP A vs TNF α, $r = 0.372$; $P < 0.001$). Multiple regression analysis showed PAPP A (RR, 4.27; 95% CI, 1.03–17.60; $P = 0.044$) and

TNF α (RR, 5.6; 95% CI, 1.43−21.83; $P = 0.013$) to be predictors of CAN, while PAPP A (RR, 6.4; 95% CI, 1.24−33.11; $P = 0.027$) and CRP (RR, 6.05; 95% CI, 1.21−29.74; $P = 0.028$) were predictors of cardio vascular events.

Rotondi et al[95] examined the role of chemokine CXCL10/IP 10 in graft failure attributed to both acute and chronic rejection. Pre transplant sera obtained from 316 deceased donor kidneys was retrospectively assayed for serum CXCL10 and CCL22/MDC levels by ELISA. Cyclosporine based immunosuppression was used in 93% of the recipients. The median follow up time post transplant (including patients who experienced graft failure) was 39 months. Patients with stable graft function had significantly lower median pre transplant serum CXCL10 levels than recipients who subsequently endured graft failure (93.0 vs 157.4 pg/mL; $P = 0.0007$). No differences for serum CCL22 levels were observed in the same groups of patients. Patients were grouped based on percentiles of pre transplant serum CXCL10 levels: 0−25th (< 64 pg/mL, $n = 80$), 25th−50th (> 64 and < 97 pg/mL, $n = 78$), 50th−75th (> 97 and < 157 pg/mL, $n = 78$) and 75th−100th (> 157 pg/mL, $n = 80$). Death censored 5 year survival rates for grafts in each percentile group were 97.5%, 93.6%, 89.7%, 78.7% ($P = 0.0006$). Pre transplant serum CXCL10 levels did not influence patient survival. The frequency of acute rejection in the first post transplant month was also increased based on the percentile of pre transplant serum CXCL10 levels in the four groups ($chi^2 = 11.412$; $P = 0.009$). Patients with pre transplant serum CXCL10 levels > 75th percentile (> 157 pg/mL) had an increased frequency of acute rejection vs patients with serum CXCL10 levels < the 75th percentile (34.8% vs 21.4%; $P = 0.01$). Multivariate analysis demonstrated that CXCL10 was the most predictive of graft loss (RR 2.787) among the variables analyzed.

4.3. Genetic markers

McLaren et al[96] assessed the frequency of five polymorphisms in ICAM 1, E selectin and L selectin in four groups of patients: renal allograft recipients with chronic allograft failure ($n = 62$); their matched donors, where available ($n = 33$); kidney allograft recipients with graft survival of greater than 10 years ($n = 110$); and a group of UK controls ($n = 101$). A variant allele in exon 4 of ICAM 1 (R241) was significantly more frequent in recipients with chronic allograft failure vs long term survivors and UK controls (19.4 vs 10.0 and 9.4% respectively, $P = 0.015$ and 0.025).

Stratification by time to graft failure demonstrated that more rapid failure was associated with another ICAM 1 variant in the recipient (E469) in exon 6 ($P = 0.033$). No significant association was detected between the selectin polymorphisms studied and chronic allograft failure.

Human beta defensin 1 (HβD 1) is a 36 amino acid with antimicrobial properties that is found in the loop of Henle, distal tubules, the female genitourinary tract and plasma.[97] HBD 1 was found to be chemotactic for T cells and dendritic cells through the CCR6 chemokine receptor.[98] Alpha 1 antichymotrypsin (ACT: 4.4 kDa) is a 'serpin' or serine protease inhibitor[99] found in liver, kidney[100] and plasma, that may be a potential biomarker of acute liver transplant rejection.[101]

4.4. Urine biomarkers

O'Riordan et al[102] assessed urinary peptides, HβD 1 (4.7 kDa) and α 1 antichymotrypsin (ACT: 4.4 kDa), as biomarkers of acute rejection in renal allografts. The paper includes clinical details of 73 patients although the authors indicated that the number of patients included in the different analyses varied due to sample availability. Samples were collected pre biopsy and before treatment was initiated and all cases of acute rejection were confirmed by renal biopsy. The majority (27/34) of acute rejections occurred within 6 months of transplantation. The mean time from urine sampling to biopsy was 1.7 ± 1.6 days. Urine was also collected from patients with clinically stable transplant function, judged by steady serum creatinine during follow up, as controls. Patients with acute allograft rejection had significantly reduced HβD 1 and increased α_1 anti chymotrypsin ($P < 0.05$) vs clinically stable transplants. Using both peptides combined, the area under the curve for the ROC curve for the diagnosis of acute rejection was 0.912. Urinary HβD 1 levels, quantified by radioimmunoassay, were 176.8 ± 122.3 pg/mL in stable patients vs 83.2 ± 52.2 pg/mL in recipients with acute rejection, for an ROC AUC of 0.749 ($P < 0.01$).

5. BIOMARKERS OF POLYOMA VIRUS INFECTION

Polyomaviruses are members of the Papovaviridae virus family, and are named for their ability to induce a variety of tumors in newborn mice.[103] The human polyomaviruses BK virus and JC viruses were named with the initials of the patients from whom they were first isolated.[104,105] They are

non enveloped viruses with a circular, double stranded DNA genome of 5300 bp and a diameter of 45 nm.[106] JC and BK polyomaviruses share 70% sequence homology with each other and with simian virus 40 (SV40).[106] The viruses are widespread in immunocompetent hosts in both the United States and Europe, with reported seroprevalence rates of 60–80%.[107,108] Complications of polyoma virus infection typically occur in immuno compromised hosts. BK virus is more commonly associated with the urogenital tract and can cause hemorrhagic cystitis,[109] renal allograft dysfunction and graft loss.[110] JC virus has been associated with neurological complications including progressive multifocal leukoencephalopathy,[105] but can cause renal allograft dysfunction. Drachenberg et al[111] prospectively evaluated polyoma virus infection in a cohort of 103 renal allograft recip ients. Evidence of BKV, JCV, or BKV + JCV shedding was found in 56.3%, 27.2%, and 16.5%, respectively. BK viruria was strongly associated with polyoma virus nephropathy (48%, $P = 0.01$) and graft loss ($P = 0.03$), whereas JCV viruria tended to be asymptomatic ($P = 0.002$). The overall incidence of BKV polyoma virus nephropathy was 5.5% compared with an incidence of 0.9% for JCV polyoma virus nephropathy. Both viruses responded to reduction in immunosuppression.

Polyoma virus nephropathy is therefore an important cause of graft dysfunction[112] and is best diagnosed by biopsy. For prospective moni toring of transplant recipients, however, allograft biopsy would be impractical. Investigators have therefore assessed biomarkers of impend ing polyoma infection and nephropathy to guide when to biopsy and management.

Nickeleit et al[112] characterized typical changes cause by polyoma virus in five cases seen within an 8 month period. PCR evidence of BK virus but not JC virus was found in urine samples from all five patients. Urinary decoy cells were also found in patients with persistent polyoma virus disease. Decoy cells were characterized by ground glass type intranuclear inclusions that were positive for polyoma virus by immunohistochemistry and electron microscopy. The specificity of decoy cell excretion was examined in urine collected from 483 renal allograft recipients, including five patients with polyoma virus disease. Abundant urinary decoy cells were found in 28 recipients (6%), while scant urinary decoy cells were found in a further 72 (15%) allograft recipients. Of the 28 patients with abundant urinary decoy cells, five had polyoma virus disease (18%) recipients, while the remaining 23 (82%) had no cytopathic evidence of polyoma in allograft biopsies by light microscopy or IHC.

In a follow up study, Nickeleit et al[106] retrospectively investigated whether BK virus DNA could be found in the plasma of renal allograft recipients with BK virus nephropathy using polymerase chain reaction (PCR). PCR for BK virus was performed on plasma samples from: nine renal allograft recipients with BK virus nephropathy; 41 recipients without nephropathy (16 of whom had urinary decoy cells), and urine; and 17 subjects with human immunodeficiency virus type 1 (HIV 1) infection who had not undergone transplantation. The latter served as immunocom promised controls. BK virus DNA was found in the plasma of all nine patients with BK virus nephropathy (diagnosed histologically), in two of the 41 renal allograft recipients without nephropathy, and in none of the subjects with HIV 1 infection. Three of the six patients with nephropathy were followed during their post transplant course. BK virus DNA was initially undetectable but was subsequently found 16−33 weeks prior to the biopsy diagnosis of BK nephropathy.

Batal et al[113] examined the consequences of increased immunosup pression in 32 allograft recipients with BK viruria, a biopsy diagnosis of acute cellular rejection, and negative in situ hybridization for viral DNA ($n = 50$). Type IA rejection was seen in 24 recipients, type IB in 24 and type IIA in 2 recipients. The presence of high urine viral load ($>$ 1.0E+05 copies/mL) was associated with development of viremia after antirejection treatment (5/9 (56%) vs 0/24 (0%) in patients with low urine viral load, $P < 0.001$).

Urinary BKV replication, detected as either decoy cells or DNA PCR antedates BKV viremia by a median of 4 weeks, and biopsy proven BKV nephropathy by a median of 12 weeks.[114]

Drachenberg et al[111] investigated the frequency and clinical correlation of BKV and JCV replication in a cohort of 103 kidney transplant recipients with urinary decoy cells. Evidence of BKV or JCV DNA by real time PCR was found in 56.3% and 27.2% of subjects respectively. A minority of subjects (16.5%) had BKV and JCV co infection. Subjects with persistent urinary decoy cells ($>$ 2 months) or an increase in serum creatinine of greater than 20% underwent allograft biopsy. Subjects with urinary BKV alone had a significantly higher serum creatinine at the time of the biopsy ($P = 0.002$) and at the end of follow up ($P = 0.05$). BKV viruria was significantly more likely to be associated with viremia at the time of the biopsy compared with pure JCV shedding (93.1% vs 14.3% respectively; $P \leq 0.0001$). The absolute level of blood viral copies was less in patients shedding JCV vs BKV (mean of 2.0E+03 JC copies/mL vs mean of

2.3E+06 BKV copies/mL). JCV viremia was also shorter lived and never persisted beyond 1 month. Polyoma virus associated nephropathy (PVAN) was more likely if BKV viremia levels were $\geq$ 10E4 copies/mL ($P \leq 0.0001$), whereas biopsies were more likely to have normal parenchyma if BKV viremia was $<$ 10E4/mL (81% vs 20% in patients with viremia of $\geq$ 10E4, $P < 0.0001$). Polyoma virus associated nephropathy was more common with BKV (5.5%) vs JCV (0.9%).

Hirsch et al[115] prospectively examined whether BKV replication was associated with nephropathy in a prospective, single center study involving 78 renal transplant recipients. Urine was collected at routine monthly outpatient visits for the first 6 months post transplant, and whenever patients required hospitalization, required an allograft biopsy or experienced graft dysfunction. Nested PCR assay was used to measure plasma BKV DNA whenever urinary decoy cells were found. BKV DNA was also measured at 3, 6 and 12 months after transplantation. Twenty three recipients had urinary decoy cell shedding at a median of 16 weeks post engraftment (range, 2–69 weeks). BKV viremia was found in 10 patients at a median of 23 weeks (range, 4–73 weeks), and BKV nephropathy was diagnosed in five recipients at a median of 28 weeks (range, 8–86 weeks). By Kaplan–Meier analysis, the probability of decoy cell shedding was 30% (95% CI, 20–40%), the probability of BKV viremia was 13% (95% CI, 5–21%) and the prob ability of BKV nephropathy was found to be 8% (95% CI 1–15%). The sensitivity and specificity of decoy cell shedding for the diagnosis of BKV nephropathy was 100% and 71% respectively, while the positive predictive value (PPV) was 29% and the negative predictive value (NPV) was 100%. The sensitivity and specificity of BKV viremia was 100% and 88% respec tively, with a PPV of 50% and a NPV of 100%. Subjects with biopsy proven BKV nephropathy had a significantly higher mean plasma viral load compared with subjects without histological evidence of nephropathy (28,000 copies/mL vs 2000 copies/mL; $P < 0.001$). On serial testing, BK viral load increased to $\geq$ 7700 copies/mL in all subjects who developed BKV nephropathy.

Viscount et al[116] examined whether detection of urinary BK virus by PCR and urine cytology could identify patients with PVAN. Biopsy confirmed BK PVAN was diagnosed in four out of 114 patients (3.5%). Using a cutoff value of $>$ 1.6E+04 copies/mL, BKV viremia had a sensi tivity and specificity of 100% and 96% respectively, and a PPV and NPV of 50% and 100% respectively. A BKV viruria cutoff of $>$ 2.5E+07 copies/mL had a sensitivity and specificity of 100% and 92% respectively, and a PPV and

NPV of 31% and 100%. Urinary decoy cells performed less well. Sensitivity and specificity were 25% and 84% respectively, and a PPV and NPV of 5% and 97% respectively for the diagnosis of concurrent PVAN.

The preceding studies therefore suggest that the absence of decoy cell shedding or viremia reliably exclude the diagnosis of PVAN.

Singh et al[117] hypothesized urinary Haufen was a biomarker of BKV nephropathy. The authors discovered the presence of urinary cast like polyoma virus aggregates by electron microscopy that they named 'Haufen' after the German word for 'cluster or stack'. Urine samples from control patients ($n = 194$ samples from 139 patients) and patients with BK polyoma virus nephropathy ($n = 143$ samples from 21 patients) were examined for the presence of Haufen, and correlated histology, BK viruria and BK viremia. Urinary Haufen correlated with biopsy proven BK nephropathy, with a concordance rate of 99%. All urinary samples from controls were Haufen negative, despite the presence of viremia (in 8%) or viruria (in 41%) of control samples. Fifty four percent (77/143 urine samples) from all 21 patients with BK PVAN contained Haufen. The detection of Haufen had a specificity and sensitivity of 99% and 100% respectively, and a negative and positive predictive value of 100% and 97% respectively.

Current guidelines suggest that screening for polyoma virus replication may allow PVAN to be detected earlier and graft loss prevented. Recommendations include screening patients at least every 3 months for the first 2 years and annually thereafter until the fifth post transplant year. However, urine screening is complicated as variations in micturition intervals and urine content may result in interassay variations. The use of urine supernatants, cell pellets or re suspended urine may also cause variations in polyoma viral load measurements. Furthermore, polymerase chain reactions may be inhibited in urinary specimens.[114]

6. SUMMARY

The preceding sections highlight a number of excellent studies that have attempted to advance the use of biomarkers in kidney transplantation. In general, most of the studies employed biomarkers that satisfied the characteristics suggested by Parikh,[1] Bakay[2] and Sandler[3] discussed in the introduction. Since the field is relatively new, there are a number of limitations in these analyses. In general, the studies reported tended to be small, and included selected patient populations with significant heterogeneity. Another problem is subjectivity in the clinical diagnoses studied. For example, the definition of

delayed graft function can vary, and studies of acute rejection may include patients diagnosed clinically, without histological confirmation. A number of studies discussed in the chapter suggest the possibility that genetic influences and differences in the quality of the donor tissue may affect outcome. The latter are rarely, if ever, included in the multivariate studies of post transplant biomarkers. Therefore, the reader is always left to wonder whether a putative post transplant biomarker is truly associated with outcomes. There is also the difficult issue of accounting for variations in transplant immunosuppression. It is rare for a population of patients to be on exactly the same immunosup pression, and even rarer for immunosuppressive levels to be the same. Whether variations in the overall level of immunosuppression could account for the reported differences seen with putative biomarkers is unknown. The field of transplantation has an excellent track record of conducting large, well powered clinical trials with fairly homogenous patient populations. It may be advan tageous for future clinical studies to include specimen collection for the express purpose of further defining the role of biomarkers in transplantation. Despite the limitations and the need for refinement, it is clear that studies of biomarkers hold promise. Whether biomarkers ever evolve to the point that they can replace traditional diagnostic methods remains to be seen. The ultimate role of biomarkers may primarily be as an adjunct in guiding which diagnostic procedure is best.

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CHAPTER 7

Cystatin C as a Biomarker in Kidney Disease

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Contents

1. STRUCTURE AND FUNCTION OF CYSTATIN C

The occurrence in urine from patients with tubular damage and in normal cerebrospinal fluid of low amounts of an alkaline protein was described in 1961.[1 3] Although several trivial names, e.g. γ trace, post γ globulin, post gamma protein, gamma CSF, high alkaline fraction (HAF), δaT and γ_c globulin[4] were used for the protein, its function as a cysteine protease inhibitor was not revealed until the primary[5] structure of its single poly peptide chain of 120 amino acid residues was described in 1982 and 3 years later[6,7] found to be homologous to the sequence of chicken egg white cystatin,[8] a known inhibitor of papain.

Presently, 12 human proteins are known to contain polypeptide chains with sequences homologous to that of cystatin C and form the human cystatin protein superfamily of cysteine protease inhibitors.[9] Cystatin C is the strongest human inhibitor of important cysteine proteases such as cathepsin K and H and is, in contrast to the other cystatins, present in significant concentrations in all investigated human body fluids.[10]

Although the location of the disulfide bridges of cystatin C was easy to establish,[11] the 3D structure of the monomeric protein present in body fluids

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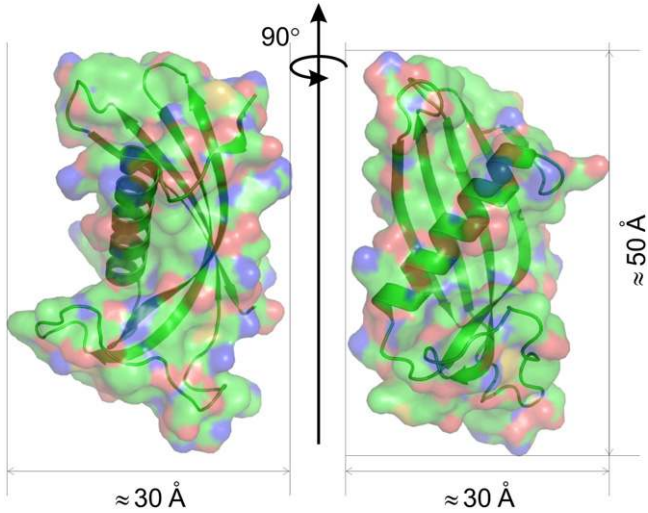

Figure 7.1 ***Dimensions (in Å) of monomeric human cystatin C.*** As emphasized in the left panel, the molecule is folded as a five-stranded antiparallel beta-sheet gripped around a long alpha helix. In a perpendicular orientation (right panel), the alpha-helix is seen to run across the concave face of the beta-sheet. The molecule has a slab-like appearance, with the longest dimension running from the papain-binding epitope (top) to the poorly structured appending segment (bottom). The dimensions have been calculated for all the non-hydrogen atoms, i.e. including side chains, which are visualized as a molecular surface (coloured according to atom type), surrounding the underlying cartoon representation of the main chain. The atomic model excludes the N-terminal peptide (residues 1−11), which is normally disordered and could not be traced in the electron density maps. Figure prepared in PyMOL.

was difficult to determine, because the conditions required for crystallization of the protein always resulted in crystals containing dimers of cystatin C.[12] However, stabilizing the monomeric structure of cystatin C by introduction of an extra disulfide bridge in the protein[13] recently allowed crystallographic studies of monomeric cystatin C.[14] Monomeric cystatin C has a slab like appearance with axes of approximately $30 \times 30 \times 50$ Å (Figure 7.1).

2. CYSTATIN C GENE STRUCTURE AND CYSTATIN C PRODUCTION

The sequence of the mRNA (cDNA) for the precursor of human cystatin C[15] was published in 1987 and the structure of the cystatin C gene[16] on chromosome 20[17] in 1990. The structures of the cystatin C gene, comprising three exons and two introns, and of its promoter, indicate that the gene is a so called housekeeping gene suggesting a stable rate of

production of the protein in all nucleated cell types.[16] The presence of a hydrophobic leader sequence in precystatin C strongly indicates that the protein normally is secreted and the leader sequence cleaved off in the process.[15,16] Indeed, immunochemical and Northern blot studies of human tissues and cell lines have demonstrated that cystatin C is present in and secreted from virtually all investigated cell types.[18 22] However, although cystatin C seems to be produced by all nucleated cells, the production rate may vary between different cell lines and tissues.[22] Subsequent studies of the promoter of the cystatin C gene have shown that it contains glucocorticoid responsive elements,[23] upregulating the cellular synthesis of cystatin C.

3. CATABOLISM OF CYSTATIN C

As a low molecular mass protein (13.4 kDa) cystatin C is mainly catabolized by free filtration in the glomeruli followed by virtually complete tubular reabsorption. Indeed, direct studies of the handling of human cystatin C in the rat have shown that the plasma renal clearance of cystatin C is 94% of that of the frequently used glomerular filtration rate (GFR) marker ^{51}Cr EDTA and that cystatin C thus is practically freely filtered in the glomeruli.[24] Figure 7.2 shows the rat plasma concentration of intact human

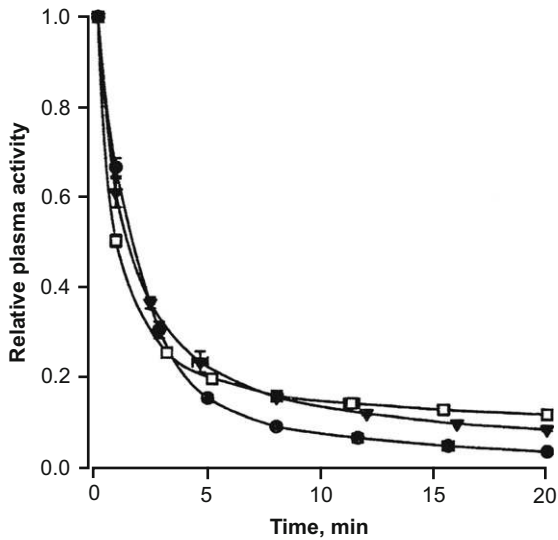

Figure 7.2 ***Plasma concentration of intact ^{125}I-cystatin C (●), ^{51}Cr-EDTA (□) and ^{131}I-aprotinin (▼) relative to the initial plasma concentration after intravenous injection in 12 rats.*** Error bars show +/− 1 SEM, when larger than the symbols. Aprotinin is a 6.5 kDa microprotein with a pI of 10.5.

^{125}I cystatin C and ^{51}Cr EDTA relative to the initial concentrations after intravenous injection. These studies also indicated that at least 99% of the filtered cystatin C was found to be degraded in the tubular cells. The plasma disappearance of cystatin C in normal and nephrectomized rats indicated that the renal plasma clearance of cystatin C is about 85% of the total plasma clearance (renal + extrarenal).

When the GFR of a set of rats was variably lowered by constricting their aortas above the renal arteries, the renal plasma clearance of cystatin C correlated strongly with that of ^{51}Cr EDTA with a linear regression coef ficient of 0.99 and with the intercept not being statistically different from 0.[24] This observation clearly implied an insignificant peritubular uptake of cystatin C. Immunohistochemical and Northern blot studies of human kidneys have also strongly indicated that human cystatin C normally is degraded by the proximal tubular cells after its passage through the glomerular membrane.[25]

4. CYSTATIN C AS A MARKER FOR GLOMERULAR FILTRATION RATE

Cystatin C was first suggested as a new marker for GFR in 1979, when it was observed that the plasma level of cystatin C was up to 13 times higher in patients on hemodialysis than in healthy persons.[4] The method developed in 1979 for determination of the cystatin C level in body fluids was enzyme amplified single radial immunodiffusion.[4] Although this procedure was slow and had a coefficient of variation of 11%, it was useful for identification of cystatin C as a GFR marker at least as good as creatinine, since the corre lation coefficients for the relation between the serum levels of cystatin C and GFR, determined by a gold standard method (plasma clearance of ^{51}Cr EDTA), were somewhat higher than that between creatinine and GFR.[26,27] However, development of automated, rapid and precise methods for determination of the serum or plasma level of cystatin C was required for the use of cystatin C as a marker for GFR in the clinical routine. The first method of this type, a particle enhanced immunoturbidimetric method, was developed in 1994[28] and applied for determination of the serum cystatin C levels in a cohort of 51 patients with GFR measured by a gold standard procedure. ROC curve analysis demonstrated that in this cohort of patients serum cystatin C had a significantly better diagnostic performance than serum creatinine (Figure 7.3). Since then several automated, rapid and precise methods for determination of cystatin C have been developed[29–34]

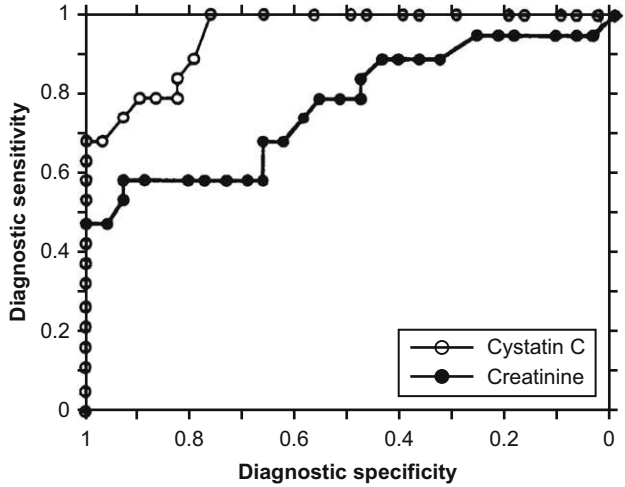

Figure 7.3 ***Non-parametric ROC plots for the diagnostic accuracy of serum concentrations of cystatin C and creatinine in distinguishing between normal and reduced GFR ($\geq$ and $<$ 80 mL/min/1.73 m^2, respectively) in 51 patients with various renal conditions.***

and the information on cystatin C as a GFR marker substantially increased. Entering 'cystatin C' AND 'glomerular OR renal' in the search field of www.ncbi.nlm.nih.gov/pubmed in June 2010 generated 1112 hits. Based upon the information in these and the use of cystatin C as a GFR marker in the clinical routine at my hospital since 1994, my understanding of the present status of cystatin C as a GFR marker is summarized below.

The main advantage of cystatin C compared to creatinine as a GFR marker is that cystatin C is less dependent upon the body composition of a patient than creatinine. For example, whereas the muscle mass strongly influences creatinine, it does not, or only marginally, affects cystatin C.[35 37] Muscle loss of a patient, e.g. by paralysis, low mobility, involuntary or voluntary (anorexia) malnutrition, will strongly impair the use of creatinine as a GFR marker, but not that of cystatin C.[38,39] Figure 7.4 shows ROC curve analysis of cystatin C and creatinine as markers for reduced GFR in a population of patients with spinal cord injury.

The age of a pediatric patient ($>$ 1 year) does not significantly influence his cystatin C level in contrast to his creatinine level (Figure 7.5), and cystatin C is therefore a more suitable GFR marker in pediatric populations since a uniform reference interval independent of age can be used.[40 44]

With increasing age the muscle mass and GFR of a person decrease. This means that both the production and elimination of creatinine decrease in

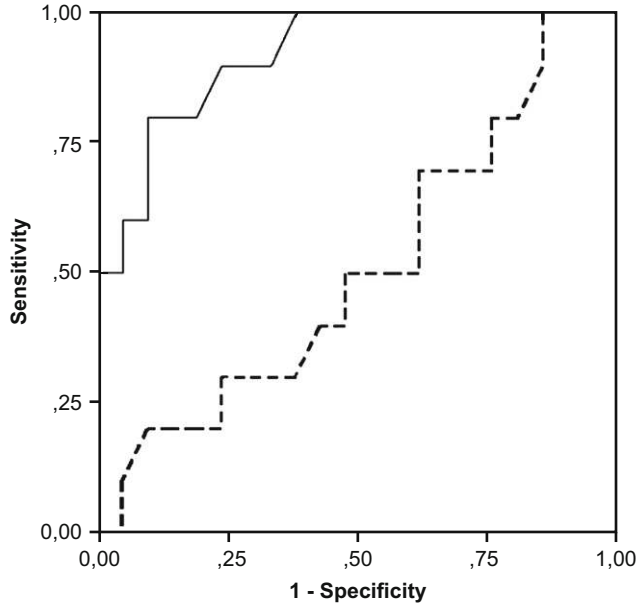

Figure 7.4 ***Non-parametric ROC plots for the diagnostic accuracy of serum concentrations of cystatin C and creatinine in distinguishing between normal and reduced GFR ($\geq$ and $<$ 80 mL/min/1.73 m^2, respectively) in 31 patients with spinal cord injury.***

parallel, which impair the usefulness of creatinine to identify a decrease in GFR in elderly people. But the cystatin C production is not strongly influenced by muscle mass and cystatin C will therefore increase with age due to the decrease of GFR with age.[45,46] Cystatin C therefore seems to be more useful than creatinine to demonstrate the normal and abnormal decrease in GFR in the elderly.[47–55]

In a number of investigations of GFR markers in liver failure, cystatin C has been shown to be a better marker than creatinine.[56–60]

Several studies of cystatin C and creatinine as markers for GFR in diabetes have indicated that cystatin C is the best marker in this condition[61–67] but occasional studies have not shown superiority for cystatin C.[68]

Two meta analyses comparing cystatin C and creatinine as GFR markers arrived at similar conclusions and agree that cystatin C is superior for demonstrating a reduction in GFR in the interval 60–79 mL/min/1.73 m^2.[69,70]

The biggest drawback with cystatin C as a GFR marker is that treating a patient with moderate and high glucocorticoid doses will result in an increased synthesis of cystatin C and an increase in cystatin C levels, falsely

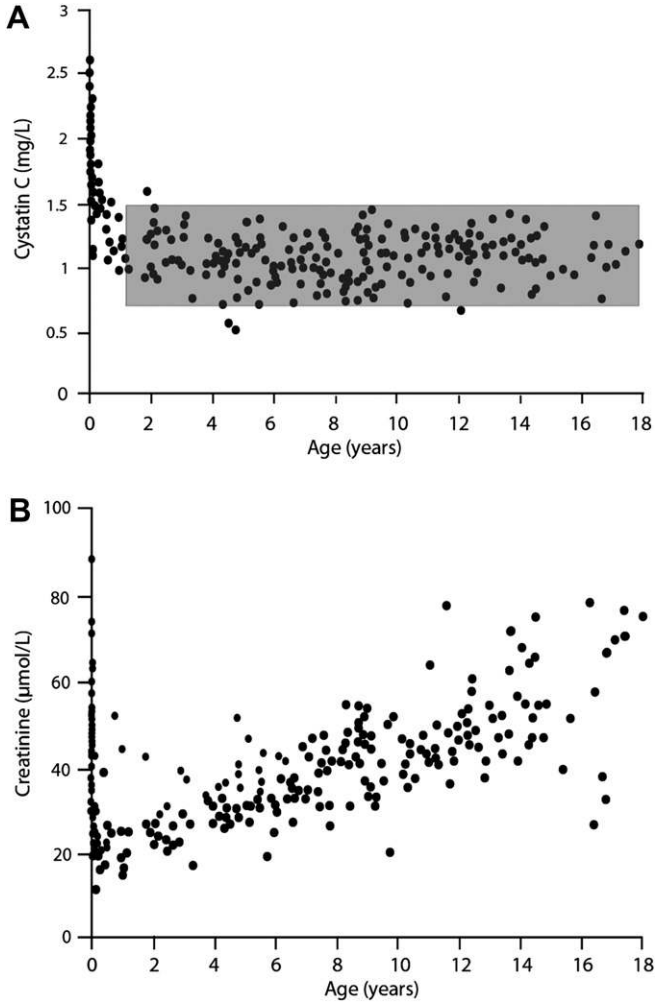

Figure 7.5 ***Serum cystatin C (A) and creatinine (B) in relation to age in a population of 258 children, 1 day to 18 years old, and without evidence of kidney disease.*** The boxed area represents the serum cystatin C reference interval for children over 1 year.

indicating a reduction in GFR.[23,71 74] The increase in the cystatin C level is related to the dose of the glucocorticoid used and small doses do not seem to significantly interfere with the use of cystatin C as a GFR marker. From a practical point of view, topical administration of glucocorticoids does not usually interfere with the use of cystatin C as a GFR marker but peroral and parenteral administration of glucocorticoids do.

Hyperthyroidism and treatment with thyroxine increases the cystatin C level, whereas it decreases the creatinine level.[75] [78] Although it has been shown that the decreased level of creatinine might be due to an increased tubular secretion of creatinine,[79] the reason for the raised level of cystatin C is unknown. Thus, caution should be exercised in the use of creatinine and cystatin C as GFR markers in hyperthyroid (and hypothyroid) states, particularly since a case report indicates that creatinine based GFR prediction equations overestimate and cystatin C based GFR prediction equations underestimate the GFR measured by gold standard procedures in hyperthyroid patients.[78]

In population studies a significant correlation between the levels of C reactive protein (CRP) and cystatin C has been noticed and it has therefore been suggested that systemic inflammation will increase the cystatin C level.[80] However, recording the variations of the levels of CRP and cystatin C for individual patients for extended periods of time does not show any correlation, strongly indicating that systemic inflammation does not influence the cystatin C level (A Grubb et al, submitted)[81] and that thus the correlations noted in population studies are not based upon a causal relationship between inflammation and cystatin C level.

The cost for analyzing cystatin C is higher than that for analyzing creatinine. However, the recent development of many automated analyzing systems by several diagnostic companies has meant a decrease in the cost for analysis of cystatin C. Our laboratory presently charges about 2€ for analysis of cystatin C and about 1€ for analysis of creatinine using an enzyme based specific method.

Although available evidence indicates that cystatin C alone generally is a better GFR marker than creatinine alone, it should be considered that the drawbacks of creatinine alone as a GFR marker are mainly due to the fact that creatinine is strongly dependent upon the body composition of a person in addition to his GFR. This drawback can, at least partly, be compensated for by using creatinine based GFR prediction equations employing, in addition to creatinine, anthropometric data, e.g. age, sex, ethnic group.[82]

5. CREATININE- AND CYSTATIN C-BASED GFR-PREDICTION EQUATIONS

Since creatinine alone has clear drawbacks as a GFR marker, it is widely considered that it should be replaced by GFR prediction equations based not only upon creatinine, but also upon anthropometric data such as sex,

age and ethnicity, which compensate for the influence of muscle mass on the creatinine level.[82] Most of the well founded, generally used and recommended creatinine based GFR prediction equations, e.g. the MDRD and CKD EPI equations,[82 86] implicitly use the mean muscle mass of a person of a specified age, sex and ethnic origin in the population employed to derive the equation, to compensate for the muscle mass influence on the creatinine level used for prediction of GFR. If a person's muscle mass deviates from the mean of that of persons of his/her age, sex and ethnic origin in the population, the GFR prediction equation will not be accurate for this person. This is an important reason for the remaining imprecision in the creatinine based GFR prediction equations. It also explains why different creatinine based equations are required for maximal diagnostic peformance in different populations of individuals, for the relation between muscle mass, age, sex and ethnicity, differs between different populations. For example, the MDRD equation generally underestimates the GFR of healthy people by 29%[87] and its application in a Japanese population requires a Japanese specific coefficient of 0.763.[88] Nevertheless, in many clinical situations the creatinine based GFR prediction equations estimate GFR at least as well as cystatin C alone.[89] One drawback with presently available creatinine based GFR prediction equations is that they usually do not work for persons below 18 years of age for which specialized prediction equations, e.g. those of Schwartz and Counahan Barratt, have to be used.[90,91] However, recently a creatinine based GFR prediction equation (the LM equation), which works for both adults and children, has been described.[92,93]

Since the level of cystatin C is less dependent upon anthropometric data than that of creatinine, simpler cystatin C based GFR prediction equations of the type $GFR = A \times \text{cystatin C}^{B}$ can be used both for adults and children.[42,94 97] Although cystatin C generally seems to be significantly less dependent upon anthropometric data than creatinine,[98] this must be verified for patient and ethnic groups not yet studied. It should also be considered that whereas cystatin C alone and cystatin C based GFR prediction equations are less influenced by muscle variation than creatinine alone and creatinine based GFR prediction equations, the usefulness of cystatin C based prediction equations are impaired in the same way as cystatin C alone by moderate and high doses of glucocorticoids.

A considerable number of creatinine or cystatin C based GFR prediction equations have been described.[82 99] The reasons for the present high number of equations are the use of different calibrators, the use of

non accurate methods for determinations of creatinine or cystatin C, the use of different patient or ethnic populations and the use of different mathematical models to generate the prediction equations. These factors must be carefully considered before a GFR prediction equation is selected for use in a particular patient population. For example, when a prediction equation, based upon a specific cystatin C calibrator and determination method, is used to estimate GFR from the cystatin C levels produced using another cystatin C calibrator and determination method, large errors in the resulting GFR estimates may result, even for similar patient populations. One way of reducing the problems associated with the selection of a suitable GFR prediction equation is to produce international calibrators for creat inine and cystatin C, and to use them not only to secure the use of stan dardized calibrators in different methods, but also to develop and secure accurate methods for both cystatin C and creatinine. The use of validated international calibrators and accurate methods for determination of creati nine and cystatin C will decrease the number of validated equations and simplify the selection of an equation suitable for a specific patient pop ulation. An international calibrator for creatinine is already available[85] and one for cystatin C (ERM DA471/IFCC) has recently been produced.[100]

Although some creatinine or cystatin C based GFR prediction equa tions produce estimated GFR values 80–85% of which are between +/ 30% of GFR measured by invasive gold standard methods in some studies, the highest percentages of estimated GFR values between +/ 30% of measured GFR values are obtained using GFR prediction equations based upon both cystatin C and creatinine.[89,98,102 106] Such equations might produce estimated GFR values 90–91% of which are between +/ 30% of GFR measured by gold standard methods.[89,105] The imprecision of all gold standard procedures means that even if a gold standard procedure is per formed twice within a short interval on patients with stable kidney function, less than 100% of the second determination will be within +/ 30% of the first. Thus, a GFR prediction equation producing GFR values 90–91% of which are within +/ 30% of GFR measured by gold standard methods is close to what is theoretically attainable. It should, in addition, be considered that in evaluations of GFR values produced by GFR prediction equations, it is automatically assumed that the imprecision of the gold standard procedure used is 0%. This means that the calculated percentage of estimated GFR values between +/ 30% of the measured GFR values always is lower than the true one, since the imprecision of the gold standard procedure increases the number of estimated GFR values outside the +/ 30% interval.

6. THE LUND MODEL: GFR-ESTIMATION WITH AN INTERNAL QUALITY CONTROL

Although GFR prediction equations based upon both cystatin C and creatinine clearly seem to have better diagnostic performance than prediction equations based upon only one of these GFR markers, such combined equations do not perform optimally in a number of clinical situations. For example, if it is known that a patient suffers from paralysis and has a very low muscle mass, the combined prediction equation will perform worse than a prediction equation using only cystatin C. In a clinical situation where the patient is treated with high doses of a glucocorticoid, the combined prediction equation will perform worse than a prediction equation using only creatinine and anthropometric data. A strategy for GFR estimation based upon automatic use of a combined prediction equation using both creatinine and cystatin C will therefore have a worse diagnostic performance than a strategy that not only uses cystatin C and creatinine as GFR markers but also takes clinical data into account.

In Lund, where cystatin C has been available in the clinical routine since 1994,[28] the following strategy for estimation of GFR has been developed.[107,108] It is built upon the use of three sources of information: the plasma levels of cystatin C and creatinine and knowledge of the clinical context. Age and sex of the patient is always available, since they can be inferred from the unique identity number (Swedish personal number) used to identify all patients. Relative GFR (mL/min/1.73 m^2) is estimated both by a GFR prediction equation based upon only cystatin C and by a prediction equation based upon only creatinine and anthropometric data. Theses two estimates are then compared with each other and, if they agree within specified limits, the average of the two estimates is used. It has been shown that the average value of the two estimates performs diagnostically at least as well as more complex ways of combining the two estimates.[105] The specified limits for agreement between the two estimates can either be applied automatically or the physician can decide for himself what level of agreement is required for the patient under study. A higher degree of agreement is required, for example, when the estimated GFR is to be used for dosing of medicines with potential adverse side effects than for deciding whether a patient has a normal GFR or not. If the two estimates agree, the average value is a very reliable estimate of GFR. As a matter of fact, during the 15 years we have been using cystatin C in parallel with creatinine as a marker for GFR, we have had about ten cases for which the GFR estimates based upon cystatin C and creatinine

agreed, but disagreed with GFR measured by our invasive gold standard procedure (plasma clearance of iohexol). In all cases, it turned out that the error had to do with some technical problems in executing the gold standard procedure. We therefore consider that, in practice, agreeing cystatin C and creatinine based estimates of GFR are at least as reliable as GFR measured by invasive gold standard procedures.

If the GFR estimate based upon only cystatin C does not agree with that based upon creatinine, the clinical situation is considered, e.g. concerning the presence of an abnormal muscle mass of the patient or use of high doses of a glucocorticoid. If clear reasons for not using either the cystatin C or the creatinine based estimates are found, only the appropriate prediction equation is used for estimation of GFR.

If no clear reasons can be found for the discrepancy between the GFR estimate based upon only cystatin C and that based upon creatinine, GFR is measured by an invasive gold standard procedure.

When the GFR of a patient has been estimated according to this strategy, changes in GFR can securely be monitored by determination of creatinine, since the strategy has connected a reliable GFR value to the creatinine level of that particular patient. But if the muscle mass of the patient significantly changes, the strategy involving two GFR estimates has to be applied again.

The strategy outlined above does not require the use of any particular cystatin C based or creatinine based prediction equation. Characterization of the population served by a hospital may be required to select the best prediction equations for that hospital. In Lund we have chosen a cystatin C based equation working for both children and adults[95] and a creatinine based equation that also works for both adults and children,[92,93] so it has been technically simple to implement the strategy. The strategy is described at www.egfr.se and this site can also be used to implement it and to calculate absolute GFR from relative GFR, which might be required, e.g. for dosing of medicines cleared by the kidneys.

7. ABNORMAL GLOMERULAR FILTRATION QUALITY: A NEW MARKER FOR KIDNEY DISEASE. USE OF CYSTATIN C TO IDENTIFY IT

GFR is defined as the volume of glomerular filtrate produced per unit of time, e.g. mL/min. Although GFR is a good general indicator of renal disease, it will not detect renal diseases in which the composition of the glomerular filtrate is deranged in the presence of a normal GFR. For

example, if the glomerular filtrate contained only substances with a molecular mass below 6 kDa the GFR would be normal, since water (18 Da) is the predominant component of all types of glomerular filtrate, but the patient would be seriously sick, inter alia because of significant distur bances in the catabolism of low molecular mass signaling and regulatory peptides and proteins. Such a disturbance in the glomerular filtrate composition would not be possible to detect using any of the substances used for invasive gold standard determination of GFR (iohexol, inulin, ^{51}Cr EDTA, ^{99m}Tc diethylenetriaminepentaacetic acid, ^{125}I iothalamate), since all of them have a molecular mass below 6 kDa. But occasional studies involving measurement of the plasma clearance of dextrans of different molecular size have shown that the composition of the glomerular filtrate changes in certain conditions, e.g. in pregnancy, in the presence of a normal GFR.[109] The cystatin C level in plasma seems to identify the altered filtration spectrum in normal pregnancy, since it increases with gestational age with the highest levels in the third trimester[110 112] in the presence of a normal GFR as determined by the plasma clearance of iohexol. In addition, other low molecular mass proteins, e.g. beta$_2$ microglobulin (12 kDa) and beta trace protein (23–29 kDa) display a similar increase with gestational age in normal pregnancy.[111,112] The genes for cystatin C, beta$_2$ microglobulin and beta trace protein are located on different chromosomes and have different promoters, so it is highly unlikely that the parallel increase in the levels of the three proteins would be an effect of increased production brought about by the hormonal changes occurring in pregnancy. The increase of cystatin C, beta$_2$ microglobulin and beta trace protein with gestational age is steeper in preeclamptic than in normal pregnancy[112] and these increases thus seem to be associated with the pathological glomerular processes, e.g. glomerular endotheliosis and swelling, occurring in preeclampsia.[113] Interestingly, the increased levels of cystatin C, beta$_2$ microglobulin and beta trace protein can be used to detect preeclampsia before a decrease in GFR occurs[112] and before the creatinine levels start to increase.[112 114] It is of additional interest that the diagnostic performance to detect preeclampsia is improved with increasing molecular size of the three proteins (the level of beta trace protein being the best) as demonstrated by ROC curve analysis.[112] A possible explanation to the above mentioned observations would be that the functional glomerular pore size decreases both during normal and pre eclamptic pregnancies, with the highest decrease rate in preeclampsia, and that the initial decrease therefore is first identified by the largest protein (Figure 7.6).

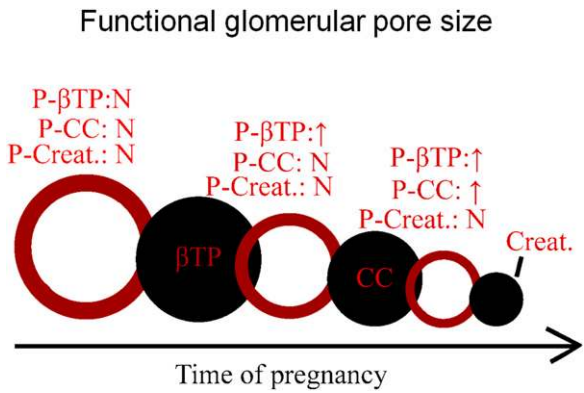

Figure 7.6 ***Schematic drawing showing the relation between functional glomerular pore size and the size of plasma proteins increasing in concentration with gestational age and useful as markers of preeclampsia.*** CC, cystatin C; βTP, beta-trace protein; Creat, creatinine.

It cannot be excluded that several renal diseases are characterized by a decrease in functional glomerular pore size, before a decrease in GFR occurs or can be detected. The plasma levels of proteins normally passing the glomerular filter to a significant extent (e.g. cystatin C, beta$_2$ micro globulin, beta trace protein, alpha$_1$ microglobulin) would in such disorders be increased in spite of a normal GFR and useful as markers for the abnormal glomerular filtration quality in such disorders.[114,115] It is also possible that the observed earlier increase of cystatin C compared to that of creatinine in some disorders may at least partly be explained by a decreased functional pore size rather than a decrease in GFR. The use of cystatin C, beta$_2$ microglobulin, and beta trace protein to characterize the elimination patterns of different dialysis modalities has already been described.[116]

8. CYSTATIN C AND AGING SUCCESS

Cystatin C has been shown to be a stronger predictor of the risk of death, cardiovascular events and hospitalization than creatinine in virtually all populations investigated, e.g. in elderly persons, in persons with peripheral arterial disease, in persons with chest pain, in persons with chronic kidney disease and in the general population.[117–130] These observations might also conveniently be expressed by saying that cystatin C is a stronger predictor of aging success than creatinine.[127] The background to these observations is not fully elucidated. Although cystatin C generally is a better marker for

GFR than creatinine, cystatin C has been shown to be a marker for increased mortality also in patient cohorts with normal GFR as estimated by creatinine based GFR prediction equations.[130] It has therefore been suggested that other causes than reduced GFR might contribute to the success of cystatin C as a marker for aging success. For example, it has been suggested that high levels of cystatin C might be toxic and that increased levels of cystatin C might indicate not only reduced GFR but also the presence of inflammatory processes.[80,117,118,120 126] It has also been shown that high levels of cystatin C are directly associated with arterial stiffness in older adults.[126] An additional way in which increased levels of cystatin C might be associated with an increased risk for cardiovascular events in the presence of a normal GFR is that a raised cystatin C level might indicate an abnormal filtration process (resulting in an abnormal glomerular filtration quality) before the GFR is decreased.[111] Recent investigations of individual patients for extended periods of time do not support that cystatin C is a marker of systemic inflammation (A Grubb et al, submitted).[81] Studies using gold standard determinations of GFR are still required to unequivocally demonstrate that cystatin C has an advantage over measured GFR as a predictor of cardiovascular events. To summarize, although a raised level of cystatin C might indicate a decline in GFR due to significant atherosclerosis in the renal arteries and thus an increased probability of simultaneous significant atherosclerosis in other vital arteries, e.g. the coronary and brain arteries, other mechanisms explaining the usefulness of cystatin C as a predictor of aging success might be involved.

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CHAPTER 8

Biomarkers of Renal Cancer

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Contents

1. RENAL CANCER

Renal cancer is the tenth most common cancer in adults, with approxi mately 200,000 new cases and 100,000 deaths worldwide each year. The incidence of renal cancer has been steadily rising, with, for example, a 100% increase in females over the past 25 years in the UK. Peak incidence is in the sixth and seventh decades with a male to female ratio of 3:2. Risk factors for the development of renal cancer include smoking, obesity and hyperten sion, as well as end stage renal failure patients on dialysis who develop acquired renal cystic disease.

The most common type of kidney cancer is renal cell carcinoma (RCC), amongst which the conventional (clear cell) histological subtype is the most common, accounting for 70—80% of all cases. A further 10—15% are papillary tumors, 4—5% chromophobe tumors, as well as collecting duct (< 1%) and the benign oncocytomas (2—5%), each arising from different areas of the kidney and with distinct underlying genetic changes, morphology and clinical features.

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In this chapter, the term RCC is used to imply the clear cell phenotype, unless stated otherwise. It is generally accepted that conventional RCC originates from proximal tubules, based on immunohistological and ultrastructural anal ysis. However, this remains a matter for contention and evidence for a distal tubular origin also exists. The sarcomatoid variant, which can occur with any histological subtype, is associated with a significantly poorer prognosis. RCCs are graded based on nuclear features, with the most commonly used grading system ranging from 1 (well differentiated) to 4 (poorly differentiated).[1]

1.1. Biology

Like several other tumor types, RCC can occur in both a sporadic and hereditary form. The Von Hippel–Lindau (VHL) tumor suppressor gene represents an important gene in this regard. It underlies the rare inherited VHL syndrome, but perhaps more significantly, it is also implicated in most sporadic conventional RCC tumors. Early studies of familial RCC localized the genetic defect to the short arm of chromosome 3, with the specific identification of the gene as VHL at 3p25–26 being identified in 1993.[2]

The most well characterized function of the VHL gene product relates to its role as a substrate binding element in a complex with elongin B, elongin C, Cul2 and Rbx1 which targets proteins for ubiquitination and subsequent proteasomal degradation. The main identified substrate for VHL binding is the transcription factor hypoxia inducible factor (HIF) family. HIF is a heterodimeric transcription factor consisting of an unstable alpha subunit and a stable beta subunit. This targeted destruction and regulation of HIF occurs normally but with loss of VHL function or in hypoxia, HIF α accumulates, binds to HIF β, and transcriptionally activates genes whose promoters contain hypoxia response elements. Approximately 100 HIF responsive genes have been described, many of which are involved in the adaptation to acute or chronic hypoxia. These include vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), platelet derived growth factor (PDGF), glucose transporters (e.g. GLUT1) and transforming growth factor α. It is evident that these proteins are involved in critical processes such as angiogenesis, cell growth and cell survival and hence loss of VHL function underpins many critical early steps in the development of most RCCs. It should be noted that the VHL protein is known to be involved in an increasing number of other cellular pro cesses, such as cell cycle regulation, extracellular matrix assembly and cyto skeleton organization. These insights into RCC biology are now being

exploited to produce a number of novel therapeutic targeted agents that are discussed below.

1.2. Diagnosis and treatment

The majority of patients have few, if any, symptoms at diagnosis. The widespread use of imaging techniques such as ultrasound (USS) and computerized tomography (CT) has led to a sharp rise in the number of incidental diagnoses although this is not thought to account, solely, for the rise in incidence. Rates of incidental RCC diagnosis in our local population are approximately 35% (Banks, unpublished data), although in parts of Europe (where USS is more routinely available in the primary care setting) rates are $> 60\%$. The classic triad of hematuria, flank pain and abdominal mass are seldom seen. Patients may present with non specific symptoms such as weight loss or general malaise, or, more rarely, secondary to para neoplastic syndromes causing hypercalcemia, polycythemia or pyrexia.

Once patients present to their physician, diagnosis currently relies on expert radiological review of CT and/or magnetic resonance (MRI) imaging. For larger tumors this is very reliable and patients usually undergo surgical resection without the need for a preoperative biopsy. Smaller renal masses (≤ 4 cm), however, are more difficult to accurately diagnose and it is estimated that 20–25% of such lesions are benign.[3]

Surgery remains the mainstay of treatment for patients with RCC. Radical nephrectomy, now often performed laparoscopically, is the gold standard of care in patients with localized and locally advanced tumors, and represents the only means of potential cure. In patients presenting with metastatic disease, removal of the primary tumor should also be considered in carefully selected patients, although this has not been validated in patients treated with more recently introduced therapies. The optimal management of smaller renal masses remains uncertain. Options include surgical excision, radiofrequency ablation or watchful waiting, with each approach carrying its own risks and benefits.

Renal tumors are characteristically resistant to standard chemothera peutic agents. For the past 25 years biological agents in the form of inter feron α (IFN), and interleukin 2 (IL 2) have been used as standard first line treatment. Single agent IFN carries modest response rates of approximately 10%, with median survival, even in carefully selected patients, of 19 months in the most recent trials.[4] Advances in our understanding of renal cancer biology have recently led to a revolution in the treatment of this disease.

In the past 3 years the Food and Drug Administration (FDA) have approved four new drugs, namely sorafenib, sunitinib, temsirolimus and bevacizumab, that are now used in the treatment of patients with metastatic RCC. Whether tyrosine kinase inhibitors (TKIs) given after surgery, in the adjuvant setting, improves overall survival is unknown and remains the focus of ongoing randomized clinical trials.

Sorafenib (Bayer Healthcare) and sunitinib (Pfizer) are small molecule TKIs that have activity against several receptor kinases including VEGFR1, 2 and 3, PDGFRβ, c KIT and RET. They are licensed for the treatment of patients with metastatic RCC based on randomized Phase III data. Sunitinib doubled median progression free survival (PFS) in comparison to single agent IFN from 5 months to 11 months ($P < 0.001$) in the first line setting.[5] Median overall survival was greater than 2 years in the trial arm.[6] Sorafenib similarly improved PFS from 2.8 months to 5.5 months in a second line placebo controlled trial.[7]

Temsirolimus (Wyeth Pharmaceuticals) is an inhibitor of mammalian target of rapamycin (mTOR) kinase, a component of intracellular signaling pathways. It plays a central role in the phosphoinositide 3 kinase (PI3K)/Akt pathway, which is often aberrantly regulated in cancers, including RCC. Temsirolimus has also demonstrated its superiority over IFN in a randomized phase III setting in poor prognosis disease, and is the only novel agent to show a statistically significant improvement in overall survival (OS).[8] Everolimus (Novartis), also an mTOR inhibitor, is the only drug to show a significant improvement in PFS in patients with metastatic renal cancer who have progressed on sunitinib or sorafenib, in the setting of a randomized phase III trial.[9]

Finally, bevacizumab (Roche), a humanized monoclonal antibody to VEGF, has also recently been reported to show improvement in PFS when used in combination with IFN, compared to IFN alone in a randomized phase III study, doubling time to progression from 5.4 to 10.2 months.[4]

1.3. Staging and prognosis

Cancers of the kidney are characterized by their highly variable natural history and therefore predicting outcomes for individual patients can be difficult. This ability to classify patients according to risk is highly desirable however. In localized disease, such information could be used to guide the intensity of follow up and to identify high risk patients who can be targeted for adjuvant therapy trials. The recent introduction of efficacious but costly

treatments also highlights a need to be able to define and target specific patient groups.

The staging of renal tumors is, like all solid tumors, based around the TNM system, introduced and recommended by the American Joint Committee on Cancer (AJCC) in 1959. In this classification, T denotes the extent of the primary tumor, N the extent of regional nodal disease and M whether metastatic disease is present. The current (6th) edition of the TNM system was published in 2002[10] (Table 8.1) and, although validated in terms of allowing the classification of patients in terms of survival, the need to continually modify the TNM system, as new evidence from large multi center studies emerges, has been highlighted.[11,12]

In addition to stage, tumor size, nodal status and histological grade are established independent prognostic factors in RCC. Several prognostic models or algorithms have been proposed, variably incorporating these and other factors.[13] Elements such as nuclear grade are, however, subject to intra and inter observer variability[14] and additionally the prognostic

Table 8.1 Renal cell carcinoma (RCC) TNM staging

Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1a	Tumor 4 cm or less in greatest dimension, limited to the kidney
T1b	Tumor more than 4 cm but less than 7 cm in greatest dimension, limited to the kidney
T2	Tumor more than 7 cm in greatest dimension, limited to the kidney
T3	Tumor extends into major veins or invades adrenal gland or perinephric tissues but not beyond Gerota's fascia
T3a	Tumor directly invades adrenal gland or perirenal and/or renal sinus fat but not beyond Gerota's fascia
T3b	Tumor extends into renal vein or its branches, or vena cava below diaphragm
T3c	Tumor extends into vena cava above diaphragm or invades the wall of the vena cava
T4	Tumor invades beyond Gerota's fascia
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastases
N1	Metastases in a single regional lymph node
N2	Metastases in more than one regional lymph node
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

validity of nuclear grading in histological subtypes other than conventional is questionable.[12] Furthermore, such models typically stratify patients into a limited number of risk groups, based on a 'risk score', meaning that estimates of risk can be wide for individual patients. For patients with localized disease, the challenge for clinicians is to identify the 30–40% of patients who will subsequently relapse following radical surgery. There is, at present, no consensus on how surveillance of such patients should be conducted.[15] The Mayo SSIGN score provides a means for determining prognosis in localized disease, using a model based on 1671 patients, and incorporates pathological T stage, N stage, tumor size, nuclear grade and histological tumor necrosis.[16] Patients are divided into three risk groups, with estimated 5 year metastases free survival rates of 97.1%, 73.8% and 31.2% in the low, intermediate and high risk groups respectively. The model is being used to stratify patients in ongoing phase III trials of adjuvant therapy.

Currently, the model most widely applied to patients with metastatic disease is that proposed by Motzer and colleagues.[17] The model stratifies patients into three risk groups, incorporating five prognostic features, namely low Karnofsky performance status (< 80%), high lactate dehydrogenase (LDH) (> 1.5 times the upper limit of normal), low serum hemoglobin (< the lower limit of normal), high corrected serum calcium (> 10 mg/dL) and time from initial RCC diagnosis to start of therapy of less than 1 year. Patients considered to have a favorable prognosis are those with no poor prognostic factors present; intermediate patients have one or two factors present; and patients with an unfavorable profile have more than two factors present. In patients treated with first line sunitinib, median overall survival was 20.7 months in the intermediate risk group, 5.3 months in the poor risk group and has not yet been reached in the favorable risk group.[6]

Recent interest has also focused on the development of preoperative nomograms that move away from the traditional reliance on histopathological criteria. Such information provides early prognostic data to patient and physician, and may guide surgical strategy and consideration of neo adjuvant systemic therapy. The most comprehensive model to date incorporates age, gender, symptoms, tumor size (by CT scan), T stage and metastasis, to predict RCC specific mortality with a high degree of accuracy.[18] Ultimately, however, currently employed prognostic tools are limited by their over reliance on clinicopathological characteristics, which belies the complexity and varied biology of RCC. Incorporation of robust,

well validated molecular markers into new or existing models is what is now required and should help to further refine prognostication for individual patients.

2. CANCER BIOMARKERS – GENERAL CONCEPTS

The potential for biomarkers to impact on the diagnosis, treatment and survival of patients with cancer is significant. Depending on the intended use, the ideal tumor marker may be a protein (or protein fragment) that can be easily and objectively measured non invasively in the serum or urine of patients. Alternatively, for differential diagnosis at the histological level, a protein that could be detected by immunohistochemistry (IHC) on tissue sections would be needed. Biomarkers that allow early detection of disease hold tremendous potential, since most solid tumors, including renal cancers, are curable if detected at an early stage. In many cancer types, biomarkers have established roles in differential diagnosis, tumor sub classification, risk stratification and disease monitoring. The advent of numerous efficacious but often expensive biological therapies in many cancers means that oncological practice is now heavily influenced by cost effectiveness. In general, in oncology, therefore, one of the most urgent needs is for the identification of novel predictive biomarkers, i.e. those that are able to identify patients that are likely to respond to a given treatment or not. When recently reviewed, less than 20 Food and Drug Administration (FDA) approved protein based cancer biomarkers were in current use.[19] Most, including proteins such as CA125, CA15–3 and carcinoembryonic antigen (CEA), are used in the longitudinal monitoring of patients for cancer recurrence and/or their response to treatment. Others such as human epidermal growth factor receptor 2 (HER2) and the estrogen receptor are used in treatment selection, whilst only two, PSA in prostate cancer and nuclear matrix protein 22 (NMP22) in bladder cancer, have any conceivable role in screening.

The advent of high throughput genomic and proteomic technologies has seen a burgeoning of novel candidate markers, yet remarkably this has been accompanied by a decrease in the number of protein biomarker approvals.[19] It is generally acknowledged that the reasons for this paradox have primarily been concerned with the lack of adequate structure in the biomarker discovery and validation process. In 2001, Pepe and colleagues defined five phases of screening biomarker development. Phase I concerns preclinical exploratory studies, Phase II clinical assay development, Phase III

involves retrospective longitudinal studies, Phase IV prospective screening studies and finally Phase V, cancer control studies.[20] Although several models of the biomarker pipeline have been defined, variably employing between three and seven phases, this stepwise progression, which draws parallels with the drug development process, clearly requires large amounts of time and expenditure. Thus many promising candidate markers have never been properly evaluated. Various initiatives have now been developed to address this, such as the establishment by the National Cancer Institute (NCI) in the USA of a consortium, the Early Detection Research Network (EDRN), to accelerate the development of biomarkers for cancer detection and diagnosis.

Historically, reports of cancer biomarkers across all cancer types, including RCC, have been plagued with issues related to inadequate sample size, poor design and analysis, and incorrect or over interpretation. Pub lished literature is heavily biased towards positive studies, with a review of a large number of articles on cancer prognostic markers concluding that only 1.5% did not report statistically significant results, without efforts to justify the importance of the marker in question in some other manner.[21] In recognition of these shortcomings, and as a joint initiative, the National Cancer Institute–European Organization for Research and Treatment of Cancer (NCI–EORTC) published guidelines for tumor marker prognostic studies, termed REporting recommendations for tumor MARKer prog nostic studies (REMARK).[22] Similar guidelines applicable to diagnostic markers (Standards for Reporting Diagnostic Accuracy (STARD)) have also been described.[23] The adoption of compliance with these guidelines by many journals as a condition of manuscript submission and acceptance should lead to better conducted studies, with earlier identification and prioritization of only the most promising candidate markers for subsequent validation.

3. RENAL CANCER BIOMARKERS

The management of patients presenting with RCC presents many chal lenges that biomarkers are ideally placed to address.[24] Currently, however, no validated biomarkers exist for patients with RCC and their development has been recognized as a priority area for research[25] (NCI Progress Review Group Report, August 2002). Such biomarkers have the potential to impact on all aspects of patient management, from diagnosis, determining prognosis and detecting recurrence to treatment selection and monitoring. Each of

these is considered below, focusing in particular on protein based biomarkers. This reflects the propensity of such markers in the literature although it is of course recognized that there are several transcriptomic studies for example, which may ultimately be mined and findings explored at the protein level.

3.1. Diagnostic markers

In terms of diagnosis, the biomarker need is both at the level of absolute disease detection of RCC through objective non invasive measurement in the patient's serum or urine, and also for differential diagnosis of the various histological subtypes with some granular variants in particular posing problems for the pathologist. Of course some markers may fulfil both criteria.

3.1.1. Circulating markers

The relatively low incidence of RCC means that screening of the general population is unlikely to be feasible and cost effective. Even if a biomarker for RCC was 100% sensitive and had a specificity of 99.4%, the positive predictive value for men older than 65 years would be only 10%.[26] Thus, screening would need to be targeted at those at high risk of disease, such as those with previous RCC, end stage renal disease, kidney transplant recipients or familial RCC. A pan urological malignancy biomarker panel is an alternative approach, reducing the demands placed on any individual protein.

Efforts to identify circulating diagnostic protein markers for RCC have been few and have been met with limited success. This reflects both the challenge of profiling complex fluids such as plasma and urine, as well as the heterogeneity and biological complexity of RCC. The overriding challenge in proteomic serum analysis is the vast dynamic range of protein concen tration, starting with albumin at approximately 40 mg/mL down to cyto kines at 1–10 pg/mL. This is a range spanning at least 10 orders of magnitude and far exceeds the analytical range of any proteomic technology. Additionally, just 22 proteins constitute 99% of the entire serum protein content[27] and pre fractionation/enrichment strategies are essential. Urine presents its own analytical challenges in terms of both low protein concentration and high salt content.

The main tool used to profile fluid samples for diagnostic purposes in RCC has been surface enhanced laser desorption ionization (SELDI) mass

spectrometry used for both urine and serum samples.[28,29] This is a relatively low resolution mass spectrometric profiling technique which is a variant of MALDI mass spectrometry, incorporating a selective chip surface to bind specific types of molecule in the samples such as hydrophobic or positively charged molecules. Essentially, a series of mass profiles can be generated and with limits due to ionization, the main analytes detected are the lower molecular proteins or peptides. The most commonly adopted approach has been to use these profiles from a training set of samples to develop algo rithms for sample classification with the model generated then being applied to test samples for subsequent classification. Issues related to lack of repro ducibility, sample stability, inadequate quality control and difficulty identi fying candidate peaks have been highlighted by our group and others, although these are now starting to be addressed in some cases.[29] [31] Using this approach, serum amyloid A (SAA) has been identified as being cleaved in some patients and has reproducibly been found to be increased in the sera of a subset of patients with RCC, but, given that SAA is an acute phase protein, the specificity of such a marker for diagnostic purposes is likely to be questionable.[31] Xu and colleagues have recently reported a SELDI based decision tree capable of differentiating serum samples from small RCC tumor patients and healthy volunteers, with a sensitivity and specificity of 81.8% and 100% respectively. Eukaryotic initiation factor 2B delta subunit was additionally identified as being highly upregulated in cancer patients. The numbers in the test set were very small, but, nevertheless, the results are promising and demonstrate the potential of the technique when conducted in a robust manner.[28] The ultimate question of such profiling methods, however, is their robustness and the applicability of such analysis and clas sification processes in a routine clinical chemistry laboratory, which is unlikely in their current formats.

Urine is an attractive source of biomarkers for tumors of the urinary tract with the potential for direct shedding of proteins into it and a less complex matrix. Approximately 70% of the urinary proteome is thought to consist of kidney derived proteins.[32] The nuclear protein matrix protein 22 (NMP22) forms the basis of the NMP22 Bladder Check ® Test, in the form of a point of care kit, to aid in the diagnosis of bladder cancer in at risk patients. A small number of studies have also examined NMP22 urine levels in patients with RCC, demonstrating that levels are significantly increased in patients with RCC compared to those with benign kidney disease or healthy controls.[33,34] However, these studies were conducted some years

ago and were small (largest $n = 65$) and no further studies have been reported since.

3.1.2. Histopathological diagnosis

The differential diagnosis of the various subtypes of RCC is important as they vary considerably in their prognosis and treatment. Papillary carci nomas have a less aggressive course than conventional clear cell tumors but respond less well to sunitinib. Two categories are recognized, Type 1 and Type 2, with the latter group of tumors displaying higher nuclear grade, eosinophilic cytoplasm and a worse outcome than Type 1 tumors. Chro mophobe RCCs are thought to arise from the intercalated cell of the col lecting duct and also carry a better prognosis than conventional RCC. Many renal epithelial neoplasms can be diagnosed reliably by experienced pathologists on the basis of morphology alone on routine hematoxylin and eosin stained slides. However, eosinophilic renal tumors, characterized by their high content of mitochondria, span the full spectrum between benign (oncocytoma) and malignant (conventional or chromophobe RCC vari ants) tumors and provide even experienced pathologists with a challenge. Additionally, markers would be useful for diagnosis of metastases in those cases when the primary site is unknown and would be of use in core biopsies where the range of tissue architecture viewed is more limited.

Several markers are being evaluated (for a review see Skinnider and Amin[35]) but one of the difficulties is that the comparison is made against the 'gold standard' of morphology alone and ideally studies should also incor porate genetic classification and long term outcome into the diagnostic classification against which to compare the potential markers. Such markers include CD10, a cell surface metalloproteinase localized to the proximal nephron of normal kidney, RCC antigen (RCCma) developed as a monoclonal antibody to gp200 glycoprotein expressed on normal human kidney proximal tubule, kidney specific cadherin, an adhesion molecule expressed by distal tubular nephron cells, the intermediate filament protein vimentin, the KIT tyrosine kinase, CK7 and the proto oncogene product Ron. Promising results have been found, for example KIT and K cadherin are absent in many papillary and conventional RCC samples and positive in the majority of chromophobe and oncocytomas,[36,37] but many studies are small and none of the markers are absolutely diagnostic for any specific RCC subtype. However, as mentioned above, the comparator is against morphology alone, which is a limitation. Based on the available evidence,

it is likely that, at present, a panel of markers may be most informative in any classification.

The speckle type POZ protein (SPOP) has been recently reported as a highly promising marker of RCC, originally identified through gene expression analysis in *Drosophila*. Of 20 tumor types tested, RCC was one of only three that showed positive staining for SPOP. Following construction of a tissue microarray (TMA) composed of almost 500 RCC and normal kidney sections, 77% of tumors stained positively for SPOP using IHC, with universal negative staining in normal tissue. On subtype analysis, 99% of the clear cell RCCs and 86% of the chromophobe RCCs showed positive staining for SPOP, but only 22% of papillary type RCCs and 6% of oncocytomas, suggesting that the marker may be useful in differentiating RCC subtypes. Furthermore, amongst tissue sections ($n = 87$) from RCC metastatic lesions, 97% stained for the protein, indicating that SPOP may also have a role in identifying RCC as the site of the primary tumors in cases of metastases from unknown origin.[38]

Whilst the focus of this chapter is on protein based biomarkers, the VHL gene is of such importance in RCC that it would be difficult not to mention it briefly at this point. Rates of involvement of the gene in sporadic RCC are now approaching 100%, suggesting that VHL loss is a pre requisite for tumor development.[39] Work from our group has recently examined VHL status in 177 RCCs. Loss of heterozygosity (LOH) was demonstrated in 89.2%, mutation in 74.6% and methylation in 31.3% of evaluable tumors; evidence of biallelic inactivation (LOH and mutation or methylation alone) was present in 86.0% whilst no definite involvement of VHL was found in only 3.4% of samples.[40] Analysis of the tumors in this way is not readily achievable routinely however, but if protein markers can be developed reflecting these findings they may be useful in supporting a diagnosis of conventional clear cell RCC in suspected cases.

3.2. Prognostic markers

Many molecules have been proposed as potential prognostic markers of RCC. Few, however, have been taken beyond single studies, often involving relatively small numbers of patient samples, and, as such, none are yet in routine clinical use. The majority of such markers described to date are tissue based, reflecting both the ready availability of tissue and the challenges of working with serum and urine. A summary of RCC protein markers published within the past 3 years is presented in Table 8.2. In the following section, details of some of the most promising prognostic markers are presented.

Table 8.2 Prognostic protein biomarkers of RCC published in the past 3 years

Protein	Reference	Year	Number of patients	Material	Subtype	Technique	Result
B7 H3	44	2008	743	Tissue	Clear cell	IHC	Independently predictive of CSS (HR1.38; $P = 0.029$)
sB7x (member of B7 CD28 family)	101	2008	101 RCC101 controls	Serum	Clear cell	ELISA	Serum levels significantly more likely to be detectable in patients. Levels associated with tumor stage, lymph node status and metastatic disease
CAIX	76	2008	91 RCC32 controls	Serum	Clear cell	ELISA	Mean levels > in metastatic RCC > localized > healthy controls. AUC ROC 0.776. Higher preoperative levels associated with higher recurrence rate
CAIX and VEGF	102	2008	122	Tissue	Clear cell	IHC	Low CAIX and high VEGF expression associated with worse outcome. CAIX independently. Co expression also independent for CSS ($P = 0.0002$)
Cathepsin D	78	2009	149 RCC90 controls	Urine	Clear cell	ELISA	Preoperative urine levels significantly associated with OS ($P = 0.005$) and CSS ($P = 0.013$) on univariate analysis

(*Continued*)

Table 8.2 Prognostic protein biomarkers of RCC published in the past 3 years—cont'd

Protein	Reference	Year	Number of patients	Material	Subtype	Technique	Result
Claudin 1	103	2008	318	Tissue	87% clear cell 9% papillary	IHC	Preferentially expressed in papillary RCC. Associated with CSS on multivariate analysis when considering localized clear cell RCC ($P = 0.03$)
CD24	104	2008	328	Tissue	Clear cell	IHC	High expression associated with higher grade and larger tumor size. Weak independent association ($P = 0.043$) with PFS
CXCR3	105	2008	154	Tissue	Clear cell	IHC	Low expression associated with worse outcome. Independent predictor of disease free survival
Cytokeratin 7 and 19	106	2008	209	Tissue	Clear cell	IHC	Expression of either marker associated with better CSS. Only CK19 independently prognostic. Also associated with genomic stability
HSP70	107	2007	145	Tissue	Clear cell	IHC	Decreased expression in tumor and with increasing grade but no association with survival

Insulin like growth factor II mRNA binding protein 3 (IMP3)	46	2008	716	Tissue	Clear cell	IHC	Strong, independent predictor of metastases and death in localized disease (HR 4.7; $P < 0.001$). Independent validation confirming previous data
Ki 67	58	2007	741	Tissue	Clear cell	IHC	Expression associated with worse outcome, independent of tumor necrosis and SSIGN score
MMP 7	81	2008	97 RCC50 controls	Plasma	71% clear cell	ELISA	Plasma levels independently prognostic for CSS ($P = 0.003$)
MMP 10	108	2007	103	Tissue	Clear cell	IHC	Expression significantly correlated with pT stage and grade. Not independently prognostic
Na,K adenosine triphosphatase alpha$_1$ subunit	109	2007	317	Tissue	Clear cell	IHC	Alpha$_1$ subunit (not beta) significant and independent predictor of CSS
Osteopontin	110	2007	80 RCC52 controls	Plasma	69% clear cell	ELISA	Median levels raised in patients with mRCC compared to localized disease and controls. AUC ROC 0.888. Independent predictor of survival

(*Continued*)

Table 8.2 Prognostic protein biomarkers of RCC published in the past 3 years—cont'd

Protein	Reference	Year	Number of patients	Material	Subtype	Technique	Result
p21	111	2007	396	Tissue	86% clear cell	IHC	Higher levels of nuclear expression associated with better prognosis in clear cell RCC; in patients with metastatic disease at diagnosis higher levels of nuclear and cytosolic p21 associated with *worse* survival
pI3 kinase	112	2008	176	Tissue	Clear cell	IHC	Activation of PI3K protein independently associated with worse outcome ($P = 0.03$). Decreased survival correlated with low PTEN and high p Akt
Tenascin C	113	2008	137	Tissue	Clear cell	IHC	Expression associated with stage, grade and CSS ($P = 0.0017$) on univariate analysis. Not independently prognostic
Thrombospondin 1	114	2009	160	Tissue	Clear cell	IHC	Expression inversely associated with grade and stage. Independent prognostic factor for CSS

CSS, cancer-specific survival; IHC, immunohistochemistry; ELISA, enzyme-linked immunosorbent assay; OS, overall survival; PFS, progression-free survival; RCC, renal cell carcinoma.

3.2.1. Tissue-based markers

B7-H family

The B7 family of T cell co stimulatory and co inhibitory molecules form some of the most promising prognostic RCC biomarkers to date. Each member has different, although overlapping, functions in controlling the priming, proliferation and maturation of T cells, which can lead to immune suppression and evasion of host immune surveillance. A correla tion between RCC patient survival and expression of three members of this family has been described to date.

B7 H1 is a cell surface glycoprotein expressed on most human tumors including RCC and implicated as a potent negative regulator of tumor immunity. Using fresh frozen tissue sections from 196 RCC patients, expression of B7 H1 was examined, demonstrating that patients with positive tumors were at significantly increased risk of cancer specific mortality.[41] Extension of the study to paraffin embedded sections from 306 patients, with a median of 10 years follow up, confirmed that the 24% of patients positive for B7 H1 were at significantly increased risk of death (hazard ratio (HR) 2.37; $P < 0.001$), with 5 year cancer specific survival (CSS) rates of 42% vs 83%, in those with and without B7 H1 expression respectively. Furthermore, it was found to be an independent predictor of mortality on multivariate analysis.[42] B7 H4 has also been implicated as a negative regulator of T cell mediated immunity. Positivity for B7 H4 was correlated with cancer specific survival (HR 3.05; $P = 0.002$) in a study of 259 RCC patients. Patients positive for both B7 H1 and B7 H4 were more than four times more likely to die from RCC compared to negative or singly positive tumors (HR 4.49; $P < 0.001$).[43] B7 H3 has also been shown to have a relatively weak association with CSS, with a HR of 1.38 ($P = 0.029$) in a study involving 743 patients.[44] It is apparent therefore that B7 H1 and B7 H4 serve, by as yet incompletely understood mechanisms, in protecting tumor cells from immune destruction. As well as forming strong candidate prognostic markers, a greater understanding of their functioning may also generate a novel approach to anti cancer therapy.

IMP3

Insulin like growth factor II (IGF II) mRNA binding protein 3 (IMP3) is an oncofetal RNA binding protein, not normally expressed in adult tissues. It is thought to function in the regulation of IGF II production. Aberrant expression of IMP3 in renal cancer correlates with survival in patients with localized RCC. In an initial study examining IMP3 expression in

371 primary tumors, 5 year survival was significantly longer (82% vs 27%; $P < 0.0001$) in patients whose tumors did not express the protein compared to those that did. The result was highly statistically significant and IMP3 expression was shown to be a strong, independent predictor of survival on multivariate analysis.[45] These results have since been externally validated in a further cohort of 629 patients with localized renal cancer. A quarter of patients' tumors expressed IMP3 and, again, this was associated with a significantly increased risk of both development of distant metastases and death from RCC. The results were most striking for patients with stage I disease, with positive IMP3 expression associated with a sixfold increased risk of progression to distant metastases (HR 6.46: 95% CI, 3.33–12.53; $P < 0.001$).[46] A prognostic model based on quantitative IMP3 and tumor stage (QITS) has since been proposed.[47] The model, based on a relatively small number of patients ($n = 369$) with localized RCC, combined IMP3 expression (using a computerized image analyzer) and TNM stage in rela tion to outcome. The model stratifies patients into four groups: patients in QITS group IV ($n = 33$), defined as high level positivity for IMP3 and any TNM stage or low level positivity and TNM stages 2 or 3, had 5 and 10 year overall survival rates of 14% and 4%. The power of the model appears to be in identifying a very high risk population who would not otherwise be recognized using TNM stage alone. Whether the addition of IMP3 expression to existing nomograms is beneficial remains to be determined.

In one of few studies to evaluate expression of prognostic markers specifically in chromophobe and papillary RCCs, IMP3 was also found to be an independent prognostic marker in this subset of tumors. The study was large ($n = 334$) considering the rarity of these tumors and suggests that IMP3 positivity is associated with a greater than 10 fold risk of developing distant metastases (HR 13.45; 95% CI, 6.00–133.14; $P < 0.001$).[48]

CAIX

The most studied molecular marker of RCC to date is carbonic anhydrase IX (CAIX). Its expression was originally described in 1986, using a monoclonal antibody (G250), which was noted to bind to RCC but not normal proximal epithelium.[49] It was later shown that the target antigen of G250 was CAIX.[50] CAIX is a metalloenzyme that contains carbonic anhydrase activity and is capable of catalyzing the reversible hydration of CO_2 to form HCO_3 and H^+. Unlike other members of its family, CAIX is a multidomain transmembrane protein composed of: (1) a small intra cytoplasmic tail; (2) a short transmembrane segment; (3) an extracellular

catalytic domain; (4) a proteoglycan like domain, involved in cell adhesion; and (5) a signal peptide. Regulated by HIF 1 α, it is thought to play a role in the adaptation of tumors to hypoxic conditions, by regulating intra and extracellular pH.

The ectopic expression of CAIX in many human neoplasms (including RCC) but not in corresponding normal tissues has been demonstrated by several groups.[51] In renal cancer, immunohistochemical studies have shown that 94–100% of conventional RCC tumors express CAIX at the plasma membrane, with uniformly negative staining in normal kidney tissue.[51,52] Overexpression is associated with tumor aggressiveness and poor outcome across many cancer types, except for RCC, where the opposite appears to be true.

The first large study examined CAIX expression in relation to outcome using a TMA of 321 RCC specimens. A staining percentage of $> 85\%$ positive cells was used to stratify patients as high ($n = 255$ (79%)) and low ($n = 66$ (21%)) expressers. Amongst the 149 patients with metastatic disease, low CAIX expression was associated with a significantly worse CSS (median 5.5 months vs 24.8 months; HR 3.10; $P < 0.001$) and was independently prognostic on multivariate analysis, considering T stage, grade, nodal status and ECOG performance status. Amongst patients with high risk localized disease (T stage ≥ 3; grade ≥ 2), low CAIX expression was also associated with poorer outcome but was not independently prognostic.[52] The same authors replicated these results in a study of 224 patients, again using a TMA. Low CAIX expression was again associated with a significantly worse CSS (22 months vs 67 months) when considering all patients, and was independently prognostic (HR 1.87; $P = 0.006$).[53] More recently, however, a large study of 730 tumor specimens employing the same antibody (M75) but using whole tissue sections found that although on univariate analysis an association with low CAIX expression (again defined as $\leq 85\%$) and poor outcome was demonstrated, this was lost on adjustment for even just one other variable, namely nuclear grade.[54] The reasons for the discrepancy are unclear, but may relate to differences in patient population and the use of TMAs, since expression within larger sections was found to be variable across the tissue.

Whether CAIX is independently prognostic or not, it is clear that a high CAIX expression is associated with a better outcome in RCC. The reasons for this are uncertain. Patients with tumors expressing low CAIX and absence of VHL mutation have been shown to have a significantly worse outcome compared to those with high expression and VHL mutation

($P = 0.0002$). Since VHL mutation is a hallmark of RCC (and is implicated in the regulation of CAIX expression), it is postulated that this underlying VHL inactivation, rather than the functional consequence of intra tumoral hypoxia, leads to high CAIX expression and improved survival in patients with RCC.[55]

Finally, the low level of expression of CAIX in normal tissues makes it an attractive target for imaging and therapy. The chimeric monoclonal antibody WX G250 (Rencarex), targeted to CAIX, has reached phase III trials. Essentially the antibody binds to RCC cells and targets them for destruction by antibody dependent cell mediated cytotoxicity. Supported by promising phase II data, the ARISER trial has recently completed recruitment of more than 850 patients, randomizing high risk post nephrectomy patients to WX G250 or placebo in the adjuvant setting with an interim analysis expected soon. A radiolabeled version of this antibody is also being used in imaging studies.

Ki-67

Ki 67 is a nuclear protein that serves as a marker of proliferation. Its expression has been correlated with survival in many cancers. In RCC, several studies have been published demonstrating this association.[56,57] The two largest studies to date have both reported strong independent prognostic ability. The first examined 224 tumors, reporting high Ki 67 expression correlating with increasing tumor stage and grade, with a risk ratio of 2.10 ($P < 0.001$) on multivariate analysis for CSS.[53] Subsequently, in a study of 741 tumors, Ki 67 was independently prognostic for CSS, with a HR of 3.43 (95% CI 2.64−4.45; $P < 0.001$). The marker retained independent prognostic ability even when controlling for tumor necrosis, which had previously been proposed by others as a surrogate marker for Ki 67 expression.[58]

Survivin

Survivin is an antiapoptotic protein that belongs to the inhibitor of apoptosis protein (IAP) family. It is over expressed in almost all human malignancies. In RCC, 31% of patients were found to have high survivin expression in a study of 312 patients. On multivariate analysis, expression of the marker remained significantly associated with CSS (HR 2.4; 95% CI 1.5−3.8; $P < 0.001$).[59] Similar results were reported in a smaller study of 85 patients.[60] Interestingly, the relationship between survivin and B7 H1 expression has been examined in a study of 298 patients with RCC. Both markers were

confirmed to be independently prognostic for CSS. One hundred and seventy seven (59.4%) tumors were classed as low or negative and 41 (13.8%) as positive or high for expression of both markers. Five year CSS rates were 89.3% and 16.2% in these two groups of patients respectively.[61]

Markers in combination

Ultimately, any single protein marker is unlikely to successfully determine prognosis across all patients. Instead, combining markers to create a molecular signature of disease is the most promising way forward. Incorporation of such markers into existing prognostic models, which are currently based on standard clinical criteria, is now starting to be explored with promising results.

In a recent large study of 634 patients with RCC, tumors were examined for expression of B7 H1, survivin and Ki 67 using whole tissue sections. Each marker was first confirmed to have independent prognostic ability for CSS, even after adjustment for the other two proteins. The panel was then used to create a prognostic algorithm, termed BioScore, based on high or low expression of each protein within each tumor. Patients with a high score were observed to be five times more likely to die from their cancer compared to those with a low score (HR 5.03; 95% CI 3.82–6.61). Most importantly, the BioScore was shown to add prognostic value to three established models, including the SSIGN score, based on tumor stage, size, grade and necrosis.[16] BioScore added little to patients deemed at low risk by the model, but was able to further stratify those deemed at moderate and high risk.[62]

In metastatic RCC, a panel of tissue markers has been successfully combined with clinical features to improve standard scoring systems. Using results from 150 patients, a model, consisting of expression of CAIX, PTEN, vimentin and p53 as well as T stage and performance status, again outperformed the UISS staging system (accuracy 0.68 vs 0.62; $P = 0.0033$).[63] A later study focusing on localized disease and analyzing a panel of eight proteins in a TMA based on 170 patients found that five, namely Ki 67, p53, endothelial VEGFR 1, epithelial VEGFR 1 and epithelial VEGF D, were independent prognostic indicators of disease free survival (DFS). The five markers alone predicted DFS with an accuracy of 0.838 (95% CI 0.813–0.863). This was more accurate than an existing nomo gram, the UCLA Integrated Staging System (UISS), which performed with an accuracy of 0.780 (95% CI 0.776–0.784). The authors then constructed a nomogram incorporating the five markers, as well as performance status

and T stage which predicted DFS with an accuracy of 0.904 (95% CI 0.875−0.932).[64]

3.2.2. Circulating markers

Circulating markers that can be measured objectively in patients' serum or urine are highly desirable. They can be measured relatively non invasively and may provide prognostic information early, prior to nephrectomy. Few such examples in RCC currently exist however, and are generally limited to single studies. As for diagnostic markers, this is likely to reflect challenges in serum and urine marker discovery,[65] a sparsity of robust clinical samples with associated clinical data for subsequent validation and the requirement for appropriately optimized protein assays.

Routine hematological and biochemical parameters

A number of hematological and biochemical parameters, that are often routinely measured in patients, have demonstrated an association with outcome in patients presenting with RCC. Such parameters make attractive markers, since they are easily and cheaply measured, as well as being widely available. Serum LDH, calcium and hemoglobin are currently used to determine prognosis in patients with metastatic disease as part of the MSKCC nomogram.[17]

Several studies have demonstrated that thrombocytosis is a poor prog nostic feature in patients with RCC. Platelet counts were examined in a mixed population of 804 RCC patients prior to nephrectomy, with 126 (15.7%) patients having metastatic disease. A high platelet count (defined as $> 450{,}000/mm^3$) was found to be independently prognostic for poor outcome, in a model containing stage, grade and performance status, with 5 year survival of 70% vs 38% in patients with a low and high platelet count respectively.[66] In another large study of 700 patients with metastatic RCC, baseline platelet count ($>$ or $< 400{,}000/mm^3$) was again reported to be of independent prognostic value (HR 1.65; 95% CI 1.36−1.99; $P < 0.001$). A quarter of patients were deemed to have thrombocytosis, with a median survival of 8.4 months in this group compared to 14.6 months ($P < 0.001$).[67] In the most recent and comprehensive study to date, the associ ation of baseline platelet count with CSS was determined in 1828 patients with RCC prior to nephrectomy. The population was again heterogeneous, derived from France and the United States, with 508 (27.8%) patients presenting with metastatic disease. The analysis was conducted considering platelet count as a continuous variable, dichotomized around $450{,}000/mm^3$

and using most informative cutoffs. Only 9.4% of patients had a platelet count above 450,000/mm^3. On multivariate analysis, using most informative cutoffs, platelet count achieved independent predictor status. However, adding platelet count to a model composed of age, tumor size, TNM stage, PS, grade and histological subtype, increased its accuracy by only 0.3% (from 85.3% to 85.6%). No extra benefit was observed when considering only patients with localized or metastatic disease. Thus the authors argue that whilst thrombocytosis is a poor prognostic feature in patients with RCC, it does not in fact add any information above and beyond other, more standard, prognostic criteria.[68]

The underlying biology behind thrombocytosis and RCC survival is uncertain. It is, however, not unique to RCC and applies equally to patients with other tumor types. It is likely therefore to reflect a non specific, tumor related, inflammatory response although interleukin 6 (IL 6) production by RCC cells may contribute. C reactive protein (CRP), a well described acute phase protein, is often raised in patients with cancer and circulating levels have been correlated with outcome. In RCC, raised CRP levels have been correlated with poor survival in a number of studies, in both the early and advanced settings. It has been shown to be independently prognostic and may be of additional value to currently used nomograms.[69] In a study of 313 patients undergoing nephrectomy for RCC, 66 (21%) of whom had metastatic disease, CRP was independently prognostic for CSS ($P = 0.003$). CRP was treated as three categorical variables, identified using most informative cutoffs, namely ≤ 4.0 mg/L, 4.1−23.0 mg/L and > 23.0 mg/L. Importantly, the addition of CRP to a pre existing prognostic model (UISS) improved its accuracy by 3.8% at 5 years ($P < 0.001$).[70] A simple scoring system based on CRP and TNM alone, termed TNM C score, has recently been described based on 249 RCC patients with advanced and localized disease.[71] The investigators dichotomized CRP as $<$ or ≥ 0.5 mg/dL and combined this with TNM to generate four risk groups. The model was externally validated in 290 patients, with a concordance index of 0.865. The study has a number of limitations however, including the small numbers of patients in the higher risk groups and the small number of events.

Such studies have measured CRP prior to nephrectomy but then tested the marker against postoperative nomograms. It is perhaps, therefore, of more relevance to determine whether CRP adds to recently published pre operative models.[18] Data from our own group, based on 286 RCC patients

(84% clear cell), have demonstrated CRP (dichotomized as $\leq$ or $>$ 15 mg/L) to correspond to 5 year survival rates of 72% (95% CI 65−78%) and 33% (95% CI 23−44%) respectively and to be a strong, independent prognostic factor for OS ($P < 0.006$) and CSS ($P < 0.001$) on multivariate analysis, when considering factors incorporated in either pre or postoperative nomograms (Jagdev et al, personal communication). Conventionally, rising CRP levels have been attributed to a liver derived response to circulating cytokines such as IL 6 and IL 10. Interestingly, however, CRP has also been shown to be produced by tumor cells themselves.[72]

Compelling evidence exists demonstrating increased neutrophil count as an independent poor prognostic factor for patients with metastatic RCC. Elevated peripheral blood neutrophils appear to predict a poor outcome following treatment with cytokines or VEGF targeted therapy (see Section 3.2.3 below). In addition, we have recently described serum neutrophil/lymphocyte ratio as a strong independent predictive factor of survival in RCC patients with localized and metastatic disease (Jagdev et al, manuscript submitted). Intra tumoral neutrophils also correlate with poor outcome in patients with metastatic and localized disease. In a recent study of 121 patients with localized RCC, presence of intra tumoral neutrophils was independently prognostic for both recurrence free (HR 3.0; 95% CI 1.7−5.4; $P < 0.0001$) and overall (HR 3.1; 95% CI 1.9−5.0; $P < 0.0001$) survival. The presence or absence of neutrophils was able to successfully further stratify patients identified as low or intermediate risk using the Leibovich score, with a fourfold higher risk of recurrence if present. The concordance index improved from 0.74 to 0.80 with the addition of intra tumoral neutrophils to the Leibovich model.[73]

Other biochemical parameters with evidence of prognostic value include serum alkaline phosphatase, erythrocyte sedimentation rate (ESR) and serum sodium. This latter marker, reported by our own group, was identified from a large number of biochemical and hematological variables examined in 212 patients with RCC. Preoperative serum sodium was found to be independently prognostic for DFS and OS when considered as both a continuous variable and when dichotomized to above and below the median value (139 mmol/L) (HR = 0.44; 95% CI 0.22,0.88; $P = 0.014$). The majority (92%) of patients had serum sodium values within the labo ratory reference range, but patients with values above the median value had significantly increased survival compared to those patients with levels equal to or below the median.[74] The explanation for these findings is uncertain and this is a single study that requires further validation.

Carbonic anhydrase IX (CAIX)

CAIX is known to be shed from the cell surface, therefore making it a potentially attractive circulating biomarker. Its presence has been detected in conditioned medium from RCC cell lines and primary cultures, and in the serum/urine of RCC patients. Two studies to date have examined serum levels in patients with RCC. Using an enzyme linked immunosorbent assay (ELISA), levels were initially measured in a small number ($n = 30$) of patients with localized RCC. CAIX was barely detectable in healthy controls although levels in RCC patients varied widely (20 pg to 3.6 ng/mL in serum) and showed no correlation with tumor size.[75] More recently, in a larger study of 91 patients with RCC, mean serum CAIX levels were reported to be significantly higher in patients with metastatic versus localized disease ($P = 0.004$) and versus healthy controls ($P = 0.001$). Higher serum levels were correlated with tumor grade, size and stage and were associated with a higher rate of relapse in patients with localized disease.[76] Clearly numbers are small but further confirmation is warranted.

Cathepsin D

Cathepsin D is a lysosomal protease and is one of only two urinary markers in RCC described to date. Increased tissue expression of cathepsin D has been correlated with poorer outcome in a number of other cancers, except RCC, where the opposite relationship has been reported.[77] In a recent study, conducted by our group, renal cancer cell lines were used to first identify cathepsin D as a candidate biomarker, followed by validation in 239 RCC preoperative urines. High urinary cathepsin D was found to be significantly associated with poorer overall survival (HR 1.33; 95% CI 1.09−1.63; $P = 0.005$) on univariate analysis and approached significance on multivariate analysis using preoperative variables ($P = 0.056$).[78]

Serum amyloid A (SAA)

SAA, as described earlier, has been suggested as a possible diagnostic marker for RCC although specificity is very likely to preclude this. Recently, Ramankulov and colleagues examined SAA levels in 98 RCC patients using an ELISA based method. SAA levels were no different between patients with early stage disease and healthy controls, although they were significantly elevated in patients with metastatic disease. This is not specific to RCC however, and applies equally to patients with other advanced cancer types. Of interest, the authors also went on to demonstrate independent prognostic values for the marker in terms of CSS (HR 2.51; 95% CI

1.09–5.78; $P = 0.030$).[79] We have employed SELDI profiling of sera from 69 healthy controls and 119 RCC patients, to identify six peaks significantly associated with CSS. One peak, at 1528 Da, was found to represent the C terminal 13 amino acids of SAA. Levels of the peptide were significantly negatively correlated with total SAA and total SAA was also of independent prognostic significance (Wood et al, personal communication).

Matrix metalloproteinase-7 (MMP-7)

MMP 7 is one member of a family of zinc containing enzymes capable of degrading various components of the extracellular matrix. At a tissue level, MMP 7 has been shown to be over expressed in RCC, correlating with higher tumor stage and grade and independently predict for poor CSS (HR 8.61; 95% CI 1.10–67.28; $P = 0.04$).[80] Plasma levels in 97 RCC patients were subsequently evaluated using a commercial assay, capable of measuring both pro and mature forms of the protein. Levels were significantly elevated in patients with metastatic (but not localized) disease, although they did not correlate with burden or site of metastases. On multivariate analysis, levels were independently prognostic for CSS (HR 2.70; 95% CI 1.39–5.24; $P = 0.003$).[81]

Immunosuppressive acid protein (IAP)

IAP was first described as a potential serum prognostic marker in RCC almost two decades ago. Since then there have been a number of studies supporting its role in both staging disease and determining prognosis in metastatic patients. In a study of 44 patients with recurrent metastatic RCC, 3 year survival was 42% and 0% in patients below and above 800 μg/mL respectively. Similar findings were reported amongst the 40 patients pre senting with metastatic disease at diagnosis.[82] More recently, IAP doubling time in patients with recurrent RCC has been shown to be independently prognostic for survival ($P = 0.0026$). Levels were measured longitudinally from before detection of recurrent disease ($n = 78$). Three year survival rate in patients with a doubling time of greater or less than 200 days was 58.9% and 12.5% respectively.[83]

3.2.3. Predictive markers

The goal of tailoring treatment to individual patients has long been held. The ability to predict prior to (or soon after) starting treatment, the subgroup of patients destined to respond carries obvious benefits. Non responders can be considered for alternative, potentially more effective,

therapies, avoiding unnecessary toxicity. Treatments that are often very expensive can be reserved for responders, which carries important economic implications. Indeed, it is likely that in future, novel anticancer agents will require their so called 'companion diagnostic' to have been developed in parallel, prior to their approval. Care must be taken when interpreting biomarker studies examining survival in patients on therapy however, as some markers which may appear to be predictive may actually also be prognostic and their apparent success related to that.

With the introduction of several efficacious yet expensive biological therapies, identifying predictive biomarkers in renal cancer treatment has never been so relevant. The treating oncologist now has a number of therapeutic options for patients with advanced disease and, therefore, defining the optimal sequence of drugs for individual patients is important. Whilst the newer agents have largely superseded previously used therapies such as IFN α and IL 2, these drugs still occasionally have their place. Some patients are not suitable for or have progressed on TKI based therapy, other biological agents may not be accessible within the available health system, and IL 2 is still the only drug that has shown long term complete responses. Therefore biomarkers that have been implicated in predicting response to these cytokines are also discussed below.

Interferon-alpha and interleukin-2

IFN α, given subcutaneously three times a week, represented the standard of care in advanced renal cancer for over two decades. Associated with significant toxicity, objective response rates are in the order of just 15%, and are usually short lived. IL 2, when administered at high dose intravenously, can induce complete responses in small numbers of patients that are durable for over 10 years. However, such treatment is associated with significant morbidity and even patient death.

Thus, both these treatments are associated with responses in a small percentage of patients, meaning that the majority suffer toxicity with no benefit. Clinical features that have been correlated with a better response to cytokines include prior nephrectomy, performance status, sites of metastatic disease and interval from presentation to the development of metastases. A prognostic model for determining response and survival in patients with metastatic RCC treated with low dose IL 2 following nephrectomy has been described. The nomogram, termed 'survival after nephrectomy and immunotherapy' (SANI), provides a score based on lymph node status, constitutional symptoms, location of metastases, histology (sarcomatoid

features or not) and thyroid stimulating hormone levels. Response rates in the low , medium and high risk groups defined using the SANI score were 43%, 27% and 15%. The model also determined survival and is thus prognostic as well as predictive.[84] Four independent factors predictive of rapid progression on cytokine therapy have been described based on 782 patients with metastatic RCC. Patients with at least three of: hepatic metastases, elevated neutrophil count ($> 7.5 \times 10^3$/L), < 1 year from renal tumor to metastases and two or more metastatic organ sites had a $> 80\%$ probability of progressing rapidly (within 3 months) on cytokine treat ment.[85] Others have since confirmed the predictive value of neutrophil counts in determining response to cytokines. Amongst 495 metastatic RCC patients, those with neutrophil count $< 6.5 \times 10^3$/L had a response rate of 30% and median survival of 22 months. Amongst the 78 (16%) with neutrophil count $> 6.5 \times 10^3$/L, response rate was 18% with a median survival of 9 months ($P \leq 0.001$).[86]

Pathological criteria predicting response have been best defined by Upton and colleagues in a study examining 231 RCC patient tumors treated with IL 2. Clear cell RCC patients with more than 50% alveolar features and no granular or papillary features had a 39% response rate (good risk) compared to just 3% amongst patients with $> 50\%$ granular or any papillary features (poor risk). Response rates were equally poor in patients with non clear cell histology.[87]

CAIX expression has been correlated with response to IL 2. Amongst 86 patients treated in one study, all complete responses (8%) were observed in the high CAIX staining group and the overall response rate was 27% vs 14% in the high and low expressers respectively.[52] A model based on CAIX expression and pathological criteria (as defined by Upton and colleagues) was subsequently described on a relatively small number of patients ($n = 66$) with metastatic RCC. Patients with good risk pathology alone, or intermediate risk pathology and high CAIX expression, were defined as good risk, whereas the poor risk group contained patients with poor risk pathological features alone or inter mediate risk pathology and low CAIX expression. Amongst the clear cell tumors, all 26 responders were in the good risk group.[88] The model requires independent validation and The Cytokine Working Group has launched the SELECT trial to determine prospectively whether a group of patients can be identified using the model that has a higher response rate to high dose IL 2 in comparison to a historical, unselected patient population.

Few studies have examined the expression of molecular markers as predictors of response to cytokines. In a recent study of 40 patients with metastatic RCC, expression levels of 10 markers was examined and correlated with response to combination IFN α and IL 2. Expression of both Ki 67 and Bcl 2 were significantly associated with response, such that weak expressers were more likely to respond ($P = 0.013$ and $P = 0.022$ respectively).[89]

Sunitinib, sorafenib and bevacizumab

There is currently much interest in identifying biomarkers that predict response to the anti angiogenic drugs sunitinib, sorafenib and bevacizumab. Despite the fact that these agents function through a well described and common mechanism targeting the VEGF receptor, the identification of a surrogate marker of activity has, to date, proven elusive.

Clinical parameters to define outcome have focused on prognosis rather than on prediction of response and, as stated above, care must be taken in differentiating these two categories of marker. For example, from data based on the registration trial of sunitinib, patients could be stratified for overall survival based on the MSKCC nomogram. However, all patients, regardless of risk group, benefited from sunitinib equally.[6] The applicability of the nomogram in determining overall survival has been confirmed in a retro spective study of 645 mRCC patients treated with sunitinib, sorafenib or bevacizumab. The study did not, however, examine response.[90] Two studies have, however, examined PFS in such patients, which may be of more relevance in selecting patients destined to benefit. The first study examined 120 patients with metastatic RCC treated with anti VEGF therapy. Five factors were identified as independently associated with poor outcome that were included in a prognostic model: PS ($\geq$ 1 vs 0), time from diagnosis to current treatment ($<$ 2 years vs $\geq$ 2 years), abnormal baseline corrected serum calcium ($<$ 8.5 mg/dL or $>$ 10 mg/dL vs 8.5–10 mg/dL), high platelet count ($>$ 300,000 mm^3), high absolute neutrophil count ($>$ 4.5 K/μL vs $\leq$ 4.5 K/μL). Three prognostic groups were identified using the model. Patients in the favorable risk group had a median PFS of 20.1 months (95% CI 19–22.3 months), intermediate risk group 13 months (95% CI 8.6–17.6 months) and those in the poor risk group had a median PFS of just 3.9 months (95% CI 1.8–7.2 months). However, patients received any one of four different therapies (84% sunitinib or sorafenib) making the results difficult to interpret.[91] In the second study, examining 375 patients treated within a phase III trial of sunitinib,[5] a nomogram based on a large

number of variables that included corrected calcium, number of metastatic sites, hemoglobin, prior nephrectomy, lung metastases, liver metastases, PS, thrombocytosis, time from diagnosis to treatment, ALP and LDH, was constructed to predict probability of 12 month PFS. The concordance index for the model was 0.633. Patients with higher scores were predicted to have just a 10% or less chance of remaining progression free after 12 months of treatment.[92] Whether such models apply specifically to patients on TKIs or, in fact, simply reflect poor tumor biology destined to progress quickly regardless of treatment, remains uncertain.

HER2 exemplifies moving from a tissue biomarker to a soluble form present in clinical fluids and serum HER2 levels may be of value in breast cancer patients. A limited number of analogous candidate circulating biomarkers have been examined to date in RCC. VEGF and the soluble form of the VEGF receptors have been most studied to date. The TARGET study compared sorafenib with placebo in the second line setting and examined baseline serum levels of VEGF in 712 patients with RCC. Higher baseline VEGF levels were associated with a worse OS and were independently prognostic amongst both placebo ($P = 0.04$) and sorafenib treated ($P = 0.02$) patients. Patients were then stratified by median VEGF level (131 pg/mL), demonstrating that both groups in fact benefited equally to sorafenib, in terms of PFS. However, in an exploratory analysis using the 25th and 75th percentiles, patients in the high VEGF group demonstrated a trend towards higher response.[93]

Changes in the levels of VEGF and its receptors in response to therapy have also been examined. In a phase II study of sorafenib, involving 63 patients, serum VEGF and placenta growth factor (PlGF) levels increased relative to baseline, whilst soluble VEGFR 2 (sVEGFR 2) and VEGFR 3 (sVEGFR 3) concentrations decreased in response to treatment. Levels of each marker tended to return to near baseline levels after 2 weeks off treatment, suggesting a drug dependent effect. Baseline levels did not predict for response, although the magnitude of change in levels of VEGF, sVEGFR 2 and sVEGFR 3 was larger in the 25 patients who achieved a radiological PR.[94] An increase in serum levels of VEGF A and PlGF and decrease in sVEGFR 3 and VEGF C has also been demonstrated in patients treated with sunitinib. The study, in 61 patients with bevacizumab refractory metastatic RCC, also reported that patients with lower baseline levels of sVEGFR 3 and VEGF C had a higher response rate to sunitinib and a longer PFS.[95] The applicability of these findings to other patients remains unknown.

The expression of targets such as VEGF and PDGF are under the transcriptional regulation of HIF1 and HIF2, which are in turn regulated by the VHL protein. Levels of HIF1α and HIF2α have been examined in tumor lysates by Western blot analysis from 49 patients with metastatic RCC treated with sunitinib. Patients with tumors containing higher levels of HIF1α ($P = 0.003$) or HIF2α ($P = 0.001$) were significantly more likely to achieve a favorable response (CR or PR) to sunitinib.[96] The results are certainly intriguing and warrant further investigation. Studies examining VHL gene status have not shown such strong correlations with response. In the largest such study to date, examining 123 patients with metastatic RCC treated with VEGF targeted therapy, response rates were no different in those with VHL inactivation compared to wild type ($P = 0.34$). However, in an exploratory subgroup analysis, patients with loss of function mutations did demonstrate a higher response rate than those with wild type VHL (52% vs 31%; $P = 0.04$). Thus, patients with intact VHL protein appear to have a clinically useful response rate to such drugs, highlighting the complexity of the biology involved and suggesting that downstream mediators of VHL (such as HIF) may be more relevant to study.[97]

mTOR inhibitors

The mTOR inhibitors are an increasingly important class of anti cancer agent in renal cancer. mTOR is a highly conserved serine/threonine kinase involved in several signaling pathways, and, as such, has a major role in regulation of cell growth and proliferation. Patients with non clear cell histology have typically been excluded from large trials using sunitinib and sorafenib and have not been adequately evaluated for response.[5] However, currently, patients with chromophobe or papillary RCC are thought to respond poorly to TKIs. It is therefore encouraging that temsirolimus has been reported as showing equivalent response rates in clear and non clear cell RCCs. The data are derived from the registration trial of temsirolimus,[8] which included 124 (20%) patients with non clear cell RCCs.[98] Whether mTOR inhibitors represent a better first line therapy than TKIs for patients with non clear cell RCCs is unknown and will require testing in a prospective manner.

Patient tumors from the same phase III trial have been examined for expression of PTEN and HIF1α on tissue sections using IHC. No correlation between expression of either protein and response to temsirolimus was demonstrated however.[99] Expression of pAKt and phospho S6 has been correlated with response, although this was a small study of just 20 patients.[100]

4. CONCLUSIONS

Cancer biomarkers have revolutionized our approach to patient care in many cancer types. They have major potential benefits for patients, particularly in contributing to 'personalized' medicine, and improved biomarkers should ultimately lead to improvements in outcomes and more efficient, safe, cost effective and evidence based use of health resources. In RCC, no such markers are in routine clinical use. Much interest is focused on identifying prognostic markers, which can identify high risk patients who should be targeted for trials of adjuvant therapy. These trials are underway and, if positive, will lead to large numbers of patients receiving TKI based therapy, in addition to those currently treated in the metastatic setting. Thus, biomarkers that predict response to these therapies are of equal importance and are also urgently required. The role of neo adjuvant therapy is also now beginning to be explored and markers that determine outcome preopera tively will be required to guide management decisions in this setting.

Currently employed nomograms are limited by their reliance on stan dard clinicopathological criteria. The development of accurate prognostic and/or predictive models that are universally applicable will require a concerted effort from the international community. In 2004, an Inter national Kidney Cancer Working Group was established to identify inde pendent, validated predictors of survival, by collecting data on $>$ 4000 previously untreated RCC patients. Clearly several potential markers have been identified and more are likely to emerge from the proteomic and genomic initiatives. However, key to their successful exploitation and translation will be the establishment of the necessary infrastructure including high quality annotated sample banks, assays development and design of multicenter trials to evaluate them with evidence based progression through this pipeline to the clinic.

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CHAPTER 9

Urinary Proteomics and Candidate Biomarker Discovery for Diabetic Nephropathy

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Contents

Diabetes mellitus (DM) is a complex and systemic disease, with complications that result from dysregulated metabolic pathways as well as genetic susceptibilities.[1–4] DM is a prevalent disease with current estimates by the American Diabetes Association using 2007 data suggesting that there are 17.5 million diagnosed and 6.6 million undiagnosed diabetics residing in the United States.[5] DM and its attendant macrovascular and microvascular complications represent a significant societal and economic burden. The medical costs of common microvascular complications of uncontrolled DM, diabetic nephropathy and diabetic retinopathy, account for 29% and 15% respectively of the $116 billion expenditures associated with diabetes.[6] A substantial gap in knowledge exists regarding the natural history of these complications; who as well as how, when and why. Clearly, there is a driving need for biomarkers that can accommodate the diagnostic complexity of this metabolic landscape. Proteomics or the proteomic paradigm has evolved into a high throughput, analytical discipline used to analyze complex biological datasets. These open ended, hypothesis generating approaches,

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when appropriately designed and interpreted, are well suited to the study of the pathogenic mechanisms of diabetic microvascular disease. In support of this position, we will review here the evolving role that proteomics has played in expanding our understanding of the natural history of diabetic nephropathy.

1. INTRODUCTION

Diabetic nephropathy (DN) is a severe microvascular complication occur ring in individuals with diabetes. Unfortunately, our understanding of diabetic complications is primarily based on a list of empirically and observationally based susceptibility risk factors (e.g. *hyper*glycemia, *hyper*-lipidemia, and *hyper*tension) and not from concrete insights into compli cation specific, pathogenic, molecular mechanisms that determine cellular or tissue fate. Given the role of proteins as regulators of cellular responses, unbiased methods that provide qualitative and quantitative information of protein abundance could be useful for understanding the pathogenesis of diabetic complications. The proteomic method for scientific analysis is an approach to rapidly survey the proteome (complete inventory of proteins expressed within a biological sample). With this method, biological samples such as urine, plasma, serum or lysates of renal parenchymal cells are systematically analyzed with the intent of identifying, quantifying and discerning the function of all observable proteins. The application of the proteomic method for comparison of disease and control samples allows for the rapid development of a hypothesis used to understand or explain aspects of disease biology such as disease initiation, progression or remis sion.[7] Recent mass spectrometric and bioinformatic advances have driven many current developments in a set of core technologies, including methods to separate complex mixtures of proteins and peptides,[8] soft ionization approaches used to characterize biological molecules by mass spectrometry[9 11] and advanced computer assisted data analyzes approaches capable of handling complex datasets.[12] The insights gained from proteomic experiments should optimally allow for a better under standing of disease state genesis or evolution of the condition. While the proteomic approach to discovery is a relatively recent development for medicine and science, its limited application to the study of renal disease and the progression of DN has yielded notable results.[13]

The NIH Biomarker Definitions Working Group has provided a consensus definition for biomarkers as "… cellular, biochemical, and

molecular (genetic and epigenetic) alterations by which a normal or abnormal biologic process can be recognized or monitored. Biomarkers are measurable in biological media, such as in tissues, cells, or fluids."[14] Some candidate biomarkers originally observed as differentiating healthy controls and disease samples are unsuccessfully validated not due to the disease but the nature of the disease complication. In the case of markers of DN, an example would be the wash out of the candidate following a demonstration of the marker's experimental scoring equally strong to chronic renal disease samples (such as IgA nephropathy, focal segmental glomerular sclerosis, membranous glomerular nephritis and minimal change disease) as to DN samples. Another example would be the study of type 2 (T2) diabetes and the unknowing assignment of candidate biomarkers to T2DN in the absence of clinical data to filter out unas sociated non T2DN biomarkers of the dysmetabolic syndrome. The foresight of some groups with prospective collection and biorepositing of clinical samples has been one key approach to circumventing this problem. Many of these samples, such as those bioreposited from the First and Second Joslin Studies on the Natural History of Microalbuminuria and Diabetes, have provided the opportunity to use proteomic methods to compare urine, plasma and serum samples from patients years or decades before the development of renal insufficiency.

A significant amount of energy continues to be expended for iden tification and development of diagnostic, 'gold standard' biomarkers of diabetic complications. The goal of these expenditures is the development of panels of sensitive and specific biomarkers for use within diagnostic assays. A secondary goal for these efforts is the discovery of diagnostic biomarkers that may yield insight into disease mechanisms. The need for an improved understanding is more important given two changes in our thinking regarding DN. First, there is a change occurring in our understanding of the natural history of DN as it relates to micro albuminuria. Second, there is new insight into the possibility that the pathophysiology of T1DN may correlate with but a portion of T2DN patients.[15] As will be highlighted later, many of the currently identified candidate biomarkers of diabetic nephropathy are derived from extracel lular and cellular proteins. For these candidates to provide insight into mechanisms, it would be beneficial if a correlation between identity and function existed. To assist with a rational analysis of candidate biomarkers, a framework for early and late pathological renal abnormalities can be useful to guide interpretation of proteomic findings.

Early models of DN centered on hyperglycemia and hemodynamic alterations. The metabolic insult, hyperglycemia, perpetuated increases in the relative concentrations of activated protein kinase C, polyol pathway intermediates, advanced glycation end products, reactive oxygen species. The early changes in type 1 diabetic patients are characterized by a glomerulopathy that includes thickening of the glomerular basement membrane, mesangial expansion and progressive loss of glomerular filtra tion surface area. In many diabetic patients, glomerular changes are often paralleled by arteriole, tubule and interstitium alterations that become more pathologically important after significant glomerular damage. The correlation of renal pathology in T2DM patients is made difficult by the co incident renal pathology occurring with advanced aging and compo nents of the metabolic syndrome. However, it appears that patients with T2DM can have renal histology that is normal, similar to T1DN changes and atypical changes characterized by arteriolar hyalinosis and tubular dropout.

2. URINARY PROTEINS AS CANDIDATE BIOMARKERS OF DIABETIC NEPHROPATHY

Normal urine and urine from microalbuminuric diabetic patients repre sents a dilute solution containing proteins derived from a number of sources – predominately plasma proteins and to a lesser extent renal cell (visceral epithelial, glomerular and tubular origins) types.[16] Urine collected from macroalbuminuric and nephrotic patients is a concentrated protein solution dominated by a few high abundant proteins, primarily albumin and IgG. In as much as most urinary biomarker investigations focus on high molecular weight proteins, urine is also a complex mixture of proteins and also protein fragments (peptides). To understand the diabetic phenotype and identify candidate biomarkers for DN, a complete understanding of all protein identities that are sourced into the urine is vital. Therefore methods such as electrophoresis and liquid chromatography (LC) are used to reduce the analytic complexity (proteins and peptides) of the sample prior to protein identification using mass spectrometry (MS) based methodology. As is highlighted here, significant work has been performed using these two separation methods. Electrophoretic approaches to proteome analyzes provide the ability to start with large masses of protein, observing protein isoforms and distinguishing specific protein spot data that include isoelectric point (pI), mass (Mr) and spot volume. LCMS methods can be

conducted in a top down (analysis of intact proteins and peptides) or bottom up (analysis of trypsinized proteins) approach. These MS methods can be conducted using multidimensional chromatography, require lower mass loads given the high sensitivity ion counting detectors and direct analysis of the analyte. A significant benefit of methods such as capillary electrophoresis (CE) MS or LCMS, which directly couple the sample separation with MS, is that larger observational data sets can be developed in shorter amounts of time.

Electrophoresis continues to play a role as a separations modality for proteomic analysis of the high molecular weight urinary proteome. Three reports discussed here highlight the application of one and two dimensional electrophoresis methods for development of candidate biomarkers for DN. In a purely proteomic approach to biomarker discovery, Thrailkill et al utilized gel electrophoresis to separate pooled 24 h urine samples from healthy controls, T1DM with normoalbumi nuria, and T1DN with microalbuminuria. The urine samples (protein normalized to 1000 μg urine creatinine) were separated, gel bands were excised, digested with trypsin and analyzed using label free (spectral counting) quantitative LCMS methods to identify 150 significantly ($P < 0.05$) differentially expressed urinary proteins. The basis for this approach to proteomics is a spectral counting approach used to identify proteins from the tryptic digests of the excised gel pieces. The MS/MS data are analyzed by matching the experimentally acquired MS/MS spectra to a database of theoretical peptide spectra derived from known protein sequences. The proteins are identified by correlating deduced peptide sequences to known protein sequences. Several studies have previously demonstrated a correlation between both peptide hits and numbers of observed spectra per peptide.[17 21] Therefore, in these label free quan titative MS approaches, each spectra assigned with high confidence to a particular peptide can be assigned a relative expression level following normalization to total numbers of spectra acquired in each LCMS experiment. The corresponding peptides are matched across multiple LCMS experiments. The number of times a discriminatory peptide spectrum (counts) has been observed in different biological samples is normalized across all measured peptides for each protein quantified.[21] Following this normalization, the same peptide data or protein data can be compared across sample analyzes and changes in expression levels inferred. This direct analysis approach was used to identify enhanced excretion of megalin and cubilin (endocytic multi ligand receptors) as

well as megalin and cubilin ligands in the urine of microalbuminuric type 1 diabetics.[22] While no other follow up validation experiments were conducted, it was speculated that these data support the conclusion of others[23] that impaired proximal tubular uptake of urinary proteins plays an important role in the development or progression of early nephrop athy in type 1 diabetics.

More classical quantitative two dimensional gel electrophoresis (2DE) proteomic experiments were used by Jiang et al to identify an acute phase reactant protein, orosomucoid, and a soluble form of E cadherin (a calcium dependent cell cell adhesion glycoprotein) as independent risk predictors (former) and biomarkers (latter) for diabetic nephropathy.[24,25] In these studies, 2DE was used as a discovery platform to identify candidate biomarkers in small numbers of samples. Following their discoveries, anti body based methods (immunoturbidometric analysis or enzyme linked immunosorbent assay, ELISA) were used to validate the finding in larger sample sets. In the first DN urinary proteome 2DE study, 46 protein spots were significantly regulated with 70% upregulated and 30% downregulated with DN. One strikingly upregulated protein was identified using MS methods as orosomucoid; also known as alpha 1 glycoprotein − an acute phase reactant protein synthesized by the liver. This protein was increased greater than eight fold in DN patients. Using immunoturbidometric analysis to extend their initial observation of urinary orosomucoid levels, Jiang et al determined the urinary orosomucoid excretion rate in an expanded sample set of 120 urine samples. The urinary orosomucoid excretion rate was slightly increased in normoalbuminuric patients but greatly elevated in micro and macroalbuminuric patients. Using a Pearson's correlation analysis, the urinary orosomucoid excretion rate was positively correlated with albumin excretion rates, serum creatinine and C reactive protein. In the second study and using differential in gel two dimensional gel electrophoresis, Jiang et al identified E cadherin in addition to several other proteins ($n_{\text{total}} = 21$) including orosomucoid as regulated (two fold expression change) in T2DM and T2DN. Using ELISA analysis for soluble E cadherin normalized to urinary creatinine (sE cadherin/Cr) in an expanded urine samples set ($n_{\text{total}} = 160$), the authors found a positive correlation of sE cadherin/Cr with nephropathy (urinary albumin excre tion rates normalized to creatinine). To gain some pathophysiological insight, the authors used immunohistochemical staining of normal and diabetic renal biopsies to discern a decreased cortical staining for E cadherin in diabetics' samples.[26]

3. URINARY PEPTIDES AS CANDIDATE BIOMARKERS OF TYPE 2 DIABETIC NEPHROPATHY AND CHRONIC KIDNEY DISEASE

Due to the increasing prevalence of T2DM and the aging population, an expectation for candidate DN biomarkers is the applicability to diagnose nephropathy in adult populations. To be successful, candidate biomarkers for DN, especially T2DN, should be specific to the exclusion of the effects of aging. The onset of diabetic nephropathy is characterized by a rise in albumin excretion rate and in most individuals occurs coincident to a transient increase followed by a precipitous decrease in glomerular filtration rate (GFR). Several cross sectional and longitudinal studies of normal populations of patients have documented functional changes such as decreased GFR occurring coincident with aging. As with diabetes, age dependent changes in renal function can be associated with genetic and environmental factors. Given these points, two recent studies (by Zürbig et al[26] and Rossing et al[27]) are noteworthy. Zürbig utilized CE MS methods to identify patterns of prevalent urinary polypeptides in 324 normal individuals aged 2–73. Upon first review, no individual or small group of peptides was discerned as differentially abundant. With a higher ordered analysis by ANOVA, the relative urinary abundance of 325 of more than 5000 peptides or approximately 6% of the urinary peptidome was regulated with age. The largest component of change could be ascribed to changes with the urinary peptidome of subjects between 11 and 18 years of age. The data for 218 patients with an age range of 19–73 years were reanalyzed. Using the same statistic approach, a grouping of 49 peptides' expression was specific to the aging process in adults. A generalized trend in the data was a decrease in observed peptides in the very old. A targeted analysis using tandem MS methods identified fragments of collagen I α I, collagen III α I, fibrinogen β chain and psoriasis susceptibility 1 candidate gene 2 protein. The specific collagen I α I fragment had been identified in previous CE MS studies by this group. Those previous studies had estab lished urinary peptide patterns associated with chronic kidney disease including DN, IgA nephropathy, focal segmental glomerular sclerosis, membranous glomerular nephritis, vasculitis and minimal change disease. The observed panel of chronic kidney disease peptide panels overlapped with 73.5% of aging specific peptides. These findings lead to the re analysis of the original data considering the patients in age cohorts of 19–30 years old ($n = 96$) and 51–73 years old to discern markers of biological renal age.

Thirteen peptides were significantly correlated with renal age. The targeted analysis of these peptides in the urinary peptidome datasets for diabetic patients found the kidneys of macroalbuminuric diabetics to be consistent with older or aged renal peptidome. These data suggested that the lesions in diabetic nephropathy are similar to aging induced renal lesions.

Using CE MS methods similar to Zürbig et al, Rossing et al conducted biomarker discovery experiments to detect differences in the urinary pep tidome of type 1 diabetic patients with normo ($n = 30$), micro ($n = 29$) and macro ($n = 30$) albuminuria with a cohort of age matched controls ($n = 30$). The questions raised and addressed by Rossing et al were: can the urinary peptidome define candidate biomarkers for: (1) early diabetes; (2) early diabetic nephropathy; and (3) diabetic nephropathy in the face of chronic kidney disease? A first review of the CE MS data by Rossing et al noted that discrete differences were not observed between urinary pepti dome profiles derived from normals, normo and microalbuminuric patients; however, large differences were observed in the urinary peptidome of macroalbuminuric T1DN patients as compared to the other groups. These observations then support the findings of Zürbig et al that the urinary peptidome of macroalbuminuric patients is significantly different from that of healthier kidneys. To address candidate biomarkers of early diabetes, the urinary peptidomes of healthy controls and T1DM with persistent nor moalbuminuria were compared by maxT testings (a statistical t testing approach that corrects for large numbers of simultaneous comparisons). A total of 40 peptides were observed at P values less than 0.05. These same T1DM patients compared to T1DM patients with macroalbuminuria using maxT testing yielded 102 statistically significant peptides ($P < 0.05$). An abbreviated list of peptides ($n = 65$) was found to classify the normo vs macroalbuminuric T1DM patients with 93% sensitivity and 97% specificity at cross validation. Twenty four of the original 102 peptides were identified as fragments of extracellular matrix proteins (collagens I and III), serum proteins (albumin, α 1 anti trypsin, transthyretin, α 2 HS glycoprotein, serpin peptidase inhibitor, fibrinogen β chain), uromodulin, beta$_2$ micro globulin, psoriasis susceptibility 1 candidate gene 2 protein, and membrane associated progesterone receptor component 1. To address candidate biomarkers of diabetic nephropathy in the face of chronic kidney disease, the biomarker panel developed previously was used to evaluate urine samples from biopsy proven IgA nephropathy ($n = 57$), focal segmental glomerular sclerosis ($n = 30$), membranous glomerular nephritis ($n = 35$) and minimal change disease ($n = 25$). More than two thirds of the chronic

kidney disease patients scored positively for diabetic nephropathy. This then indicates that the patterns of diabetic kidney disease detected throughout the study may largely reflect chronic renal damage. To address this, the study then evaluated the non diabetic renal disease urine samples against urine samples of T1DM patients with macroalbuminuria applying similar statis tical methods (support vector machine based model, SVM BM). A total of 17 peptides were identified that correctly identified 42 of 44 diabetic samples and 98 of 104 non diabetic renal disease samples. These data might suggest that there are common pathways of renal damage but that DN specific peptides still exist.

4. URINARY PEPTIDES AS CANDIDATE BIOMARKERS OF EARLY PROGRESSIVE RENAL FUNCTION DECLINE IN TYPE 1 DIABETIC NEPHROPATHY

In addition to diagnosis of existing disease, we and other groups have sought to identify biomarkers which might be prognostic of DN and its progres sion. These studies illustrate the remarkable value of the well characterized, clinically curated patient samples of the First Joslin Study on the Natural History of Microalbuminuria and Type 1 Diabetes. Using samples from the Joslin study, we recently utilized a top down LC MALDI TOF MS approach to identified components of the urinary peptidome whose abundance correlated with future renal function decline in T1DM patients with microalbuminuria.[28] Furthermore, these peptides, used as guides to select renal proteins for follow up immunohistochemical and confocal microscopic analysis, suggested specific roles for the cellular stress response pathway in development of renal function decline.

In this study, the characterization or curation of the clinical samples was paramount. To determine onset and levels of microalbuminuria, patients were followed from 1991 to 2007 and the albumin excretion rate (in micrograms per minute) was estimated from the albumin to creatinine ratio in random urine samples. Within 4 years after the initial evaluation of the cohort in 1991, new onset microalbuminuria developed in 109 of the 943 patients with normoalbuminuria. Eighty six patients were followed until 2007 and 61 of these patients met the following criteria and were included in the analysis: (1) greater than 8 years of biennial follow up examinations after the onset of microalbuminuria until 2007 in order to measure serial estimates of the glomerular filtration rate; and (2) sufficient stored urine specimens (at least one 6 mL aliquot of urine per examination per patient)

for analysis of peptide components taken within 5 years of the onset of microalbuminuria. For all patients the earliest available urine sample after the documentation of microalbuminuria onset was used for isolation of the urinary peptidome.

Two cohorts of patient samples were analyzed and included T1DM patients with microalbuminuria who: (1) demonstrated stable or age equivalent loss of renal function (referred to as controls or non decliners) and (2) demonstrated early progressive loss of renal function (referred to as cases or decliners). Assignment to these cohorts was based on serum cystatin C estimates of the GFR. The GFR in mL/min was approximated numerically by the reciprocal of cystatin C (in mg/L) multiplied by 100 (cC GFR) and a regression slope fitted to serial measurements of cC GFR over several years was used to accurately track the trend in renal function over that time. Data available from the Baltimore Aging Study were used to establish the reference distribution for evaluating whether a negative slope or trend in renal function qualified as an abnormal rate of decline (desig nated 'early renal function decline' or 'ERFD'). Nineteen subjects (cases) had renal function loss of 3.3% or more per year (slopes ranging from −3.3 to −16.1% per year). The remaining 42 patients without such rapid renal function loss (slopes ranging from +1.9 to 3.2% per year) were designated as controls.

To avoid the introduction of systematic bias in our analysis, the order of case or control sample handling during all stages of peptide isolation and MALDI TOF MS data acquisition was randomized. To address both the problems of sample matrix complexity (urinary salts and osmolytes) as well as urinary peptidome complexity, peptides were isolated from the urine using first ultrafiltration (isolating the lower than 10,000 Dalton peptidome fraction) followed by desalting with solid phase extraction (SPE) methods. Each isolated peptidome was fractionated into 45 components based on hydrophobicity using one dimensional reversed phase capillary scale HPLC column. Each fraction was then analyzed by MALDI TOF MS.

The statistical analysis of the LC MALDI TOF MS datasets presented several challenges. To focus effort on the most promising peptides, we imposed several stringent criteria for selecting peptides for further analysis. At the expense of eliminating true positive but to decrease the number of false positive associations between peptide expressions and early renal function decline, we eliminated 3364 peptides that were detected in less than 20% of the specimens. Next, using a modification of the approach of Rossing et al, discussed previously,[27] we required that there be at least a 50%

difference in the frequency of a peptide between case patients and control subjects and that this difference be statistically significant; reducing the peptidome from 825 to six. For these six peptides, we compared their urinary abundances using the peptide peak's characteristics. The peak characteristics were defined from the integrated signal area for the peptide isotopic series. Three peptides were present more frequently in urine of case patients in comparison with urine of control subjects, and three were present less frequently in urine of case patients in comparison with urine of control subjects. Interestingly, the abundance and detection frequency of two peptides 983.534 and 1190.638 m/z were completely correlated whereas the others were less correlated or not correlated at all among themselves. The association of these peptides with early renal function decline was studied further using logistic regression analysis controlling for the effects of other covariates such as HbA1c and albumin excretion rates. Urinary presence of peptides 983.534, 1190.638 and 1838.851 m/z was strongly and independently associated with the presence of early renal function decline (ERFD). The adjusted odds ratios (ORs) varied from 4.4 to 4.9 (95% confidence interval (CI) 1.2 to 20.0). Conversely, urinary presence of peptides (i.e. 1841.811, 2195.965 and 2315.018 m/z) was protective against ERFD. Adjusted ORs varied from 0.2 to 0.4. The 95% CI for the 1841.811 m/z was less than 1.0 but for the two others slightly above 1.0, indicating that after adjustment for other covariates, the negative association of these peptides with ERFD had only borderline significance. Analysis of contemporaneous plasma samples from the same patients by similar methods established that the observed differences in these peptides were specific to the kidney and not derived from filtered, differentially abundant plasma peptides. Therefore these peptides were now considered to be candidate biomarkers for early renal function decline in T1DM patients with microalbuminuria.

To begin to understand a role of the candidate biomarker in the pathophysiology of early progressive renal function decline, we undertook MS/MS studies to discern amino acid sequences to the six peptides. The three more abundant peptides were fragments of the cadherin like protein FAT tumor suppressor 2, zona occludins 3 (ZO 3), and inositol pentakis phosphate 2 kinase (IPP2K). The three peptides that decreased in the early renal function decline specimens were fragments of extracellular matrix proteins – tenascin X, α I (IV) collagen and α I (V) collagen. The analysis of the MS/MS data for the 1838.851 m/z peptide, assigned to IPP2K, was consistent with a glycyl glycyl post translational modification to the epsilon

amino group of the internal lysine, which would be presumed to result from ubiquitination of the parent protein IPP2K.

To extend the value of these peptides in better understanding the evolution of early renal function decline in T1DM, we then examined whether the candidate urinary peptides might provide insight into disease induced changes in renal protein expression. Unlike urine samples, few studies are approved for prospective renal biopsy collection. Therefore using renal biopsies from normal individuals and patients with diabetes, we examined the tissue expression of IPP2K and ZO 3 using immunohisto chemistry. To maintain the focus on early renal function decline, the biopsies were from patients with diabetes, minimal albuminuria and serum creatinine levels of 1.2−1.9 mg/dL. Patients with DN had increased IPP2K expression in renal tubules and glomeruli. ZO 3 had increased expression in biopsies from patients with DN as compared with control subjects. Furthermore, the ZO 3 staining was less linear, not confined to the cell periphery, and increased in the cytoplasm when compared with normal biopsy sections. This staining pattern of ZO 3 is similar to that of another zona occludens protein, ZO 1, whose expression has also recently been demonstrated as altered in cultured podocytes incubated in high glucose medium.[29]

The finding for IPP2K was intriguing. IPP2K has been shown to be a constituent of mRNA containing granules responsible for protein trans lation arrest in stressed cells.[30] These cytoplasmic inclusions, referred to as stress granules, are observed in cells subjected to environmental stress, including heat, irradiation, oxidative conditions and hyperosmolarity.[31] Because we observed increased intact IPP2K in the kidney and IPP2K urinary fragments, we examined whether stress granules are present in DN. To establish the stress granule, we determined the expression of a constitu tive stress granule protein, T cell intracellular antigen 1 (TIA1). We observed increased TIA1 staining in DN. TIA1 was primarily localized to the cytoplasm and in a granular pattern, indicative of stress granules. To confirm the presence of stress granules in DN, we determined the extent to which TIA1 and IPP2K co localize in renal tissue from patients with diabetes. Renal biopsies from normal donors and patients with diabetes were stained with TIA1 and IPP2K antibodies. Normal biopsies stained positively for TIA1, the staining was with a cytoplasmic distribution that was far smaller than stress granules. IPP2K staining in DN biopsies was faint. DN renal biopsies stained positively for both IPP2K and TIA1 in large granular structures consistent with the expected diameter and distribution of stress granules.

This study achieved the goals of identifying urinary peptides that predict progressive early renal function decline and establishing an association of the observed urinary peptides with changes in the renal parenchyma. These peptides reflect changes in both tubular and glomerular protein expression that are associated with the formation of stress granules and may define a new cellular mechanism by which DN is initiated or progresses. As has been illustrated for some of the studies reviewed here, the usefulness of these discriminating peptides as biomarkers of diabetes associated renal function decline must be determined in additional rigorous studies in a larger patient population. These results provide the hope that candidate biomarkers can provide insight into the mechanisms of diabetic kidney disease.

5. A TARGETED PROTEOMIC ANALYSIS FOR CANDIDATE BIOMARKERS OF EARLY RENAL FUNCTION DECLINE IN TYPE 1 DIABETIC NEPHROPATHY

In addition to the known T2DM risk factors of obesity, hypertension and genetics, many studies have demonstrated a correlation of T2DM and a chronic, low grade inflammatory state. In addition to the prominent role of the glomerular lesion, this chronic low grade inflammatory state is postulated to be pathologically involved with the development of compli cations such as DN.[32 35] Correspondingly few studies have addressed the association of inflammation and T1DM with the future risk of developing progressive renal function decline leading to T1DN. Two recent targeted proteomic studies from the Joslin Diabetes Center[36,37] looked specifically at the association of serum and urinary markers of inflammation and T1DN. Using clinical data obtained from longitudinal follow up studies, encom passing in many cases more than a decade, Wolkow et al was able to assemble and test the study entry urine sample of three groups of T1DM patients recruited into the First Joslin Study of the Natural History of Micro albuminuria in Type 1 Diabetes. These patient groups were defined as T1DM patients with: (1) persistent normoalbuminuria and no renal func tion decline ($n = 74$); (2) new onset microalbuminuria and no renal function decline ($n = 43$); and (3) new onset microalbuminuria and early progressive renal function decline ($n = 28$). We should stress again here that the cohorts were assembled using longitudinal data but the urine and serum used for analysis was derived from study entry samples. Using antibody based assays, quantitative measures of a targeted set of five urinary inflam matory markers including IL 6, IL 8, monocyte chemoattractant protein 1,

interferon gamma inducible protein (IP 10) and macrophage inflammatory protein 1δ were established. The chemokines IL 8, monocyte chemo attractant protein 1, interferon gamma inducible protein (IP 10) and macrophage inflammatory protein 1δ were increased with significance ($P < 0.05$) in the urine of observed new onset microalbuminuric patients who experienced significant future renal function decline. The cytokine IL 6 was also increased in the urine of new onset microalbuminuric patients who experience progressive renal function decline but the significance value was less ($P < 0.08$). An analysis of contemporaneous serum samples for IL 8, macrophage inflammatory protein 1δ and C reactive protein did not document any significant differences, suggesting that the observed urinary differences were specific to the kidney. These differences were maintained after adjustment for urinary creatinine. A multivariate analysis was used to find association of elevated levels of more than one chemokine with the future development of early renal function decline. This analysis suggested a greater than five fold risk for developing early progressive decline of renal function with an elevation of two or more chemokines. Here a targeted proteomic approach was used to establish a risk association of low grade inflammatory processes in urine samples years before a measurable loss of renal function. Furthermore, these results support the hypothesis that renal specific, but not systemic, inflammatory processes contribute to the progression of nephropathy in patients with type 1 diabetes.

6. FUTURE DEVELOPMENTS AND APPLICATIONS OF PROTEOMICS FOR BIOMARKER DISCOVERY

Diabetes is a complex disease. The complexity of the etiology is reflected by the complexity of disease complications such as DN. Proteomic approaches have evolved to deal with disease complexity using such label free top down LCMS approaches such as we used[28] or targeted proteomic approaches used by Wolkow et al.[36] We have seen that urine based biomarkers of renal diseases involving proteinuria will likely be composed of complex protein charge form patterns of urinary resident serum proteins. Further investi gation into the enzymatic pathways producing these biomarker patterns can perhaps yield more relevant mechanistic information into renal glomerular and/or tubular pathophysiology. The variability of the human urinary proteome has to be addressed or offset before any meaningful advances occur. Nonetheless, as label free MS methods of protein quantification permeate general proteomic research fields we should begin to see these

methods successfully applied toward the study of DN. Perhaps most exciting of all is the possibility that urine peptide analysis may allow us to gain insight into the proteins that mediate damage to the renal parenchyma in diabetes.

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CHAPTER 10

Biomarkers in Glomerular Disease

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Contents

Glomerular diseases are a major cause of end stage renal disease. Many glomerular diseases are treatable if the specific diagnosis is known. A renal biopsy is required to make a definitive diagnosis of the cause of the disease in most cases. Although renal biopsy is the gold standard for diagnosis, it is invasive and has potential complications such as bleeding, infection and death.[1] Although serial renal biopsies might improve treatment, they are often difficult to justify because of the risk, discomfort and expense. New methods are needed to identify the cause of renal disease and prognosis without a biopsy. Urine or blood testing for biomarkers could replace renal biopsy as a simple, safe and accurate test that could be repeated to follow progression of the disease and monitor response to therapy. The clinical course of many glomerular diseases is highly variable so it is difficult to predict if any given patient will lose renal function or suffer the associated complications of renal disease. The major impediment to the development of new treatments is the inability to identify patients that would benefit from new treatments. Beneficial effects of treatment are much more likely to occur in patients who will progress rapidly. Biomarkers provide the opportunity to identify the disease, predict prognosis and predict the response to therapy in a non invasive way.

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1. BIOMARKERS IN GLOMERULAR DISEASES

A number of biomarkers have been proposed to predict the rate of progression or the underlying cause of glomerular diseases. The best characterized is urinary albumin as a predictor of progression in diabetic nephropathy. Microalbuminuria is associated with an increased rate of progression of diabetic renal disease in patients with both IDDM[2] and NIDDM.[3] Unfortunately, microalbuminuria is not an ideal marker for progression of diabetic nephropathy. A large study of patients with insulin dependent diabetes (IDDM) points out the problems with microalbuminuria as a marker for progression of diabetic nephropathy.[4] After 5 years, 33% of patients with microalbuminuria had progressed to normoalbuminuria and 19% had diabetic nephropathy. In another study of 386 patients with type 1 diabetes and persistent microalbuminuria, 58% experienced regression of the rate of albumin excretion by 50% or greater over at least a 2 year period.[5] The role of biomarkers in diabetic nephropathy is the subject of another chapter in this book. In this chapter, we will focus on biomarkers in other glomerular diseases such as lupus nephritis, membranous nephropathy, focal segmental glomerulosclerosis (FSGS), minimal change disease and IgA nephropathy. In addition, we will highlight studies using discovery techniques such as proteomics to identify novel biomarkers.

1.1. Predictors of outcome in glomerular diseases

Glomerular diseases can have a range of outcomes from complete spontaneous remission to progression to ESRD. While renal biopsy can help identify the disease, it is not as good at predicting the outcome. Biomarkers which can predict outcome would be tremendously helpful to guide treatment decisions. *N* acetyl beta glucosaminidase (NAG) is a tubular enzyme which is increased in the urine during renal injury. In patients with nephrotic syndrome, NAG concentrations above a cutoff value predicted progression to chronic renal failure better than did the level of proteinuria.[6] While this finding needs further verification, it is a promising approach to prediction of outcomes in patients with glomerular diseases. A number of other studies have been done in patients with specific glomerular diseases.

2. BIOMARKERS IN LUPUS NEPHRITIS

Treatment of lupus nephritis has met with limited success for a number of reasons. Current therapeutic regimens typically produce complete and

partial remission rates of less than 50%.[7,8] Furthermore many therapies are highly toxic. Flares of nephritis can be difficult to predict ahead of time which makes timely treatment more difficult. Finally, renal biopsy is typi cally not repeated at the time of flares which makes tailoring treatment more difficult. Biomarkers which can predict flares early, which can predict the class of nephritis present and which can help guide treatment, would be extremely useful and help improve outcomes in lupus nephritis.

2.1. Predictors of lupus nephritis class

One of the key areas of interest in the field is biomarkers that can predict the type of nephritis present. Currently, examination of urine sediment and proteinuria can be helpful but renal biopsy is required to differentiate specific classes of lupus nephritis. A number of potential candidate markers have been evaluated to differentiate between classes of nephritis. Messenger RNA levels in urine of IP 10, its receptor (CXCR3), transforming growth factor β (TGF β) and vascular endothelial growth factor (VEGF) were evaluated for their ability to discriminate between class IV lupus nephritis and other classes.[9] All four analytes had significantly different levels between class IV lupus nephritis and other classes. ROC curve analysis showed that IP 10 had the best discriminative power with an AUC value of 0.89. When individual values were plotted however, there was a significant amount of overlap between the number of copies of mRNA in patients with class IV lupus and other classes. Thus better discriminating power is needed before a test based on IP 10 message levels could be used as a rationale for treat ment with toxic agents in patients with lupus nephritis. The levels of β_1 integrin (CD29) expression on peripheral blood T cells has been compared between patients with class IV lupus nephritis and other classes of nephritis.[10] Patients with class IV lupus nephritis showed a significantly higher level of β_1 integrin expression on T cells than patients with other classes of lupus nephritis or healthy volunteers. Furthermore, there was an inverse correlation between β_1 integrin expression and serum complement levels which is an indicator of the severity of the lupus flare. In this study all patients with increased β_1 integrin expression had class IV lupus although not all patients with class IV lupus had increased β_1 integrin expression. These data are exciting in that they suggest that β_1 integrin may be a highly specific marker for class IV nephritis but will need to be confirmed in other populations. Proteomic signatures have also been used to predict the class of nephritis present.[11] Two dimensional gel electrophoresis of urine proteins

was used to develop an algorithm which could differentiate between classes of nephritis identified by renal biopsy. A combination of spots was identified using a machine learning algorithm that could predict the cause of disease with AUC values between 0.85 and 0.95. Two hundred and thirteen proteins were used in the analysis although most of the accuracy of the analysis was contributed by 10 spots. Although this represents an improvement in accu racy compared to single analytes, measurement of larger numbers of proteins is correspondingly more difficult. Furthermore, the results have not yet been independently confirmed. Overall, the existing studies do not support any currently available tests to determine lupus class without a renal biopsy. Studies that identify novel biomarkers will be useful and successful methods will likely use a combination of markers to predict the class of lupus nephritis that is present.

2.2. Biomarkers that predict renal lupus flares

A potential stumbling block to efficacious treatment of lupus nephritis is the timing of treatment. Typically, nephritis is treated after structural changes to the glomerulus have already occurred. These structural changes are indi cated by increases in proteinuria, hematuria and serum creatinine. If treatment could begin earlier, it may be more successful. A number of studies have attempted to identify biomarkers that can predict flares in renal lupus activity. Urinary FOXP3 mRNA has been proposed as a candidate marker for lupus renal disease activity.[12] FOXP3 is a regulator of the development and function of regulatory T cells. FOXP3 mRNA levels were higher in patients with active lupus nephritis than those without. Furthermore, among patients with active renal disease, levels were higher in patients with proliferative disease. Finally, levels were higher in the group of patients that did not respond to therapy (57.6 ± 69.8 copies) than in the group that did (2.4 ± 1.9 copies). This finding suggests that FOXP3 mRNA levels may be useful in predicting renal activity and response to treatment but will need to be further evaluated. TWEAK (tumor necrosis factor like weak inducer of apoptosis) is a pro inflammatory cytokine. The association of urinary TWEAK with lupus renal activity has been investigated in a multicenter cohort study.[13] Urinary TWEAK levels were higher in patients with lupus nephritis than in patients with lupus without nephritis. High levels of urinary TWEAK predicted lupus renal activity with an odds ratio of 7.36. Urinary TWEAK levels peaked during a renal flare and were significantly higher during the flare than before or after the flare. Whether TWEAK can be used to anticipate renal flares has not been shown.

Neutrophil gelatinase associated lipocalin (NGAL, also known as lipocalin 2) has been shown to be a marker of acute kidney injury (AKI) and has been proposed as a candidate marker for lupus flares. In a prospective study of emergency department patients, Nickolas et al studied the ability of a single measurement of urinary NGAL to identify patients presenting with AKI.[14] Urine NGAL was measured in 635 patients admitted through the emergency department. Mean NGAL concentration was significantly higher in patients with AKI. Based on this and other studies showing its predictive value for kidney injury in AKI (see Chapter 5), NGAL has been evaluated as a predictive marker in lupus nephritis. Anti double stranded DNA antibodies can upregulate the expression of NGAL in mesangial cells in the glomerulus. The levels of urinary NGAL have been compared between patients with lupus nephritis and those with lupus without renal disease activity.[15] Urinary levels were significantly higher in patients with lupus nephritis. Furthermore, the urinary levels of NGAL correlated significantly ($r = 0.452$, $P = 0.009$) with renal disease activity scores but not with extrarenal disease scores. NGAL has also been associated with renal flares in children with lupus nephritis.[16] In this study, urinary NGAL levels greater than 0.6 mg/dL were 90% sensitive and 100% specific for identifying childhood lupus nephritis relative to those with juvenile rheumatoid arthritis. Similarly, in an adult population higher NGAL levels were found in patients with active lupus nephritis.[15] These studies show an association with disease activity but do not necessarily demonstrate that NGAL can be used as an early marker to identify patients who have an imminent flare of lupus nephritis. However, several studies have looked at the predictive ability of NGAL. Hinze looked at urine from a group of 111 pediatric patients with systemic lupus erythematosus (SLE) to determine if NGAL levels increased before a clinical flare of lupus nephritis.[17] Urinary NGAL levels increased by as much as 104% 3 months prior to worsening of lupus nephritis. Plasma levels also increased prior to a renal flare but to a lesser extent. Rubinstein and colleagues have recently looked at a similar question.[18] Urinary NGAL levels from the previous visit were used to determine if they could predict renal disease activity at each subsequent visit. The AUC of urinary NGAL as a predictor of a renal flare was 0.76. This was greater than the AUC for anti double stranded DNA antibody titers and similar to the AUC values obtained for C3 and C4 levels. These studies demonstrate that urinary NGAL may be an early predictor for flares of lupus nephritis. New tests with better predictive value would be useful, however.

Urinary monocyte chemoattractant protein 1 (MCP 1) has also been proposed as a potential predictor of renal lupus flares.[19] Urinary MCP 1 concentration was higher at the time of a renal flare than it was at non renal flare and in healthy and non lupus renal controls. Furthermore, the increase in MCP 1 was seen 2–4 months prior to the clinical flare, indicating that it may be an early predictor. Levels of urine IL 6 have also been proposed as a marker of lupus renal activity.[20] Urine IL 6 levels correlated with disease activity as well as with active urine sediment. This study did not evaluate whether IL 6 levels could be used as an early predictor of renal lupus flare. Similar results were found for urinary vascular cell adhesion molecule 1 (VCAM 1)[21] and urine osteoprotegerin.[22] From these studies of early predictors of lupus renal disease activity, NGAL and MCP 1 appear to be the most promising. Further evaluation of these candidates as well as others will help to better guide treatment in patients with lupus nephritis.

3. MEMBRANOUS NEPHROPATHY

Membranous nephropathy is a frequent cause of nephrotic syndrome. The spontaneous outcome of membranous nephropathy can be difficult to predict which makes decisions about treatment hard. Without treatment, only about a third of patients with membranous nephropathy will have their renal function decline to the point that they need dialysis over 10 years.[23] The remaining two thirds of patients will not receive any benefit from treatment since the natural history of their disease is relatively benign. Better methods are needed to predict which patients will have progressive wors ening of renal function so that treatment can be targeted to these patients while patients that will not progress are spared from cytotoxic therapies. Protein expression in renal biopsy tissue is one potential method to predict the patient outcome regarding progression of the disease. Interstitial smooth muscle actin (SMA) was stained for on the renal biopsy to determine if it could be used as a predictor of progression.[24] SMA staining in the myo fibroblasts strongly correlated with GFR after 7 years of follow up. In a similar study, interstitial alpha smooth muscle actin staining on the renal biopsy was strongly associated with progression to end stage renal disease (ESRD) among patients with membranous nephropathy. In addition MCP 1 positive infiltrating mononuclear cells were strongly associated with progression to ESRD.[25] These studies show that findings on biopsy may be useful to predict progression of idiopathic membranous nephropathy but the findings will need to be further defined in larger and more diverse

populations. Staining of biopsies for specific proteins that can predict outcome is promising but is less likely to provide the ability to longitudinally follow the disease course in patients. Urinary levels of a number of proteins have also been used to predict the progression of renal disease in idiopathic membranous nephropathy. In these studies beta$_2$ microglobulin (β2m), IgG, urinary complement levels, NAG and L fatty acid binding protein (L FABP) have been used with varying success to predict progression of renal disease. Unfortunately, a number of definitions of progression of disease have been used and none have used hard outcomes such as progression to ESRD. Urinary β2m levels and IgG levels were used to attempt to predict outcome of membranous nephropathy.[26] All patients in this study had a baseline serum creatinine which was less than 1.5 mg/dL. The endpoint was serum creatine greater than 1.5 mg/dL or 50% rise in serum creatinine. After 53 months, 44% of the patients had met this endpoint. After multivariate analysis, urinary β2m was the strongest predictor for progression. Sensitivity and specificity to predict progression were 88% and 91%. Urinary IgG performed slightly less well. Although the sensitivity and specificity of these markers is not yet high enough to base them on the use of cytotoxic therapy, the results are encouraging. Activation of the complement system may play a role in the pathogenesis and progression of membranous nephropathy. Urinary C3dg and C5b 9 levels have been measured in patients with IgA and membranous nephropathy.[27] High urinary concentration of these two complement activation markers were found to correlate with membranous nephropathy and not IgA nephropathy. Furthermore, 66% of patients with membranous nephropathy who had high levels of urinary C5b 9 showed an unstable clinical course with deteriorating renal function compared to only 18% of those with a low level. This study demonstrates that urinary complement levels may be beneficial in predicting which patients should be treated with membranous nephropathy. However, markers that would provide better separation of progressors from non progressors are needed. Another urinary marker that has been used to predict outcome in membranous nephropathy is L FABP.[28] In a study of 40 patients, urinary levels of L FABP predicted worsening of renal function with a sensitivity of 81% and a specificity of 83%. Renal failure (defined as an increase in serum creatinine greater than 25% and exceeding 1.5 mg/dL) occurred in approximately 15% of patients with L FABP levels lower than 5.7 μg/mmol while approximately 82% of patients with higher levels of L FABP had renal failure. This separation of progressors from non progressors is slightly better than that seen with

urinary complement levels but still not adequately predictive to be used to change treatment decisions. The same authors have examined the role of urinary β2m in predicting prognosis in membranous nephropathy.[29] In this study, the endpoint was slightly different than the study of L FABP. Renal failure was defined as an increase in serum creatinine of 50% or a serum creatinine greater than 1.5 mg/dL. Renal survival was 32% at 1 year in the group with high β2m and 93% in the group with low β2m. β2m was superior at predicting progression of renal disease to NAG but similar to L FABP. In summary, a number of potential urinary biomarkers to predict progression of idiopathic membranous nephropathy have been proposed. The most promising are urinary β2m, L FABP and the complement acti vation marker C5b 9. These urinary markers have the added advantage that they can be followed sequentially during treatment, unlike findings on renal biopsy. These biomarkers have the potential to greatly improve our ability to select patients who should be treated for membranous nephropathy. The majority of the studies, however, have used small increases in serum creatinine as an endpoint. While this is an important first step since these patients are more likely to progress further, it does not definitively identify patients who will develop ESRD. Future studies should focus on identifying patients with hard outcomes such as progression to ESRD, including larger numbers of patients, using combinations of markers to predict outcome and identifying novel markers.

4. FOCAL SEGMENTAL GLOMERULOSCLEROSIS

FSGS is a common cause of nephrotic syndrome. Initial treatment is typically done with corticosteroids for a prolonged period but many patients do not respond. Markers that could predict which patients would respond could help avoid the toxicity of long term treatment in patients who ultimately will not benefit from treatment. Mastroianni and colleagues examined the value of urinary retinol binding protein as a prognostic marker in the treatment of nephrotic syndrome.[30] Urine levels of plasma retinol binding protein were measured in patients with FSGS, minimal change disease or mesangial proliferative glomerulonephritis. Patients with pretreatment levels less than 1 mg/mL were 30 times more likely to respond to treatment than those with higher levels. Furthermore, patients with higher baseline levels of urinary plasma retinol binding protein that normalized during treatment were more likely to respond to treatment than those patients that did not. This finding is promising but will need to be validated in additional patients.

5. MINIMAL CHANGE DISEASE

Minimal change disease is the most common glomerular disease in children and also a common cause of nephrotic syndrome in adults. Renal biopsy is typically not performed in children presenting with nephrotic syndrome until after a treatment attempt with steroids has failed. A method to differentiate minimal change disease (MCD) from other diseases would be very helpful in guiding treatment. Garin and colleagues measured the urine levels of soluble CD80 (sCD80) in patients with relapsed MCD as well as several other glomerular diseases.[31] Urinary concentrations of sCD80 were significantly higher in patients with relapsed MCD compared to patients in remission, patients with other glomerular diseases and normal controls. In a follow up study, the authors compared urinary concentrations of sCD80 between patients with relapsed MCD and patients with FSGS.[32] Levels were significantly higher in patients with relapsed MCD. A ROC curve showed that the AUC value for differentiating relapsed MCD and FSGS was 0.99 and the AUC value for relapsed MCD verses remission was 1.0. Further more, CD80 protein expression was present in renal biopsy tissue in 7/7 biopsies from patients with relapsed MCD while no staining was seen in biopsy tissue from two patients with FSGS and one patient with MCD in remission. These data are exciting in that they are the first to show that a single marker may be able to differentiate between two diseases that may be otherwise difficult to differentiate without a renal biopsy. Furthermore, the findings have been replicated in a separate, chronologically distinct population and there are biopsy data to support the findings. The studies suggest that urinary sCD80 may be a biomarker to differentiate FSGS from MCD. To demonstrate the usefulness of this marker, the findings should be replicated by other authors.

Other studies of biomarkers in MCD have looked at the association of proteins with relapse or response to treatment. NAG levels have been evaluated to determine their association with remission and relapse in children with MCD.[33] Levels of urinary NAG (normalized for urine creatinine) in patients in remission were the same as normal controls. In patients with relapse, urinary levels were elevated, demonstrating that NAG may be an indicator of relapse. Levels of urinary NAG have not been measured prior to clinical relapse to determine if they may be used as an early marker to predict relapse. Woroniecki evaluated the ability of a set of urinary cytokines to distinguish between MCD and FSGS as well as between steroid responsive and steroid resistant nephrotic syndrome.[34]

There were no significant differences in the levels of ICAM 1 and TGF β1 related to steroid responsiveness. Urinary concentrations of TGF β1 were significantly higher in patients with FSGS compared to those with MCD.

6. IGA NEPHROPATHY

IgA nephropathy is the most common primary glomerular disease world wide. The clinical course of IgA nephropathy can be highly variable, which makes decisions about the treatment difficult. Many patients have a long term indolent course of their disease. A number of studies have evaluated the ability of protein markers in tissue biopsies or in urine or serum to predict the progression of the disease. Renal biopsy is usually performed so evaluation of tissue can be an informative method to obtain information about prognosis. The crescentic variant of IgA nephropathy has a worse prognosis than variants without crescents. Bazzi and colleagues evaluated the ability of several different predictors of progression in crescentic IgA nephropathy after biopsy.[35] Serum creatinine alone was the best single predictor of progression with an AUC of 0.92. The fractional excretion of IgG normalized for the percent of glomeruli that were not globally sclerotic on the renal biopsy was the second best with an AUC value of 0.90. Interestingly, the combination of serum creatinine and normalized fractional excretion of IgG was able to stratify the patients into a high and low risk group in which 100% of the patients in the high risk group progressed and none of the patients in the low risk group progressed. Although this discrimination requires renal biopsy, it indicates that urinary excretion of IgG can be useful in helping determine the prognosis of patients with crescentic IgA nephropathy. Similarly, Van Es did a retrospective analysis of renal biopsies from 50 patients with IgA nephropathy to determine if GMP 17 positive T lymphocytes in renal tubules predict progression in early stages of IgA nephropathy.[36] They found that there was a positive association between GMP 17 positive cytotoxic T lymphocytes in intact renal tubules and progression of IgA nephropathy, indicating that GMP 17 positive cytotoxic T lymphocytes may be another marker of progression that can be determined using renal biopsy. A third study used findings on biopsy to predict which patients with IgA nephropathy would have a positive response to treatment with steroids. This study used the number of cells in the biopsy that were positive for fibroblast specific protein 1 (FSP1) in patients with IgA nephropathy.[37] The investigators compared the ability of serum creatinine, estimated GFR, severity of mesangial proliferation, percent of

sclerotic glomeruli, extent of interstitial damage and FSP1 positive cell number to predict the response to treatment with corticosteroids. The number of FSP1 positive cells was the strongest predictor of response. When patient biopsies had more than 32.6 positive cells/high power field, they were more likely to show steroid resistance. A similar study of FSP1 in renal biopsy showed a similar correlation with prognosis.[38] These studies demonstrate that findings on renal biopsy may be able to predict response (or lack of response).

Differentiating between IgA nephropathy and a host of other glomerular diseases can be a difficult problem. Boor compared the serum levels of platelet derived growth factor DD (PDGF DD) in patients with IgA nephropathy to patients with lupus nephritis, FSGS, membranous glomerulonephritis and ANCA associated vasculitis as well as healthy controls.[39] Only patients with IgA nephropathy had levels of PDGF DD which were significantly higher than control patients, suggesting that serum PDGF DD may be useful as part of a panel of biomarkers to distinguish glomerular diseases.

Tubulointerstitial injury occurs with IgA nephropathy and can be an important part of the injury process. The injury can be detected by renal biopsy but biomarkers can help with diagnosis. Urinary concentrations of NGAL have been compared to evidence of tubulointerstitial injury on biopsy.[40] Both NGAL and NAG were elevated in patients with IgA nephropathy and tubulointerstitial injury but increases in NGAL were seen with earlier (Lee grade II) lesions than were increases in NAG. This suggests that urinary NGAL levels could potentially be used to identify and follow tubulointerstitial injury. The prognostic value of this association has not been evaluated.

A very interesting study looked at the prognostic value of urinary interleukin 6 (IL 6) in patients with IgA nephropathy.[41] Urinary IL 6 levels were measured in 59 patients with IgA nephropathy who were followed for a mean of 8 years. IL 6 levels were significantly higher in progressors. Furthermore, patients with urinary IL 6 levels greater than 2.5 ng/day at diagnosis had a 7.8 fold higher risk of progression than patients with lower levels. Urine IL 6 is a compelling candidate marker to predict progression in IgA nephropathy but will need to be further evaluated. Serum IgA/C3 ratio has also been measured to attempt to correlate the levels with renal prog nosis. Increasing values of the ratio were associated with worsening prog nosis.[42,43] While the association was not as strong as that seen in studies of urinary IL 6, serum IgA/C3 ratio may provide important additional

information about prognosis. The ratio of epidermal growth factor (EGF) to monocyte chemotactic peptide 1 (MCP 1) in the urine has also been used to predict renal prognosis in IgA nephropathy.[44] This study was based on previous findings that EGF may modulate the renal response to injury whereas MCP 1 plays a role in progression of renal disease possibly by recruiting inflammatory cells into the interstitium. Patients were divided into tertiles based on the ratio of EGF to MCP 1. Patients in the lowest tertile had a significant decline in renal function while those in the highest tertile had a 100% renal survival at 48 and 84 months of follow up. The area under the receiver operator characteristics (ROC) curve was used to determine the quality of the tests as a predictor of adverse outcomes. MCP 1 had an area under the curve (AUC) of 0.57. EGF alone had an AUC value of 0.83 while the ratio had an AUC of 0.91. These data indicate that the ratio of EGF to MCP 1 is another compelling candidate to predict progression of IgA nephropathy. Complement factor H plays a role in regulating complement activation. The potential of urinary complement factor H as a biomarker of IgA nephropathy disease activity has been investigated.[45] The urinary levels of complement factor H were associated with disease activity as measured by serum creatinine and the amount of proteinuria. Whether this analyte can be used to predict prognosis in patients with IgA nephropathy has not been evaluated.

In summary, a number of promising candidates to predict the risk of worsening renal function in IgA nephropathy have been proposed. The numbers of GMP 17 positive T lymphocytes and FSP1 positive cells look promising as potential markers in renal biopsy. Studies of urinary IL 6 and the ratio of EGF to MCP 1 look particularly promising but will need to be further evaluated. Studies which combine measurement of urinary IL 6, EGF and MCP 1 may be particularly enlightening. Measurement of urinary values has the added benefit that the measurements can be done serially to determine if they can be used to assess response to treatment or to time the initiation of treatment.

7. ANCA-ASSOCIATED VASCULITIS

Renal function at diagnosis is a strong predictor of renal survival in ANCA associated vasculitis. Bakoush and colleagues evaluated whether urinary IgM excretion may also be a good predictor of renal survival.[46] Univariate analysis showed age, level of serum creatinine, albuminuria and urine IgM were inversely correlated with renal survival. In a multivariate analysis, only

patient age and urinary excretion of IgM were associated with renal survival. This study demonstrates that urinary excretion of IgM may be a better predictor of renal survival than serum creatinine but will need to be replicated in other populations.

8. DISCOVERY OF NEW BIOMARKERS USING PROTEOMICS

Proteomic analysis provides an additional tool to identify novel biomarkers. These novel markers can then be tested to determine their validity. In addition, combinations of biomarkers can be analyzed using statistical or informatic tools. These approaches have been used to attempt to diagnose glomerular diseases and to provide prognostic information. One of the promising techniques is capillary electrophoresis coupled to mass spectrometry (CE/MS). This approach was used to identify patterns of polypeptides that can differentiate between glomerular diseases including differentiating minimal change disease from FSGS.[47] While the approach needs to be further validated, it provides an interesting insight into the potential of such techniques. Julian and colleagues used a similar approach to characterize IgA related renal diseases.[48] In this study urine from patients with IgA nephropathy, Henoch–Schönlein purpura and IgA associated nephropathy secondary to hepatitis C virus was analyzed by CE/MS. A pattern of polypeptides was identified which was seen in 90% of patients with IgA nephropathy and Henoch–Schönlein purpura but only 1% of patients with hepatitis C related disease and no normal control patients. While these findings using CE/MS are interesting, the technology is not currently available to measure the multiple polypeptides necessary on a clinical basis. Another proteomic technique with the potential to identify novel biomarkers is two dimensional gel electrophoresis (2DE). We used 2DE to identify a set of proteins that can predict the cause of glomerular disease from among diabetic nephropathy, FSGS, membranous nephropathy and lupus nephritis.[49] Interestingly, the urinary proteins that allowed the differentiation were glycosylated charge forms of proteins. This finding implies that different glomerular diseases have different glomerular permeability to charged proteins. This finding has not yet been replicated in a different set of patients. Two dimensional gel electrophoresis has also been used to identify urine proteins which are present in patients with IgA nephropathy but not in the urine of normal controls.[50] The investigators found 82 protein spots that were increased in urine from patients with IgA nephropathy relative to controls and 134 that were decreased. Eighty four of the proteins were identified by

peptide mass fingerprinting. Although it is not known if these proteins are also differentially present in other renal diseases, it provides a list of protein candidates known to be present in the urine of patients with IgA nephrop athy. An interesting approach to determine the response to treatment with ACE inhibitors of patients with IgA nephropathy used 2DE to identify urine proteins that were different between responders and non responders.[51] Kininogen, inter alpha trypsin inhibitor heavy chain and transthyretin were identified as proteins that differed between patients with IgA nephropathy that did and did not improve with ACE inhibitor treatment. Immunoblotting was then used to confirm that patients with low levels of kininogen at baseline were less likely to respond to treatment with ACE inhibitors. Another approach to identify candidate biomarkers is to analyze proteins and peptides using surface enhanced laser desorption and ionization mass spectrometry (SELDI MS). In this technique proteins are adsorbed to a surface based on specific chemistries, unbound proteins are washed off and the remaining proteins are analyzed by mass spectrometry. Using this technique, the urine proteome of children with steroid sensitive and steroid resistant nephrotic syndrome has been evaluated.[52] The authors identified a pattern of poly peptides which could predict steroid responsiveness 100% of the time. A protein of mass 4144 Daltons was identified as the most important classifier in the group of polypeptides but the identity of the peptide was not determined. These results are promising in that they show the potential of combinations of proteins to predict outcome but will need to be validated. Another study that used SELDI MS serially examined urine from patients with lupus nephritis.[53] Urine from 19 patients was obtained at baseline, pre flare, flare and post flare. SELDI MS analysis followed by tandem mass spectrometry sequencing of relevant peptides was done. Several proteins were identified which had concentrations that peaked during or before renal flares. The 20 amino acid isoform of hepcidin was found to increase 4 months before renal flare and returned to baseline at the time of the flare. In contrast, the 25 amino acid isoform of hepcidin decreased at flare and returned to baseline 4 months after. These data about the ability to identify and use early markers for renal flare in lupus are promising but will need to be further validated. Methods using liquid chromatography/mass spectrometry to identify differentially expressed proteins that predict the identity of the disease or give prognostic information have not yet been used. These approaches are perhaps more likely to identify single proteins or combinations of proteins with diagnostic or prognostic potential. The coming years are likely to provide more of these studies.

A number of candidate markers have been identified in glomerular diseases. The most promising are markers for early prediction of flares in patients with lupus nephritis, prediction of outcome in patients with membranous nephropathy, differentiation between children with minimal change disease and FSGS and progression of IgA nephropathy. Some of these markers, such as the use of sCD80 to differentiate between children with FSGS and minimal change disease and the use of NGAL as a predictor of renal flares in lupus, may be validated to the point where they can be used clinically within the next several years. Other markers still require much validation and may ultimately not be sufficiently discriminatory to serve as useful markers. The use of discovery techniques such as proteomics may provide additional new candidate markers. Ultimately, some diseases are likely to require the use of combinations of markers in useful clinical assays.

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CHAPTER 11

Biomarkers in Preeclampsia

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Biomarkers in Kidney Disease
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1. DEFINITION AND PREVALENCE OF THE DISEASE

Preeclampsia, the most frequently encountered renal complication of pregnancy, is a leading cause of maternal and perinatal morbidity and mortality worldwide. It is a multi systemic disease that complicates 3–8% of pregnancies, and that is characterized by new onset hypertension and proteinuria after 20 weeks of gestation.[1] In the mother, the disease can progress to widespread endothelial dysfunction affecting mainly the liver, brain and kidney. In the fetus it is associated with intrauterine growth restriction and prematurity.[2] As of 2010 there are still no clinically useful tests to predict the disease, and the only known cure is delivery of the placenta.

In developing countries where access to health care is limited, preeclampsia is a leading cause of maternal mortality. Of the estimated 60,000 or more deaths from preeclampsia worldwide each year, > 90% of the deaths are in low and middle income countries.[1] In the developed world, the burden falls on the neonate, since premature deliveries are performed to preserve the health of the mother. Although technological advances in perinatal and neonatal care have reduced infant mortality due to preterm birth, morbidity remains a serious problem. These babies are at increased risk of neurodevelopment disabilities such as cerebral palsy, mental retardation, sensory deficits and behavioral impairments[3] and are also more vulnerable to metabolic disorders and cardiovascular disease (CVD) later in life.[4 6]

Preeclampsia is not only responsible for adverse pregnancy outcomes, but also predisposes to long term health complications entailing a major economic and familial burden in society. The ability to predict or prevent preeclampsia, and the development of a therapy that safely prolongs gesta tion are of critical importance and would constitute a major advance in women's health.

Many factors have been associated with an increased risk of developing preeclampsia, including familial obstetric history, preexisting medical conditions, age, and characteristics of pregnancy such as parity. In a systematic review of controlled studies, Duckitt and Harrington reported that nulliparity, multiple pregnancy, family history of preeclampsia, history of preeclampsia in a previous pregnancy, a time span of more than 10 years since the last pregnancy, maternal age above 40, raised body mass index, raised blood pressure at booking and preexisting medical conditions such as antiphospholipid antibodies, diabetes, hypertension and renal disease, were

associated with an increased risk of developing preeclampsia.[7] Nulliparity and multifetal gestations increased the risk almost threefold, and anti phospholipid antibodies over ninefold.[7] Paternal factors have also been implicated. Reproductive practices that minimize exposure to sperm, such as barrier contraception, non partner donor insemination and short dura tion of sexual cohabitation with the father before conception are associated with an increased risk of preeclampsia. Indeed, multiparous women preg nant with a new partner have a risk similar to nulliparous women. It is still not clear if this effect is due to the change in paternity per se or to the greater risk associated with increased interpregnancy interval.[8]

2. PATHOPHYSIOLOGY AND MECHANISMS

It has long been recognized that preeclampsia will not resolve until after complete placental delivery. Further, as illustrated by cases of molar and extrauterine pregnancies, while the placenta is required for developing preeclampsia, the fetus is not.[9,10] In addition, cases of postpartum eclampsia have been associated with retained placental fragments, with rapid improvement after uterine curettage.[11] Taken together, these observations suggest that the placenta is both necessary and sufficient for the development of preeclampsia. First proposed by E.W. Page in 1939,[12] it is now widely recognized that the placenta is the central culprit in the pathogenesis of the disease.

Research has focused on the placenta as the source of the disease, and has tried to unravel the mechanisms that ultimately lead to generalized maternal endothelial dysfunction. Many hypotheses have emerged that attempt to gather a causal framework for the disease, causing preeclampsia to be named the 'disease of theories.' Despite intensive investigation, its etiology and pathogenesis is not completely understood, and as of 2010 there is no cure other than delivery of the placenta. Nevertheless, knowledge in the field is progressing substantially with recent findings, opening new perspectives for the near future, specifically key discoveries about alterations in placental antiangiogenic factors in the pathogenesis of the clinical syndrome.

It has been suggested that preeclampsia is caused by placental dysfunction followed by the release of factors by the diseased placenta into the maternal circulation, inducing widespread endothelial dysfunction that heralds the classic manifestations of the disease[13] (Figure 11.1). In this regard, two antiangiogenic proteins overproduced by the placenta that gain access to the maternal circulation have become candidate molecules responsible for the

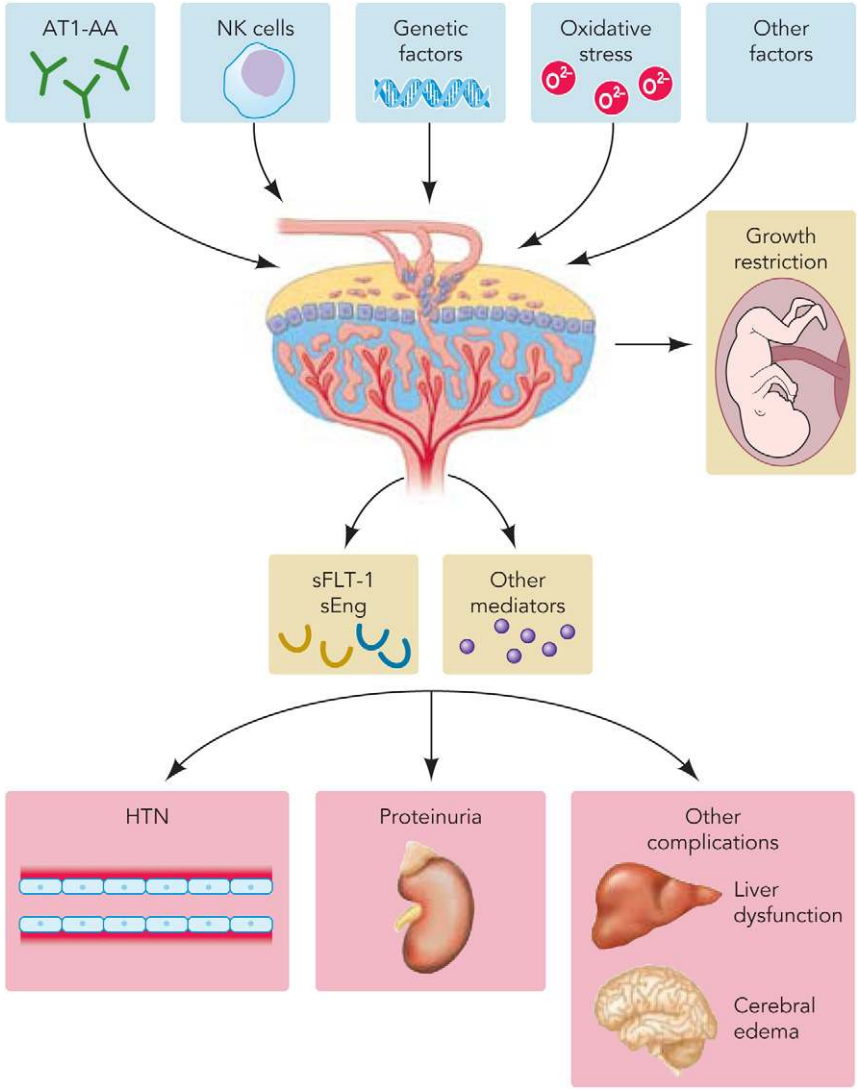

Figure 11.1 ***Summary of the pathogenesis of preeclampsia.*** Immune factors such as autoantibodies against the angiotensin receptor (AT1-AA), oxidative stress, natural killer (NK) cell abnormalities and other factors may cause placental dysfunction, which in turn leads to the release of antiangiogenic factors (such as sFlt-1 and sEng) and other inflammatory mediators to induce hypertension (HTN), proteinuria and other complications of preeclampsia. Reproduced with permission from Wang et al.[158]

phenotype of preeclampsia. Soluble Fms like tyrosine kinase 1 (sFlt 1), an endogenous inhibitor of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), and soluble endoglin (sEng), a circulating co receptor of transforming growth factor beta, have been shown to be at increased levels in the serum of preeclamptic women, as compared to normal pregnancy, weeks before the appearance of overt clinical manifes tations of the disease.[14,15] In addition, when injected into rats, these molecules produce systemic endothelial dysfunction resulting in a preeclampsia like phenotype, including severe hypertension, proteinuria, glomerular endotheliosis and features resembling HELLP syndrome (hemolysis, elevated liver enzymes, low platelets).[16]

An array of insults may contribute to placental damage that is proximally linked to the production of soluble pathogenic factors by this organ. Various pathways have been proposed to have key roles in inducing placental disease, including deficient heme oxygenase expression, placental hypoxia, genetic factors, autoantibodies against the angiotensin receptor, oxidative stress, inflammation, altered natural killer cell signaling and, more recently, defi cient catechol *O* methyl transferase.[17] Interestingly, most of these were shown to increase placental production of the antiangiogenic factors. Still, the underlying events that induce placental disease activating the cascade of placental damage and antiangiogenic factor production remain unknown.

3. CLINICAL MANIFESTATIONS

Besides hypertension and proteinuria de novo after 20 weeks of gestation, the hallmarks of the disease, preeclampsia may also be accompanied by other manifestations. Additional signs and symptoms that can occur include edema, acute renal failure, liver abnormalities, thrombocytopenia, micro angiopathic hemolytic anemia, placental abruption, visual disturbances, stroke, seizures and death. These manifestations are the result of widespread endothelial dysfunction affecting mainly the liver, kidney and brain (Figure 11.1).

Hemolysis, abnormal elevation of liver enzymes levels and low platelet count occur together as the HELLP syndrome. *H* for hemolysis, *EL* for elevated liver enzymes and *LP* for low platelets.[18] Considered by many to be a severe variant of preeclampsia, HELLP syndrome occurs in 5% of cases and can progress rapidly to a life threatening condition.[19] Another serious complication of preeclampsia is eclampsia, differentiated from the former by the presence of seizures.[20] The onset of eclampsia is often heralded by

headache, visual disturbances and epigastric pain. However, the eclamptic convulsion can occur suddenly and without warning. Once associated with a high mortality rate, improved and aggressive management have decreased the occurrence of convulsions, and nowadays maternal deaths are unusual.[21]

The clinical spectrum varies widely from preeclampsia accompanied by mild hypertension and without demonstrable fetal involvement, to preeclampsia with various organ dysfunction, HELLP syndrome, eclampsia, intrauterine growth restriction (IUGR) and preterm delivery. Women with mild preeclampsia developing at term generally have pregnancy outcomes similar to those of women with normotensive pregnancies. Some women will experience an atypical presentation of the disease, e.g. with the absence of hypertension or proteinuria, or that manifests outside the established gestational time period.[22] It has been reported that 10% of women with other clinical and/or histological manifestations of preeclampsia have minimal or no proteinuria, and that 14% of women who develop eclampsia have no proteinuria at all.[21] Moreover, either hypertension or proteinuria may be absent in 10–15% of women who develop HELLP syndrome.[19]

For clinical purposes, presentations have been classified as mild or severe, and early or late onset. In fact, mild and severe presentations are usually associated with mild and severe outcomes respectively, and early onset of the disease (< 34 weeks) is associated with greater morbidity than when the disorder presents at term. Nonetheless, these classifications can be misleading, since any preeclampsia can rapidly progress to a devastating form.[23]

3.1. The kidney

In preeclampsia, renal plasma flow (RPF) and glomerular filtration rate (GFR) decrease by approximately 25%. It is important to note that in normal pregnancy RPF and GFR generally increase from 30% to 50%. Thus, blood urea nitrogen and creatinine in preeclampsia may actually be very approximate to or slightly above those seen in the normal range for non pregnant women. The degree of proteinuria varies from minimal to the nephrotic range, and does not appear to be correlated with maternal and fetal outcomes.[24] Hyperuricemia and hypocalciuria also occur. Urinary sediment is usually bland, and red blood cells and cellular casts are rarely seen. The decrement in RPF is attributable to vasoconstriction, whereas the fall in GFR relates both to the decrement of RPF and the development of a particular glomerular lesion termed glomerular endotheliosis. Glomerular

endotheliosis is a unique pathological condition characterized by ultra structural changes in the renal glomeruli that shares some similarities with thrombotic microangiopathies, but that also shows some intriguing differ ences. Under light microscopy, the glomeruli appear relatively large and the glomerular capillary lumen appears 'bloodless' due to endothelial and mesangial cell swelling and hypertrophy. Fibrin deposition can be detected by immunofluorescence, but thrombosis is definitely unusual. Electron microscopy reveals subendothelial electron dense fibrinoid and granular deposits, as well as loss of endothelial fenestrae.[25,26]

Despite marked proteinuria, the epithelial foot processes appear rela tively intact. Nonetheless recent evidence suggests that these podocytes are also affected, as considerable podocyturia accompanies the proteinuria.[27] In rare cases, preeclampsia can lead to acute renal failure in pregnancy. After delivery, the glomerular changes usually reverse rapidly, coinciding with the resolution of hypertension and proteinuria. Focal segmental glomerulo sclerosis is seen in some cases. Clinically, even these women regain normal renal function along with resolution of proteinuria.[26]

3.2. Liver and coagulation abnormalities

Normal pregnancy is associated with a relatively hypercoagulable state which is of teleological advantage in avoiding hemorrhage after delivery. In preeclampsia, this hypercoagulability is accentuated (e.g. reduced anti thrombin III, protein S and protein C) and is usually associated with platelet activation and thrombocytopenia. These occur most likely as a result of endothelial cell damage/thrombotic microangiopathy.[26]

The extent of liver damage in preeclampsia depends on disease severity. Increases in transaminases and lactic acid dehydrogenase levels are generally seen. These are usually mild except in the setting of HELLP syndrome. Indeed, transaminases levels are a clinical marker for severity of disease. Examination of the liver can show periportal hemorrage, ischemic lesions and fibrin deposition. Bleeding from periportal lesions, or hemorrhage into infarcts, can cause an intrahepatic hematoma. Although rare, subcapsular bleeding leading to hepatic rupture can occur as a catastrophic complication of pregnancy.[26]

3.3. The brain

Besides convulsions (eclampsia), headache, blurred vision, scotoma and blindness occur as central nervous system manifestations of preeclampsia.

It can be due to retinal detachment or vascular occlusion, but more frequently is of cortical origin. Cortical blindness almost always resolves spontaneously after control of blood pressure, but blindness related to other causes such as progression of underlying diabetic retinopathy may be permanent. Stroke is a serious but rare complication. Fatal cases of preeclampsia demonstrate various degrees of cerebral bleeding, from microscopic petechiae to gross hemorrhage, ischemic brain damage, microinfarcts and fibrinoid necrosis. The cerebrovascular manifestations of severe preeclampsia are poorly understood, but may represent a form of reversible posterior leukoencephalopathy syndrome. Neuroradiography findings on computed tomography (CT) and magnetic resonance imaging (MRI) show vasogenic cerebral edema and infarctations in the subcortical white matter and in adjacent gray matter, predominantly in the parietal and occipital lobes. Interestingly, these same characteristic MRI changes have also been associated with the use of antiangiogenic agents in cancer therapy.[26]

3.4. Fetal complications

Although the acute manifestation of the disease is more ominous for the mother, the condition affects the fetus as well, imposing an increased risk of iatrogenic and spontaneous prematurity, intrauterine fetal growth restriction, oligohydramnios, and an increased risk of perinatal death. Although the exact pathogenesis of these complications is unknown, impaired uteroplacental blood flow or placental infarction are likely contributors. Many studies are now addressing the effects of preeclampsia per se on the health of these children, and whether or not these children (premature or term) are different from other premature and term children.

Children born to mothers who had preeclampsia, especially those born at term, had an increased risk of being hospitalized for a number of diseases such as endocrine, nutritional and metabolic diseases and diseases of the blood and blood forming organs.[28] In preterm IUGR children with signs of cardiac dysfunction, being born to mothers who had preeclampsia did not influence cardiac performance.[29] In addition, for those exposed to preeclampsia during pregnancy, increased blood pressure in childhood[30,31] and stroke in adult life have been reported.[32]

3.5. Long term complications

Traditionally, women have been reassured that the syndrome would resolve postpartum without long term consequences for the mother other than

a higher risk of recurrence of the disease in subsequent pregnancies. However, there is increasing evidence suggesting that these women are at an increased risk for developing end stage renal disease[33] and cardiovascular disease[34 37] later in life. Approximately 20% of women with preeclampsia develop hypertension or microalbuminuria within 7 years of a preeclamptic pregnancy, as compared with only 2% among women with uncomplicated pregnancies.[38] In a recent meta analysis, Bellamy et al showed that after a pregnancy complicated by preeclampsia, women had an increased risk for hypertension (3.7, 95% CI 2.70−5.05), ischemic heart disease (2.16, 95% CI 1.86−2.52), stroke (1.81, 95% CI 1.45−2.27) and venous thromboembolism (1.19, 95% CI 1.37−2.33).[34] The risk of death from cardiovascular and other causes is also increased in these women. Women with preeclampsia at term had a 1.65 fold higher long term risk of death (95% CI 1.01−2.70) from cardiovascular causes than women who did not have preeclampsia.[35] In particular, women with a history of preeclampsia and preterm birth had a dramatic 8.12 (95% CI 4.31−15.33) higher risk when compared to women with normal pregnancies. Mortality from all causes was also increased: 1.04 (95% CI 0.88−1.23) and 2.71 (95% CI 1.99−3.68) respectively.[35] Women with early onset, severe preeclampsia appear to be at highest risk.[36,37]

Whether preeclampsia is another manifestation of a shared pathophys iology or is an independent risk factor for CVD is still a matter of debate. Indeed, preeclampsia and CVD share many pathophysiological mechanisms and risk factors, such as obesity, diabetes and hypertension, that can lead to both preeclampsia and cardiovascular diseases at different times during a woman's life. On the other hand, preeclampsia may itself induce vascular and metabolic changes that increase the risk for developing CVD. Regardless of this debate, preeclampsia raises a red flag concerning the risk of cardiovascular disease in later life. Proper management targeting lifestyle and risk factor modification should be implemented.

4. DIAGNOSIS

Historically, there has been difficulty reaching a consensus on diagnostic criteria for preeclampsia, and the definitions have changed over time. Current criteria mandate new onset of hypertension and proteinuria after 20 weeks of gestation. Hypertension is defined as systolic blood pressure of $\geq$ 140 mmHg on two occasions or as diastolic blood pressure of $\geq$ 90 mmHg on two occasions at least 4−6 hours apart after the 20th week of gestation in women known to be normotensive before. Proteinuria is

defined as ≥ 0.3 g in a 24 h urine specimen and/or protein to creatinine ratio of > 0.30.[20]

Although the criteria presented are clear, accurate diagnosis of preeclampsia may not be straightforward. Accurate diagnosis relies on precise blood pressure and proteinuria measurements, and, of course, general agreement on the criteria. It is well recognized that blood pressure measurement is prone to inaccuracy due to observer and device error.[39] In addition, the 24 h urine specimen is not always available, and studies have shown that urinary dipstick determinations correlate poorly with the amount of protein found in 24 h urine samples.[40,41] More recently, the urinary protein to creatinine (P:C) ratio has become the preferred method for quantification of proteinuria in the non pregnant population. However, its use to estimate 24 h protein excretion for the diagnosis of preeclampsia has been controversial. Several studies have compared the P:C ratio with 24 h urine collection in this setting, with discordant conclusions. A meta analysis showed a pooled sensitivity of 84% and specificity of 76% using P:C ratio cutoff of greater than 30 mg/mmol, as compared with the gold standard of 24 hour urine protein excretion > 300 mg/day.[42]

Several clinical and laboratory findings suggest severe disease, and should prompt consideration of immediate delivery. Oliguria (less than 500 mL urine in 24 h) is usually transient; acute renal failure, though uncommon, can occur. Persistent headache or visual disturbances can be a prodrome to seizures. Pulmonary edema complicates 2–3% of severe preeclampsia and can lead to respiratory failure. Epigastric or right upper quadrant pain may be associated with liver injury. Elevated liver enzymes can occur alone or as part of the HELLP syndrome.

Preeclampsia should be distinguished from other disorders that can occur with increased blood pressure in pregnancy, including chronic hypertension and gestational hypertension. Chronic hypertension is defined as hyper tension that is present and observable before pregnancy, or that is diagnosed before the 20th week of gestation. Gestational hypertension is defined as transient hypertension during pregnancy if preeclampsia is not present at the time of delivery, and blood pressure returns to normal by 12 weeks post partum.[20] It is important to discriminate between preeclampsia and these conditions, since pregnancy management and outcome differ substantially. Most women with chronic hypertension have uneventful gestations as long as their blood pressure remains at (or is controlled to) levels considered ‘mild to moderate’. In contrast, preeclampsia is associated with many adverse maternal and fetal complications.

The wide clinical variability and presentation of the disease, including atypical presentations, make diagnosis more challenging. Preeclampsia can also appear as early as 12 weeks of gestation in the case of trophoblastic diseases such as hydatidiform mole.[20] Predicting preeclampsia weeks before its clinical presentation is crucial, and involves close monitoring, earlier recognition of the syndrome, and proper and timely intervention before life threatening complications develop. Hence, there is an urgent need for biomarkers that can identify women at increased risk of developing the disease, can accurately rule out diagnoses in suspected cases, and thus identify women at increased risk of adverse outcome. Such a biomarker would also assist the investigation of targeted strategies for prevention and treatment of preeclampsia.

5. BIOMARKERS

It is important to note that the utility of a predictive test will depend on the overall prevalence of the disease. Since the incidence of preeclampsia is relatively low, screening tests with positive test results require high likeli hood ratios (LR) in order to adequately predict the disease's probability, and tests with negative results require very low likelihood ratios to confidently exclude the disorder. Thus, useful prediction for preeclampsia would require a very high likelihood ratio (> 15) for a positive test as well as a very low likelihood ratio for a negative result (< 0.1).[43,44]

5.1. Angiogenic markers

Since alterations in absolute levels of sFlt 1, VEGF, PlGF and sEng in the maternal circulation precede the clinical onset of preeclampsia by several weeks to months, they have been proposed as a potential predictive test. Levine and colleagues performed a nested case control study within the Calcium for Preeclampsia Prevention (CPEP) trial that included 120 preeclamptic women and 120 normotensive pregnancies and measured serum concentrations of angiogenic factors (total sFtl 1, free VEGF and free PlGF) throughout pregnancy.[14] In normotensive pregnancies, sFlt 1 levels were stable during the early and middle stages of gestation, and started to rise at 33–36 weeks. PlGF concentrations increased during the first two trimesters, peaked at 29–32 weeks, and decreased thereafter. Consistent with what was previously observed, sFlt 1 levels were significantly higher in the preeclamptic group during clinical disease.[45,46] Furthermore, Levine et al observed that circulating levels of sFlt 1 began to increase 5 weeks before

the clinical onset of preeclampsia and correlated with disease severity. In parallel with the increase in sFlt 1 levels, free PlGF and free VEGF levels decreased, suggesting that those levels were the result of binding by sFlt 1. Low levels of PlGF at both 13–20 and at 21–32 weeks were predictive of preterm preeclampsia, and low levels at 33–41 weeks were predictive of term preeclampsia. Associations between sFlt 1 levels and preeclampsia were not observed until closer to the onset of disease. High sFlt 1 levels no earlier than 21–32 weeks were predictive of preterm preeclampsia, while high sFlt 1 levels at 33–41 weeks predicted term preeclampsia. In general, women who developed severe and/or early onset preeclampsia had higher sFlt 1 and lower PlGF levels at each of the time intervals.[14]

Later, prompted by the finding that sEng acts together with sFlt 1 in the pathogenesis of preeclampsia, the same group performed another nested case control study, this time with an additional focus on sEng.[15] In normal pregnancies serum levels of sEng started to rise at 33–36 weeks of gestation, but rose earlier and more steeply in women who developed preeclampsia, and reached a peak at the onset of clinical disease. sEng levels began to rise 9–11 weeks before the clinical onset of preterm preeclampsia, and began to rise 12–14 weeks before the clinical onset of term preeclampsia. High levels of sEng at 13–20 weeks and at 21–32 weeks were predictive of preterm preeclampsia, and high levels at 21–32 weeks and at 33–41 weeks were predictive of term preeclampsia. Of note, levels were not markedly elevated prior to disease presentation either in women who developed gestational hypertension, or in normotensive women delivering small for gestational age babies.[15]

The sFlt 1:PlGF ratio, an index of antiangiogenic activity that reflects both increased sFlt 1 and decreased PlGF, was also evaluated, and found to parallel sEng levels. However, multivariate analysis showed that each was independently associated with preeclampsia, and that the sFlt 1:PlGF ratio predicted preeclampsia more reliably than protein alone. Adding sEng to the equation, (sFlt 1 + sEng):PlGF ratio was more strongly predictive of preeclampsia than were individual biomarkers. Finally, when analyzed, the risk among women with high or low levels of sEng, of sFlt 1:PlGF ratio or of both, the risk of developing preeclampsia was greatest (Odds Ratio > 30) among women with higher levels of both sEng and sFlt 1:PlGF ratio, while women with high levels of a single biomarker had only small elevations in the risk of developing preeclampsia (Odds Ratio of 2.3–7.4).

These two studies reported exciting findings with promising Odds Ratio (OR), especially for preterm preeclampsia, based on biomarker levels in the

second trimester. Various other independent studies analyzed the predictive accuracy of angiogenic factors, reporting significant changes in PlGF, sFlt 1 or sEng before the onset of preeclampsia[47 59] (Table 11.1). Overall, the sensitivity, specificity and positive and negative likelihood ratios of PlGF, sFlt 1 and sEng for all cases of preeclampsia ranged between 40% and 100%, 43% and 100%, 1.4 to infinity and 0.1–0.8, respectively. For early onset preeclampsia, the sensitivity, specificity, and positive and negative likelihood ratios varied between 17% and 100%, 51% and 97%, 1.7 and 24 and 0.0 and 0.9, respectively. This wide range in diagnostic performance might be explained by differences in study design, populations included, gestational age at sampling, etc.

Changes in PlGF are also seen as early as the first trimester whereas reproducible alterations in sFlt 1 and sEng are observed only in the mid to late second trimester onward. Predictive accuracy appears higher for early onset preeclampsia. The incorporation of these angiogenic factors into a single angiogenic index has improved accuracy in the prediction of preeclampsia. The index that has been extensively studied is sFlt 1:PlGF ratio, but many others have been proposed, such as PlGF:(sEng × sFlt 1), PlGF:sEng and PlGF:sFlt 1.[59]

5.1.1. Sequential changes

Because circulating concentrations of angiogenic factors change with gestational age, it has been proposed that sequential changes in levels of sFlt 1, PlGF and sEng could be more informative in assessing the risk for preeclampsia than are time point measurements. Rana et al[60] and Vatten et al[61] described that sequential changes in angiogenic factors from first to second trimester differ in women destined to develop preeclampsia. A small increase in PlGF and a high increase in sFtl 1 were strong predictors of preeclampsia. The Odds Ratios were higher for sequential change than for each measurement alone. Interestingly, the combination of the lowest quartile of PlGF change and the highest quartile of sFlt 1 change was associated with an OR of 35.3 (95% CI 7.6–164.2) for preterm preeclampsia, and a 3.2 (95% CI 1.4–7.0) OR for term preeclampsia.[61] Sequential changes of sEng were also predictive of preeclampsia.[60] Consistent with these results, Erez et al reported that differences in concentration of sEng, sFlt 1 and PlGF between first and second trimesters were associated with increased risk of preterm preeclampsia and OR of 14.9 (95% CI 4.9–45.0), 3.9 (95% CI 1.2–12.6) and 4.3 (95% CI 1.2–15.5), respectively. A small change in the PlGF:sEng ratio conferred an increased

Table 11.1 Studies of angiogenic factors for preeclampsia prediction

Reference	Number of women	Angiogenic factor evaluated	Gestational week	Outcome	Cutoff points	Odds ratio/ relative risk (95% CI)	Sensitivity (%)	Specificity (%)	Positive LR	Negative LR	ROC area
47	39	PlGF	5–15	All	32 pg/mL	95 (7.6–1180)	91	91	9.6	0.1	–
			16–20		90 pg/mL	16 (2.4–106)	67	89	5.9	0.4	
48	80	PlGF	17	PE < 34	80.8 pg/mL	4.2 (1.35–13.1)	–	–	–	–	0.80
49	200	PlGF	10	All	12 pg/mL	14.1 (2.0–102.2)	–	–	–	–	0.69
50	3296	PlGF	22–26	All	280 pg/mL	2.6 (1.67–3.94)	69	51	1.4	0.6	–
				PE < 34	280 pg/mL	5.5 (1.98–15.1)	80	51	1.7	0.4	
51	23	sFlt 1	25–28	All	957 pg/mL	–	80	100	Infinity	0.1	–
52	88	sFlt 1	24–28	PE< 34	1560 pg/mL	–	17	97	6.4	0.9	–
			28–32		1575 pg/mL		83	95	16.6	0.2	
			28–32	PE > 34	1575 pg/mL		19	95	3.7	0.9	
			32–37		2164 pg/mL		70	97	23.3	0.3	
53	184	sEng	15–17	All	4.5 ng/mL	–	85	69	2.7	0.2	0.85
14	240	PlGF	13–20	PE < 37	87 pg/mL	9.6 (1.6–57.6)	–	–	–	–	–
			21–32		363 pg/mL	19.6 (2.3–163.8)					
			13–20	Term PE	87 pg/mL	6.7 (1.6–27.5)					
			21–32		363 pg/mL	1.2 (0.5–3.1)					
			33–41		175 pg/mL	4.1(1.4–12.2)					
		sFlt 1	13–20	PE < 37	1047 pg/mL	1.3 (0.4–5.0)					
			21–32		1131 pg/mL	4.7 (1.3–16.6)					
			13–20	Term PE	1047 pg/mL	1.5 (0.6–3.7)					
			21–32		1131 pg/mL	1.7 (0.7–4.4)					
			33–41		2191 pg/mL	7.5 (2.6–21.8)					

15	552	sEng	13–20	PE < 37	7.9 ng/mL	2.2 (1.1–4.6)	–	–	–	–	–
			21–32		7.2 ng/mL	9.4 (4.3–20.7)					
			13–20	Term PE	7.9 ng/mL	1.1 (0.6–2.3)					
			21–32		7.2 ng/mL	2.6 (1.4–4.8)					
			33–42		13.6 ng/mL	7.0 (3.4–14.4)					
		sFlt 1:PlGF	13–20	PE < 37	16.9	2.5 (1.0–6.0)					
			21–32		3.4	12.6 (5.3–30.3)					
			13–20	Term PE	16.9	1.9 (0.9–4.2)					
			21–32		3.4	1.7 (0.9–3.2)					
			33–42		11.9	12.3 (5.5–27.2)					
		(sFlt	13–20	PE < 37	110.7	6.1 (2.4–15.4)					
		+ sEng):	21–32		18.6	16.0 (6.7–38.0)					
		PlGF	13–20	Term PE	110.7	2.4 (1.1–5.4)					
			21–32		18.6	3.1 (1.7–5.8)					
			33–42		61.9	8.3 (4.0–17.3)					
55	63	PlGF	19–24	All	118.0 pg/mL	–	77	62	2.0	0.4	–
				PE < 34	118.0 pg/mL		83	62	2.2	0.3	
		sFlt 1		All	500.5 pg/mL		62	70	2.1	0.5	
				PE < 34	631.3 pg/mL		67	89	6.1	0.4	
		sFlt 1:PlGF		All	3.15		62	51	1.3	0.8	
				PE < 34	3.15		67	51	1.2	0.7	
		sFlt 1		All	631.3/81.8		77	73	2.9	0.3	
		+ PlGF		PE < 34	875.8/14.8		83	95	16.6	0.2	
56	77	sEng	19–24	All	4.14 ng/mL	2.67 (0.65–10.9)	80	43	2.1	0.5	–
				PE < 34	16.6 ng/mL	10.5 (1.1–95.9)	50	95	3.9	0.5	
		sEng +		All	4.15 + 567.2	6 (1.46–24.5)	60	89	6.2	0.5	
		sFlt 1		PE < 34	4.15 + 631.3	50 (4.6–540.4)	100	93	14.9	0.0	

(*Continued*)

Table 11.1 Studies of angiogenic factors for preeclampsia prediction—cont'd

Reference	Number of women	Angiogenic factor evaluated	Gestational week	Outcome	Cutoff points	Odds ratio/ relative risk (95% CI)	Sensitivity (%)	Specificity (%)	Positive LR	Negative LR	ROC area
57	108	PlGF	23	All	144 pg/mL	—	88	81	4.6	0.2	0.90
				PE < 34	134 pg/mL		100	76	4.2	0.0	0.90
		sFlt 1		All	614 pg/mL		96	87	7.4	0.1	0.94
				PE < 34	978 pg/mL		100	87	7.7	0.0	0.96
		sFlt 1:PlGF		All	3.92		100	85	6.7	0.0	0.04
				PE < 34	7.78		100	90	10.0	0.0	0.96
54	140	sFlt 1	14–21	All	2705.8 pg/mL	6.9 (2.3–20.7)	85	55	1.9	0.3	—
		sEng			4903.6 ng/mL	7.1 (2.3–21.7)	85	59	2.1	0.3	
		sFlt 1:PlGF			20.5	6.8 (2.4–19.4)	85	67	2.6	0.3	
		(sFlt 1 + sEng)/ (PlGF + TGFβ1)			3.0	74.8 (17.6–316.7)	85	90	8.5	0.2	
58	104	PlGF	24–28	All	382.5 pg/mL	—	92	81	4.8	0.1	0.83
		sFlt 1			16460 pg/mL		73	81	3.8	0.3	0.87
		sEng			12.1 ng/mL		81	85	5.3	0.2	0.88
		sFlt 1:PlGF			38.47		89	89	7.7	0.1	0.92

59	1622	PlGF	6–15	All	28.04 pg/mL	–	63	60	1.6	0.6	0.65
			20–25		215.04 pg/mL		52	76	2.2	0.6	0.65
			6–15	PE < 34	22.93 pg/mL		78	70	2.6	0.3	0.74
			20–25		126.42 pg/mL		100	96	24.1	0.0	0.99
		sFlt 1	6–15	All	1405 pg/mL		50	68	1.6	0.7	0.59
			20–25	PE < 34	3460 pg/mL		67	93	9.8	0.4	0.87
		sEng	6–15	All	8.59 ng/mL		40	79	1.9	0.8	0.58
			20–25		6.7 ng/mL		58	73	2.2	0.6	0.68
			20–25	PE < 34	7.85 ng/mL		100	90	9.8	0.0	0.97
		PlGF:s Eng	6–15	All	6.69		94	31	1.3	0.2	0.66
			20–25		48.28		69	61	1.8	0.5	0.68
			6–15	PE < 34	2.91		78	72	2.7	0.3	0.75
			20–25		13.44		100	98	57.6	0.0	0.99

all, all preeclampsia; CI, confidence interval; LR, likelihood ratio; PE, preeclampsia; PlGF, placental growth factor; ROC, receiver operating characteristic; sEng, soluble endoglin; sFlt-1, soluble Fms-like tyrosine-kinase-1.

risk for preterm and term preeclampsia, and OR of 7.68 (95% CI 1.7–34.74) and 2.46 (95% CI 1.15–5.26), respectively. Differences in concentrations from second to first trimester had higher OR than isolated measurements.[62] Sequential changes were also measured at later gestational ages. The rate of rise in sFlt 1 and sFlt 1:PlGF ratio assessed from 22 to 36 weeks was also predictive of overall preeclampsia risk, with areas under the receiver operating characteristic (ROC) curve of 92.4% (95% CI 86.3–98.5) and 93.8% (95% CI 88.2–99.4), respectively.[63]

Recently, Kusanovic et al reported a remarkable performance of delta and slope of PlGF:sEng ratio (from early pregnancy and midtrimester), with a positive LR of 55.6 (95% CI 36.4–55.6) and 89.6 (95% CI 56.4–89.6), respectively, for predicting early onset preeclampsia. Overall, their accuracy was better than that of individual factors. Indeed, the slope PlGF:sEng ratio performed better than any other test.[59]

5.1.2. High risk populations

Many of the studies reported are limited to healthy, nulliparous populations in Caucasians.[14] How do angiogenic factors perform in other settings, namely in high risk populations and across other populations?

Moore Simas et al analyzed the performance of angiogenic biomarkers in 94 women with at least one of the following risk factors for preeclampsia: pregestational diabetes mellitus, chronic hypertension, chronic kidney disease, obesity, systemic lupus erythematosus, antiphospholipid antibody syndrome or prior history of preeclampsia.[63] Samples were collected between 22 and 36 weeks, at 4 week intervals. In this high risk population, they showed that maternal serum sFlt 1 is significantly increased, and PlGF significantly decreased prior to disease onset in women who go on to develop preeclampsia, as compared to women who do not develop preeclampsia.[63] This suggests that these biomarkers are likely to be clinically useful in this population as well. Similar to what was shown in healthy nulliparous women,[14] levels increased earlier in women destined to develop preterm preeclampsia, and the sFlt 1:PlGF ratio was more predictive of the development of preeclampsia than sFlt 1 alone.[63] In samples taken at 22–26 weeks, the area under the ROC curve calculated for isolated sFlt 1 and sFlt 1:PlGF ratio for development of preterm preeclampsia was 90.1 (95% CI 78.0–100.0) and 97 (95% CI 90.8–100.0), respectively.

Sibai et al evaluated the performance of sFlt 1 and PlGF in 704 women with previous preeclampsia and/or chronic hypertension enrolled in a randomized, placebo controlled trial of vitamins C and E.[64] Samples were

collected at 12–19.9 weeks and at 24–28 weeks of gestation. Angiogenic factor levels at 12–19.9 weeks were not associated with term preeclampsia, but had significant associations with onset of the disease prior to 27 weeks, as did levels obtained at 24–28 weeks with onset of preeclampsia prior to 37 weeks. Although there was a significant association between these markers and the subsequent development of preeclampsia, with very good sensitivity and negative predictive value for preeclampsia developing prior to 27 weeks, the corresponding positive predictive values (PPV) were poor (6–8% at a specificity of at least 90%). Thus, the authors concluded that these markers might not be clinically useful for predicting preeclampsia in this high risk population.[64] However, one of the problems with this study was the wide gestational windows used in the analyzes of the data and the heterogeneity of the preeclampsia phenotypes studied.

5.1.3. Other antiangiogenic states

An antiangiogenic profile may not be unique to preeclampsia, but may underlie other pregnancy complications such as mirror syndrome,[65] unexplained fetal death,[66] placental abruption[67] and delivery of small for gestational age babies, without the presence of preeclampsia.[49,68,69]

Low PlGF and high sEng have been associated with an increased risk for delivery of a small for gestational age neonate[49,68,69] as well as with changes in maternal plasma concentrations of sEng, PlGF or in their ratios between the first and second trimesters of pregnancy.[62] Romero et al performed a longitudinal nested case control study to evaluate whether maternal concentrations of angiogenic factors differ prior to development of the disease between women with normal pregnancies and women destined either to develop preeclampsia or to deliver a small for gestational age (SGA) neonate.[70] Patients destined to deliver SGA neonates showed changes in maternal plasma concentration of sEng and PlGF, but not sFlt 1. These changes differed in timing and magnitude from those in patients destined to develop preterm or term preeclampsia.[70] The difference in the pattern of change reflected two distinct phenotypes of an antiangiogenic state. Patients destined to develop SGA neonates had a higher plasma concentration of sEng from as early as 10 weeks' gestation onwards, while patients who developed preeclampsia had higher plasma concentrations only after 24 weeks' gestation. sFtl 1 levels did not change between patients destined to develop SGA pregnancies versus the controls, but did increase in women who later developed preeclampsia. When compared to controls, PlGF levels were decreased both in women who delivered SGA neonates

and in women who developed preeclampsia. In both groups, this decrease was already evident at 10 weeks.[70]

In conclusion, pregnancies destined to deliver SGA neonates and those destined to develop preeclampsia presented different antiangiogenic profiles. Alterations in PlGF and sEng levels were already evident as early as 10 weeks of gestation in those pregnancies destined to deliver SGA neonates, while sFlt 1 levels were predictive only for preeclampsia.[70] Also, the profile of maternal plasma concentrations of angiogenic (PlGF) and antiangiogenic factors (sEng and sFlt 1) between the first and second trimesters is significantly different among patients who subsequently had a normal pregnancy versus those destined to develop preeclampsia or to deliver SGA neonates.[62]

5.1.4. PlGF in the urine

Free PlGF is freely filtered into urine and therefore has also been assessed as a predictive factor of preeclampsia. Levine et al evaluated the urinary PlGF levels at 13 weeks of gestation onward.[71] In normal pregnancies, urinary PlGF increased during the first two trimesters, peaked at 29–32 weeks, and then decreased. In preeclamptic pregnancies, the pattern of urinary PlGF was similar to that of normal pregnancies before the onset of preeclampsia, but beginning at 25–28 weeks, and not before, levels were significantly reduced. There were particularly large differences between the controls and the cases with subsequent early onset preeclampsia. For samples collected after 21–31 weeks, the adjusted OR was 22.5 (95% CI 7.4–67.8). The investigators concluded that decreased urinary PlGF concentrations at midgestation are strongly associated with subsequent early development of preeclampsia.[71] These findings were confirmed by others.[72,73] Recently, Savvidou et al measured urinary PlGF at 11–14 weeks. They found that in the first trimester, development of preeclampsia was not preceded by altered urinary PlGF,[74] confirming that first trimester urinary PlGF levels are not useful for predicting preeclampsia.

5.1.5. Differential diagnosis

In addition to being useful in the prediction of preeclampsia before the onset of clinical symptoms, angiogenic factors may also prove useful in diagnosing the disease and in distinguishing it from other hypertensive disorders of pregnancy, such as gestational hypertension and chronic hypertension. The clinical utility of sFlt 1, sEng and PlGF serum levels in differentiating among hypertensive disorders of pregnancy has been evaluated. The sensitivity and

specificity in differentiating preeclampsia from chronic hypertension were 84% and 95% for sFlt 1 and 84% and 79% for sEng.[75] sFlt 1 and PlGF also differentiated women with superimposed preeclampsia (i.e. chronic hypertension plus preeclampsia) from those with chronic hypertension without preeclampsia.[76] Circulating antiangiogenic factors have also been used to differentiate between preeclampsia and other causes of escalating hypertension in pregnant women undergoing hemodialysis.[77]

In a recent prospective cohort study of 1622 pregnacies, Kusanovic et al measured plasma PlGF, sEng and sFlt 1 levels in early pregnancy (6–15 weeks) and midtrimester (20–25 weeks), reporting an excellent predictive performance for PlGF:sEng ratio, its delta and its slope.[59] In early pregnancy, mean PlGF levels were significantly lower ($P = 0.01$) in patients who subsequently developed early onset preeclampsia when compared to normal pregnancies. In midtrimester, mean sEng levels and mean sFlt 1 levels were significantly higher ($P < 0.001$), and mean PlGF levels significantly lower ($P < 0.001$) in patients who subsequently developed early onset preeclampsia than in those who did not. The most informative analytes were PlGF and sEng, and the highest likelihood ratios were provided by ratios of midtrimester plasma concentrations of PlGF, sEng and sFlt 1 used in early onset preeclampsia (< 34 weeks). These same analytes did not perform well in the identification of preeclampsia as a whole; in particular, they had a poor performance in the prediction of term preeclampsia. In contrast, a combination of these analytes such as the PlGF:sEng ratio, its delta and slope, had the best predictive indices with a sensitivity of 100% for all tests, a specificity between 98% and 99%, positive predictive values between 24% and 33%, negative likelihood ratios of 0 (95% CI 0.0–0.3) for all tests, and positive likelihood ratios of 57.6 (95% CI 37.6–57.6), 55.6 (95% CI 36.4–55.6) and 89.6 (56.4–89.6), respectively, for predicting early onset preeclampsia (< 34 weeks). The authors concluded that risk assessment for preeclampsia is feasible based on the maternal concentrations of angiogenic and antiangiogenic factors. In this population the prevalence of early onset preeclampsia was 0.6%, yielding PPVs between 24% and 33%. Thus, the number of patients to be closely followed to identify one case of early onset preeclampsia is between three and four high risk cases. Moreover, a positive test result of the slope of PlGF:sEng in midtrimester increases the pretest probability from 0.6% to 35.1%, and a negative result decreases the pretest probability from 0.6% to 0.0% for early onset preeclampsia.[59] Taking into consideration the low prevalence of early onset preeclampsia, these are

remarkable results, and fulfill the criteria for a good predictive test, with positive LR much higher than 15 and negative LR below 0.1.

In conclusion, maternal plasma concentrations of angiogenic factors seem to be a promising tool for predicting preeclampsia, with their predictive performance highest for preterm preeclampsia and for samples assessed in midtrimester.

Very high positive LR and low negative LR have been reported by several studies (Table 11.1). Nonetheless the corresponding PPVs are low mainly due to the relatively low prevalence of the disease. In some cases, the combination of these analytes with other parameters may be helpful in risk assessment for preeclampsia. In addition to prediction, angiogenic factors can contribute to clinical decision making, contributing to accurate and differential diagnosis of preeclampsia, and distinguishing it from other disorders occurring with hypertension in pregnancy.

Recently, two studies[76,78] described the use of automated assays for measuring angiogenic factors. Currently, assessment relies on detection by ELISA kits, which are suitable for research purposes but not for the widespread use in the clinical setting. Automated tests will allow a fast and easy to implement assessment of angiogenic factors in the everyday clinical routine context.

In summary, angiogenic markers may be useful for the prediction of preterm preeclampsia and the recent study conducted by Kusanovic et al was a major step forward in understanding the predictive capacity of these biomarkers.[59] Several prospective longitudinal studies are in progress, including a large World Health Organization trial expecting to recruit 10,000 women, to examine the predictive value of alterations in angiogenic factors (in both urine and serum, serially obtained throughout gestation) on the subsequent development of preeclampsia, and on other adverse outcomes. Hopefully, these will provide the final evidence that is needed regarding these biomarkers.

In addition to angiogenic proteins, other molecules such as PP 13 and PAPP A have also been evaluated as predictive markers (see below).

5.2. Placental protein-13

Placental protein 13 (PP 13)[79] is a member of the galectin family,[80] predominantly expressed by the syncytiotrophoblasts that are involved in normal implantation and placental vascular development.[81] First trimester circulating levels of PP 13 are significantly lower in women who go on to

develop preeclampsia, IUGR and preterm birth.[82,83] In a prospective nested case control study involving 290 controls and 47 preeclamptic women, Chafetz et al observed that preeclamptic pregnancies had lower levels of PP 13 in the first trimester (9–12 weeks) when compared with controls. Results were expressed as multiples of the gestation specific median in controls (MoM). Using a cutoff of 0.38 MoM, the OR was 32.1 (95% CI 14.5–71.0), the sensitivity was 79% and the specificity 90%. Receiver operating characteristic (ROC) analysis yielded areas under the curve of 0.91 (95% CI 0.86–0.95).[84]

Romero et al reported a sensitivity of 100% for early onset preeclampsia and of 85% for preterm preeclampsia at 80% specificity and a cutoff of 0.39 MoM. PP 13 did not perform well for prediction of severe preeclampsia and mild preeclampsia at term.[85] Spencer et al conducted a nested case control study of 446 cases and 88 controls. At a specificity set at 80%, the sensitivity of first trimester PP 13 for all cases of preeclampsia was 40%, and was 50% for early onset preeclampsia.[86] Furthermore, a recent study has shown that there is a benefit in sequential testing with PP 13. Gonen et al measured levels of PP 13 at 6–10 weeks, 16–20 weeks and 24–28 weeks of gestation in 1366 women, reporting that PP 13 in the first trimester alone or in combination with the slope between the first and the second trimesters may be a promising marker for assessing the risk of preeclampsia. Combining MoM at 6–10 weeks and a slope between 6–10 and 16–20 weeks, the odds ratio was 55.5 (95% CI 18.2–169.2), the sensitivity was 78% and the specificity 94%.[87]

PP 13 was also predictive of early onset preeclampsia in a high risk population. At a MoM cutoff of 0.53, for a false positive rate of 10%, sensitivity was 71%. Again, it predicted early onset disease better than disease at term.[88] Second trimester levels of PP 13 (22–32 weeks) taken at a single time point are not useful in predicting preeclampsia, and its prediction did not increase when coupled with Doppler velocimetry.[89]

Combining first trimester PP 13 with other parameters may further improve predictive performance. Larger prospective studies are needed to determine whether PP 13 will be a valuable clinical marker for early prediction of preeclampsia.

5.3. Pregnancy-associated plasma protein A

Pregnancy associated plasma protein A (PAPP A) is a peptidase produced by syncitiotrophoblast with hydrolytic activity for insulin like growth factor

binding proteins.[90,91] These regulate insulin growth factors known to be important for implantation, for trophoblast invasion of maternal decidua and for placental growth.[92] It is released into the maternal circulation where it binds the eosinophil major binding protein, an inhibitor of its proteolytic activity.[91]

Decreased levels of PAPP A in the first trimester have been associated with increased risk of adverse pregnancy outcomes, including preeclampsia.[93] It is in fact an established biomarker for trisomy 21. Spencer et al described an association of a modest increase in the likelihood ratio of developing preeclampsia with decreasing levels of PAPP A. At the 5th centile of normal PAPP A (MoM 0.415) the odds ratio was increased 3.7 fold (95% CI 2.3−4.8), and at this cutoff 15% of cases of preeclampsia were identified.[94]

A recent systematic review and meta analysis determined the accuracy in predicting preeclampsia of five serum analytes, used in Down's serum screening. At the 5th centile of normal PAPP A, the positive LR was 2.10 (95% CI 1.57−2.81) and the negative LR was 0.95 (95% CI 0.93−0.98).[95] First trimester serum PAPP A was not a good predictor of late onset preeclampsia.[96]

5.4. Renal dysfunction related tests

Because the kidney is a major target organ of preeclampsia, renal dysfunction related tests were proposed as possible predictors of preeclampsia.

5.4.1. Serum uric acid

Hyperuricemia observed in preeclampsia led to studies to determine if measuring serum uric acid levels could be used to predict preeclampsia. Unfortunately, uric acid is of limited clinical utility in either distinguishing preeclampsia from other hypertensive disorders of pregnancy or as a clinical predictor of adverse outcomes.[97,98]

5.4.2. Proteinuria

Proteinuria is routinely assessed in antenatal care visits from first booking. Proteinuria measurement includes total protein or total albumin excretion during 24 h, microalbuminuria, albumin/creatinine ratio and dipsticks for spot proteinuria or albuminuria. Pooled estimates of sensitivity and speci ficity for total proteinuria were 35% (95% CI 13−68%) and 89% (95% CI 79−94%); for total albuminuria were 70% (95% CI 45−87%) and 89% (95% CI 79−94%); for microalbuminuria were 62% (95% CI 23−90%) and 68%

(95% CI 57–77%); and for albumin/creatinine ratio were 19% (95% CI 12–28%) and 75% (95% CI 73–77%), respectively.[99]

5.4.3. Kallikreins

The kallikrein kinin system is an important paracrine regulator of vessel dilatation, and consequently of blood flow. Millar et al[100] and Kyle et al[101] assessed the levels of urinary kallikrein as a predictor of preeclampsia. Kallikreinuria has been shown to be decreased in patients with preeclampsia as compared to uncomplicated pregnancies; however, it is unlikely to be useful as a screening test as the reported specificity values are quite poor.

5.5. Free fetal nucleic acids

The first description of the presence of fetal cells in the mother stems from the 19th century when a German pathologist detected trophoblast cells in the lungs of women who died of eclampsia. In 1969, male fetal cells were found in the blood of healthy pregnant women.[102] Several studies confirmed these results and led to the description of fetal maternal cell trafficking. Several investigators have described circulating nucleic acids of fetal origin in maternal blood and in relatively more abundance than fetal cells.[103,104] It is now widely recognized that there is transfer of allogeneic fetal cells into the maternal circulation and vice versa,[105] and that cell free fetal nucleic acids (DNA and mRNA) circulate in the maternal blood.[106] Except for the migration of fetal cytotrophoblasts, the exact mechanism leading to bidirectional transplacental migration of cells is largely unknown. Also, the mechanism of release of free extracellular nucleic acids into the circulation is not yet clear. Multiple lines of evidence suggest that the vast majority of the cell free fetal DNA in the maternal plasma is probably derived from the placenta through apoptosis and necrosis of cytotrophoblasts, although some could be derived from circulating cells.

The examination of fetal cells, specifically erythroblasts, and of cell free fetal DNA from the blood of pregnant women is the subject of intense research, with the aim of developing new risk free methods for prenatal diagnosis.[107,108] Cell free fetal DNA is already in use in determining fetal sex and fetal Rhesus status.[108]

In preeclampsia, fetal maternal cell trafficking is significantly altered with elevated numbers of fetal cells detected in the maternal circulation during those pregnancies.[109] Prospective studies further indicated that this perturbation occurs early in preeclamptic pregnancies.[110,111] In a similar manner, it has been shown that in preeclamptic pregnancies[112] cell free fetal

DNA is elevated long before the clinical onset of the disease.[113,114] These results were confirmed in a large case control study within the CPEP Trial. Levine et al reported a two stage increase in cell free fetal DNA in maternal sera before the onset of preeclampsia, with an initial elevation starting at 17–28 weeks (36 vs 16 genomic equivalents/mL, $P < 0.001$), and a secondary elevation beginning about 3 weeks before the onset of clinical syndrome (176 vs 75 genomic equivalents/mL, $P < 0.001$).[115] Of note, the fetal DNA was greater from 17 to 20 weeks onwards than in the controls, but was not different statistically until 25–28 weeks. In early pregnancy (between 13 and 16 weeks) there was no demonstrable difference. If preeclampsia was severe, the reported differences were greater and at an earlier gestational age, or they were associated with a small for gestational age infant.[115]

Crowley et al performed a nested case control study to quantify plasma fetal DNA before 20 weeks of gestation in pregnancies subsequently complicated by preeclampsia, in comparison to normal pregnancies. The median gestational age at sample collection was 13 weeks. The sex determining region Y (SRY) gene, which is specific to the Y chromosome, was used as a fetal marker. This gene was detected in 94% of preeclamptic women, and in 78% of normal pregnancies. However, its median levels were similar between cases and controls. The authors concluded that free fetal DNA quantification in maternal plasma before 20 weeks is not a useful predictor of preeclampsia.[116] Using whole blood samples collected at the first antenatal visit (mean 15.7 ± 3.6 weeks), Cotter et al reported a sensitivity of 39%, and a specificity of 90% for predicting preeclampsia at a cutoff of 50,000 SRY copies/mL.[117]

A downside of using these tests as screening tools for predicting preeclampsia is that, as of 2010, the analysis of fetal cells or cell free fetal DNA is still complex and costly. Owing to high maternal DNA background, detection of fetal DNA from maternal plasma is difficult. In addition, the quantification of fetal DNA is typically based on Y chromosome specific sequences, i.e. SYR and the DNA Y chromosome segment (DYS) limits the technique to pregnancies carrying a male fetus. Thus, other approaches have been used to overcome this limitation, such as the utilization of different epigenetic markers between maternal and fetal DNA,[118] and fetal RhD gene[119] as universal, gender independent fetal markers. Total free DNA has also been used, and has been reported to be increased in women who subsequently develop preeclampsia, thus overcoming the gender issue.[120] Interestingly, mRNA of placental origin has also been

identified in pregnant women, and research in the area has turned the focus on fetal free mRNA to produce new biomarkers. The advantage of using mRNAs is that it is of placental/fetal origin, is specific to pregnancy and is independent of fetal gender. Ng et al detected and quantified mRNA expression in maternal plasma of human chorionic gonadotropin and human placental lactogen, proteins which are produced exclusively by the placenta. Messenger RNA expression in these proteins was found to be pregnancy specific and to reflect relative placental gene expression.[121]

Purwosunu et al demonstrated that in preeclamptic pregnancies a panel of free mRNA of placental origin is increased in maternal plasma at gestational weeks 15–20. At a 5% false positive rate, the detection rate was 84% (95% CI 71.8 91.5), with area under the ROC curve of 0.927 ($P < 0.001$).[122] Circulating fetal cells in maternal blood and cell free fetal nucleic acids are a promising field of research. Future studies will determine whether they will turn out to be good predictive biomarkers for preeclampsia.

5.6. Uterine Doppler velocimetry

Preeclampsia is characterized by an abnormal placenta and a decreased invasion of maternal uterine arteries by cytotrophoblast cells. As a result, the normal vascular remodeling of maternal uterine spiral arteries converting them into high flow and low resistance vessels does not occur. Therefore, Doppler ultrasonography has been evaluated as a potential predictive test for preeclampsia.

The uterine artery is identified using color Doppler ultrasonography, and then pulsed wave Doppler is applied to obtain waveforms. The increased flow resistance within uterine arteries results in an abnormal waveform pattern, which is represented by either an increased resistance index, or pulsatility index, or by the persistence of a unilateral or bilateral diastolic notch. Several indices are then calculated and assessed from flow velocity waveforms, and either alone or combined have been investigated as predictive of preeclampsia. This has revealed varied results. In this regard Cnossen et al conducted a recent systematic review and meta analysis to assess the use of uterine Doppler ultrasonography to predict preeclampsia.[123] The authors concluded that an increased pulsatility index in the second trimester, alone or combined with notching, is the best Doppler index predictor of preeclampsia. In high risk patients, an increased pulsatility index with notching had a positive LR of 21.0 (95% CI

5.5–80.5) and a negative LR of 0.82 (95% CI 0.72–0.93). In low risk patients it had a positive LR of 7.5 (95% CI 5.4–10.2) and a negative LR of 0.56 (95% CI 0.47–0.71). Other Doppler indices showed low to moderate predictive value, e.g. when assessed in the first trimester.[123]

Several studies have assessed the predictive accuracy of uterine Doppler velocimetry for early onset preeclampsia. Positive LR ranged from 5.0 to 20 and negative LR ranged from 0.1 to 0.8.[44] It appears that irrespective of the index or combinations of index used, uterine artery Doppler velocimetry may be a moderate to good predictor for the development of early onset preeclampsia.

5.7. Combination of tests

Angiogenic factors along with other modalities may be combined for predicting preeclampsia. In order to identify patients at risk for severe and/or early onset preeclampsia, Espinoza et al conducted a prospective study of 3296 women to determine the role of uterine artery Doppler velocimetry (UADV), maternal plasma PlGF and sFlt 1 concentrations in the second trimester.[50] Sample collection and uterine artery Doppler velocimetry were performed between 22 and 26 weeks, and showed that the combination of abnormal UADV and low serum PlGF was strongly associated with both early onset and severe preeclampsia, with OR of 35–45. sFlt 1 did not improve prediction of Doppler combined with PlGF. For all of the cases of preeclampsia, the prediction sensitivities of maternal plasma PlGF concentration, abnormal UADV, and the combination of these tests were 61%, 35% and 27%, respectively. The corresponding specificities were 51%, 90% and 96% and positive LR 1.42 (95% CI 1.25–1.62), 3.42 (95% CI 2.60–4.49) and 7.53 (95% CI 5.27–10.75), respectively. Combination testing improved the specificity, PPV and positive LR over each test alone for the prediction of early onset preeclampsia, although with a slight reduction in sensitivity.[50]

Stepan et al performed a prospective study of 63 second trimester pregnant women with abnormal uterine perfusion. When combining the measurements of uterine Doppler with sFlt 1 and PlGF levels in the second trimester, the sensitivity and specificity of Doppler alone to predict early onset preeclampsia increased from 67% to 83%, and from 76% to 95%, respectively. The combination of parameters performed better than any parameter alone[55]. Later on, the same group[56] also demonstrated that in pregnancies with abnormal uterine perfusions that resulted in the development of preeclampsia, second trimester levels of sEng were also increased.

Combined analysis of sEng and sFlt 1 in this population with abnormal uterine Doppler was able to predict early onset preeclampsia with a sensi tivity of 100% and a specificity of 93.3%.[56]

Combination of markers in the first trimester was also evaluated. Patients who developed preeclampsia requiring delivery before 34 weeks' gestation had lower PP 13 serum concentration than did normotensive controls at 11−14 weeks' gestation. For a 90% detection rate, the false positive rate for PP 13 was 12%, and for Doppler analysis alone, performed at the same gestational age, it was 31%. For a 10% false positive rate, the detection rates would have been 80% for PP 13 alone and 90% for PP 13 combined with Doppler.[124] In the above study by Spencer et al,[86] the sensitivity of first trimester PP 13 for all cases of preeclampsia increased from 40% to 74%, and for early onset increased from 50% to 74% when combined with uterine artery Doppler velocimetry. However, serum PP 13 does not improve significantly the prediction of early preeclampsia that is provided by a combination of maternal factors, uterine artery pulsatility index (PI) and PAPP A.[125] Interestingly, PAPP A does not improve the prediction of early preeclampsia when first trimester PP 13 and second trimester PI are used together.[86] Second trimester levels of PP 13 (22−32 weeks) are not useful in predicting preeclampsia, and prediction did not improve when coupled with Doppler velocimetry.[89]

An increase in cell free fetal DNA has been described in women with an abnormal uterine Doppler and who developed preeclampsia.[126] Studies assessing the combination of Doppler with cell free fetal DNA for pre dicting preeclampsia are not available. However, assessment of this metab olite is unlikely to improve the performance of Doppler, because there is a high association between uterine artery PI and plasma cell free fetal DNA.[127]

Recently, Poon et al evaluated 7797 women with singleton pregnancies during gestational weeks 11−13. This yielded very good results using an algorithm developed by logistic regression that combined the logs of uterine pulsatility index, mean arterial pressure, PAPP A, serum free PlGF, body mass index and presence of nulliparity or previous preeclampsia. At a 5% false positive rate, the detection rate for early preeclampsia was 93.1%.[128] The calculated positive LR was 16.5, and negative LR was 0.06.[129]

In the reported studies, the combination of angiogenic factors, placental proteins and other parameters such as Doppler studies increased the sensi tivity without losing specificity. Still more studies are needed to confirm these results and assess the cost effectiveness of this approach.

6. NOVEL BIOMARKERS AND FUTURE PERSPECTIVES

With the completion of the human genome project, various high throughput techniques evolved, allowing in single experiments the simul taneous examination of thousands of genes (genomics), gene transcripts (transcriptomics), proteins (proteomics), metabolites (metabolomics), pro tein interaction (interactomics), chromatin modifications (epigenomics) and so forth.

These novel technologies have greatly increased the number of potential DNA, RNA and protein biomarkers, leading to renewed interest in the field. Analysis of a single biomarker, or a combination of only a few, is being replaced by a multiparametric analysis yielding a signature of genes, RNA or proteins. These promising new methodologies are currently being reported in almost all fields of medicine such as oncology,[130 132] nephrology,[133] cardiology[134 136] and many others, and have reported distinguishing patterns that help in early diagnosis, classification, prognosis and in the prediction of response to therapies.

6.1. Transcriptomics

The transcriptome is a description of all DNA that is transcribed into RNA (messenger RNA, transfer RNA, microRNA and other RNA species) at any given moment. It forms the template for protein synthesis, resulting in the corresponding proteome. Transcriptomics refers to global RNA assessment.

Farina et al measured a panel of seven circulating mRNAs in maternal blood from six women with preeclampsia, and from 30 controls. A different expression pattern between cases and controls was reported. Inhibin A, p selectin and vascular endothelial growth factor receptor mRNA values were higher in preeclampsia, whereas human placental lactogen, KISS 1 and plasminogen activator inhibitor type 1 were lower, as compared to normotensive controls.[137] The authors suggested that aberrant quantitative expression of this circulating placenta specific mRNA in serum from preeclamptic women might prove useful for the prediction of this disorder.[137] This was not a large scale approach. Recently, Tsui et al described the use of microarray technology for identification of new placental specific mRNA markers in maternal plasma.[138] Circulating cells of fetal/placental origin are also a source of mRNA that can be assessed as a potential biomarker. Okazaki et al performed gene expression profiling and real time quantitative reverse transcription polymerase chain reaction

(RT PCR) in the cellular component of maternal blood to identify potential biomarkers of preeclampsia. Microarray analysis was performed in five samples from women with preeclampsia, and in five matched control subjects. This was followed by RT PCR analysis in 28 blood samples from women affected with preeclampsia and 29 controls. Trophoblast glycoprotein (a trophoblast membrane protein) and pregnancy specific β1 glycoprotein (protein produced by the syncytiotrophoblasts) mRNA were increased in women with preeclampsia, and there was a direct correlation between pregnancy specific β1 expression levels and severity of the disease.[139]

Chorionic villous sampling (CVS) is a biopsy of placenta chorionic villous performed under ultrasonic guidance around 10–13 weeks of gestation for prenatal diagnosis. Founds et al followed 160 pregnant women on whom CVS had been performed. Of these, four developed preeclampsia, and their banked CVS was matched to eight control CVS of unaffected pregnancies. Microarray analysis was conducted on these samples revealing 36 differentially expressed genes between normal pregnancies and those who went on to develop preeclampsia, 6 months before the onset of clinical symptoms.[140] Consistent with these results, Farina et al also reported a different CVS gene expression profile in women who went on to develop preeclampsia, as compared to normal pregnancies. Altered expression was found among several genes, including those involved in the invasion of human trophoblasts, inflammatory stress, endothelial aberration, angiogenesis and blood pressure control. Furthermore, RT PCR analysis of peripheral blood at term showed significant differences for all the genes studied.[141]

In addition to mRNA, small RNA molecules such as microRNAs (miRNAs) are now being investigated as novel circulating markers. MicroRNAs are short (19–25 nucleotides), single stranded non protein coding RNAs that regulate gene expression by binding to the 3′ untranslated region of the target mRNAs. MicroRNAs are involved in diverse genetic pathways across human tissues including fertility regulation.[142] Pineles et al[143] and Zhu et al[144] studied the expression of miRNAs in preeclamptic placentas obtained at delivery, as compared to those from placentas of normal pregnancies. They reported a different expression profile between the two groups. A clinically useful test for risk assessment in preeclampsia should be minimally invasive.[43] In this regard, placental specific miRNAs have been shown to be secreted into maternal circulation[145] and detectable in maternal plasma samples.[146]

6.2. Proteomics

The proteome is the total complement of proteins present in any defined biological compartment such as a whole organism, a cell, an organelle or a fluid such as blood, amniotic fluid or urine. Proteomics has the advantage over transcriptomics of measuring the protein itself, i.e. the functional product of gene expression.

Blumenstein et al compared the plasma proteome at 20 weeks' gestation in women who subsequently developed preeclampsia to that of healthy women with uncomplicated pregnancies, and reported a different pattern of proteins between the two groups. The differently expressed proteins are involved in lipid metabolism, coagulation, complement regulation, extra cellular matrix remodeling, protease inhibitor activity and acute phase responses.[147]

Recently, Buhimschi et al performed a proteomic profiling of urine from pregnant women, and reported that women with severe preeclampsia requiring mandated delivery presented a unique urine proteomic finger print.[148] Furthermore, this characteristic proteomic profile appeared more than 10 weeks before clinical manifestations, and distinguished preeclampsia from other hypertensive or proteinuric disorders in pregnancy. Proteomic profiling of urine performed better than protein:creatinine ratio and sFlt 1: PlGF ratio for the prediction of preeclampsia requiring mandated delivery. Tandem mass spectrometry and de novo sequencing identified the biomarkers as non random cleavage products of SERPINA1 and albumin. Of these, the 21 amino acid C terminus fragment of SERPINA1 was highly associated with severe forms of preeclampsia requiring early delivery.[148]

6.3. Metabolomics

Metabolomics is defined as the global analysis of endogenous and secreted metabolites in a biological system. As with proteomics, studies of the human metabolome can be carried out on routine samples of urine, plasma or serum, and requires minimal specialist preparation of samples. An advantage of metabolomics is that it involves a smaller and more tractable group of compounds compared to the proteome.

A preliminary study revealed that metabolomic strategies might be appropriate for investigating the metabolic function of trophoblast or placental tissue, and to assess changes in response to altered environmental conditions. Heazell et al examined the placental metabolome under different oxygen tensions. Placental villous explants were cultured in 1%,

6% and 20% oxygen for 96 h, revealing new redox biomarkers.[149] The same group showed that conditioned media from preeclamptic explants has a different metabolic footprint when compared to conditioned media from uncomplicated pregnancies.[150] Metabolomic strategies were also applied to the plasma, and it was found that preeclamptic pregnancies have a different metabolomic profile when compared to normal pregnancies.[151] Using three of the metabolite peak variables, preeclampsia could be distinguished from normal pregnant controls with a sensitivity of 100% and a specificity of 98%.[152]

In conclusion, similar to what is occurring in other fields of medicine, the use of these novel technologies in preeclampsia appears quite promising. Although the number of studies is still scarce, they suggest that an aberrant transcriptomic, proteomic and metabolomic profile may be predictive of the disease, opening a new and exciting avenue in biomarker discovery for preeclampsia. Future studies are warranted, with the collaborative efforts of bioinformatics, biostatistics, researchers and clinicians.

In addition to predicting the presence of disease, a biomarker can also be used as an indicator of disease severity, prognosis and response to thera peutics. In this regard, gene expression profile of placentas has been shown to be different between preeclampsia and uncomplicated pregnancies and early and late onset preeclampsia.[153,154] Accordingly, proteomic analysis of placentas,[155] amniotic fluid,[156] plasma[157] and urine[148] of women with established preeclampsia revealed different proteomic profiles from those of normal controls. Similar to what has been reported for other biomarkers, these results highlight the future possibility of applying genomic and pro teomic strategies to rule out preeclampsia in complicated cases, to classify the disease in terms of severity, and to assess its prognosis. In addition, this technology could generate a very large database that could be mined by computational biologists, and could yield new pathways and molecules that may bring new insights into the mechanism of the disease. It would also stimulate hypothesis driven research, accelerating the efforts to unravel the biology of preeclampsia, and would ultimately lead to new therapies.

7. CONCLUSION

Preeclampsia can be a devastating disease. It remains a major cause of maternal and neonatal mortality. The ability to predict preeclampsia would be a major advance in maternal fetal medicine. In this regard, several biomarkers have been proposed. Angiogenic factors, PP 13, and

combinations of these and other parameters with Doppler analysis hold promise for future predictive testing for preeclampsia. Newer genomic and proteomic technologies are a rapidly emerging field that has enabled bio logical samples to be surveyed for biomarkers in ways never before possible, and promises the development of exciting new applications over the next few years.

In addition, non invasive, easily performed and inexpensive tests will be a useful alternative approach for use as screening tests in developing countries. In this regard, urinary PlGF seems to be a good candidate, because it requires no specific expertise, and could be assessed in more rural areas, that are remote from central hospitals.

It is exciting to envision the tremendous impact that an accurate biomarker for preeclampsia would have, i.e. reduction of fetal and maternal deaths, improvement in acute and long term outcomes, reduced health costs, and in addition, the acceleration of drug discovery leading to the ultimate goal: the effective treatment of preeclampsia. More prospective studies are needed to better evaluate the clinical utility of preeclampsia biomarkers.

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