Biomarkers in kidney damage
a systematic review

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1. Table of contents

2. Summary

The aim of this review is to give a comprehensive overview of the current state of biomarkers and biomarker research in kidney damage. Kidneys play an important role in the maintenance of the physiologic balance in the body. It can therefore be very detrimental if the kidneys are damaged. Kidney injuries can be divided into to classes: acute and chronic kidney injury. Especially in acute kidney injury, early detection is essential for a successful treatment. Correct diagnosis of chronic forms of injury is very important. Standard biomarkers for kidney injury (e.g. serum creatinine levels) are non-specific and only detectable after at least several days after onset of kidney injury. Fortunately, new biomarkers are under development, which are very promising for clinical use as well as for research purposes.

3. Introduction

The aim of this review is to give an overview of old and new biomarkers in kidney damage. Biomarkers are useful tools to detect kidney injuries and diagnose kidney diseases. This is very useful in clinics, drug development, and exposure research. Biomarkers for diagnosis of disease are used in clinical situations. These biomarkers need to specify which disease a patient has and how severe the disease is. This information can then be used to decide on a treatment and the biomarker might be used to monitor the disease during and after treatment.
Biomarkers for detection of kidney injuries are used in drug development. Nephrotoxicity is an unwanted side-effect of drugs. During the process of drug development, many compounds are tested and compounds with nephrotoxicity need to be excluded in the earliest phase possible. Biomarkers for the early detection of kidney injuries are used for this purpose, as they can detect kidney injuries before there are clinical symptoms.
Biomarkers for detection of kidney injuries can also be used in exposure research. These biomarkers can detect sub-clinical damage to the kidney. People exposed to low doses of a possibly harmful substance usually do not develop clinical symptoms, however this does not mean that there is no damage to the kidney. Sensitive biomarkers which can detect minor damages to the kidneys are useful for exposure research situations.
Not all biomarkers are suitable for all three purposes. Most biomarkers for kidney damage are not specific enough and can only detect severe injury to kidneys. New biomarkers carry the promise of higher specificity and early detection. This study reviews current and new biomarkers that may be more useful for therapy, drug development, and exposure research.
In order to evaluate the optimum purpose for a good biomarker, type of injury detection, advantages, and disadvantages are summarized.

The primary role of the kidneys is to maintain a stable internal environment for optimal cell and tissue metabolism. The kidneys balance water and solute excretion, excrete waste products and maintain acid-base balance of the blood. The kidneys are also involved in the hormonal regulation of the body by excreting several hormones.
The most prominent task of the kidneys is to produce urine. The formation of urine is a process which involves filtration, reabsorption and secretion by the glomeruli and the tubules of the kidneys.
The glomerulus is the first site of urine formation. Together with an afferent arteriole it forms a renal corpuscle where fluid and solutes are filtered from the blood (see

Figure 1). The efferent tubule is called the proximal tubule. Filtration is based on both active and passive mechanisms of reabsorption and secretion of molecules. The functional unit of the kidney is the nephron. This is a tubule which starts at the glomerulus and after several loops ends in the urinary duct.
The proximal tubule regulates the reabsorption of Na^+ and water by active transport through the epithelial cells. The proximal tubule is also the part of the kidney where most drugs are secreted into the urine. Because of this, the proximal tubule is affected most by drug toxicity.
The next part of the tubule is the loop of Henle. It mainly regulates cation reabsorption. The loop of Henle is followed by the distal tubule. The distal tubule regulates K^+, Na^+, Ca^+ levels and pH. These ions are balanced by active transport over through the epithelial cells. The distal tubule is almost impermeable to passive transport of water and Na^+ (McCance and Huether, 2002a).
The glomerulus and proximal tubule are most prone to damage and are thus involved in the majority of kidney injury cases.

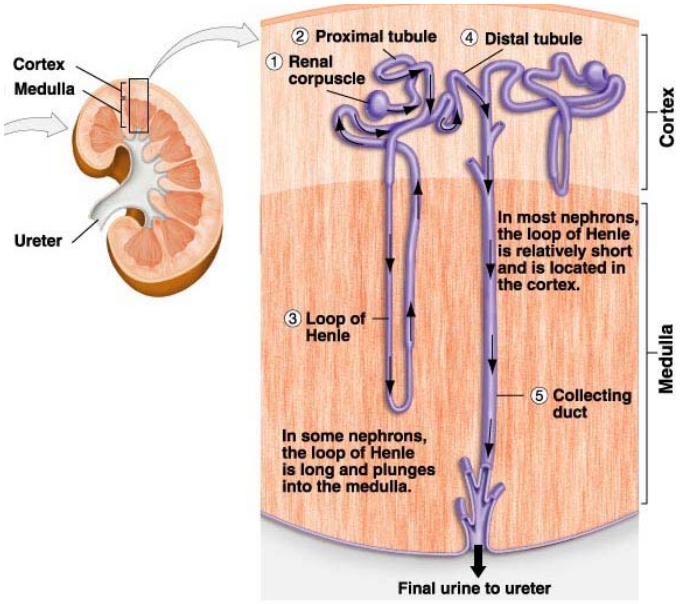

Figure 1 Kidney anatomy: kidney and a nephron.
Urine production starts with filtration at the renal corpuscle (1). Through reabsorption and secretion in the tubules (2-4) the final urine is formed and excreted into the collecting duct (5) which ends in the ureter. The ureter leads to the bladder.
Picture © of Encarta

3.1 Kidney injury

Kidneys are an important component of the body. Therefore, kidney injury is a very serious problem. There are several forms of kidney. Major forms of kidney injury are:

- acute kidney injury
- chronic renal failure
- glomerular disorders
- urinary tract infection
- renal transplant
- cancer

A short description of each is provided in this section.

3.1.1 Acute kidney injury

Acute kidney injury (AKI, also acute renal failure (ARF) or acute kidney failure (AKF)) is an abrupt reduction in renal function. The most prominent symptom is oliguria,

diminished urine output, but a normal or increased urine output can also be the case. Standard biomarkers for AKI include elevated levels of blood urea nitrogen (BUN) and serum creatinine. AKI is usually reversible if it is diagnosed early and treated properly. AKI can be caused by different clinical conditions, for example severe hypertension, vascular obstruction or glomerular disease. A combination of ischemic and/or hepatotoxic factors may also cause acute kidney injury. Acute kidney injury can be classified as prerenal, intrarenal, or postrenal. Prerenal acute kidney injury is caused by impaired blood flow. Intrarenal acute kidney injury can be caused by acute tubular necrosis (most common), cortical necrosis, acute glomerular nephritis, vascular disease, drug allergy or toxicity or contrast administration. Postrenal acute kidney injury is rare and is caused by an obstruction of the urinary tract.
The RIFLE (Risk, Injury, Failure, Loss, and Endstage) criteria can be used to quantify AKI. Risk is qualified as a creatinine increase of ≥50% from baseline; Injury is qualified as a creatinine increase of ≥100% from baseline and Failure as a creatinine increase of ≥200% from baseline. Loss is defined as persistent AKI (>4 weeks) or kidney loss and Endstage is defined as complete loss of kidney function for more than 3 months.

3.1.2 Chronic renal failure

Chronic renal failure (CRF) is a slowly progressing silent condition. CRF is usually associated to one of different diseases, such as diabetes or as a complication to systemic lupus erythematosus. Biomarkers for CRF include increased serum creatinine, increased serum and urinary potassium, increased levels of urinary urea, reduced urinary phosphate and reduced serum calcium levels. In severe cases of CRF, proteinuria and hematuria occur. Kidneys have large adaptive capabilities and CRF progresses slowly. As a result, chronic renal failure usually does not becomes apparent before renal function has declined to less than 25% of normal.

3.1.3 Glomerular disorders

Glomerular nephritis is an inflammation of the glomerulus. This inflammation can be caused by a variety of factors, including detrimental effects of drugs or toxins. Glomerular disease is the most common cause of chronic and end-stage renal failure. Biomarkers for glomerular nephritis include hematuria, proteinuria with albumin as major protein, lowered GFR and elevated serum creatinine. A biopsy is needed to provide conclusive evidence of glomerular nephritis. As a biopsy is an invasive procedure, a non-invasive biomarker test would be a useful substitution for the standard procedure.

The most common origin of glomerular disorders is the disturbance of immunological mechanisms. However, the glomerulus can also be damaged by drugs and toxins. Biomarkers that are used to identify glomerular dysfunction include elevated BUN and the presence of nephrotic and nephritic sediment. The glomerular filtration rate (GFR) is also reduced, which is reflected in the plasma creatinine concentration. Death occurs in 2-5% of acute glomerular disease patients.

3.1.4 Urinary tract infection

Urinary tract infection (UTI) is an infection of the kidney, ureter or bladder. UTI is usually caused by bacteria. This is most common in women, because of the shortness of the urethra and its closeness to the anus. Up to 30% of women may have a lower UTI at some time in their life. Marker for UTI is the presence of bacteria in the urine.

3.1.5 Renal transplant

For kidney transplant patients, allograft rejection is a serious and frequent problem. This is still a problem, despite the fact that immunosuppressive therapy and human leukocyte antigen matching have improved tremendously in recent years. Early diagnosis is very important for long-term survival. At present, serum creatinine levels are mostly used to monitor renal function after transplantation, but this is insufficient for prevention of allograft nephropathy (Shapiro et al., 2001). If there is suspicion of allograft rejection, a biopsy is taken to confirm the diagnosis.

3.1.6 Cancer

Renal cell carcinoma is the most common kidney tumor. However, this represents about 2% of cancer deaths in the US (American Cancer Society, 2000). Clinical manifestations are hematuria, flank pain, palpable flank mass and weight loss, but these occur in less than 10% of cases. No good urinary or serum biomarkers are available.

3.2 Biomarkers introduction

In general, biomarkers are tools to determine whether a certain condition is present in the body. In a broad sense this can be about determining whether a certain process is taking place, such as pregnancy, or levels of exposure to a substance. Biomarker literally means: ‘a biological compound that can be measured, which will reveal the presence and/or severity of a disease’.
Several topics are of concern when working with biomarkers, either in the development of biomarkers or in their practical application. This section will elaborate on the topics of: biomarker purposes

- general properties of biomarkers
- early and late biomarkers
- sensitivity
- specificity
- the predictive value of a positive test
- the predictive value of a negative test
- the development of standards
- the specific relevance of biomarkers in kidney damage

3.2.1 Biomarker purposes

Biomarkers can be used for different purposes. Based on the test objective, different biomarkers are appropriate. Biomarkers can be used to diagnose diseases, give a subtype classification, monitor diseases, predict the response to a certain treatment, and give a prognosis (LaBaer, 2005).

<u>Diagnosis of diseases</u>
Biomarkers are used to diagnose diseases. In the clinic, biomarkers are used to distinguish between healthy and disease and to distinguish between diseases. This can lead to a problem for researchers trying to develop a new biomarker, as in the process of study the two groups that are contrasted are healthy people and people with the specific disease. This is a useful way to find new biomarkers and to study them. In the clinic the biomarker might not have the discriminating power that was observed under study circumstances. For example the clinical biomarker oliguria is very adept at diagnosing renal failure, but oliguria is also a biomarker for dehydration, hypovolemic shock and urinary retention.

Biomarkers are needed for early detection of diseases. In the clinic, early diagnosis is relevant if early intervention is possible. In pharmaceutical research, biomarkers that are able to detect the onset of disease before the onset of (severe) clinical symptoms are very useful. In toxicological and epidemiological research, biomarkers which can diagnose sub-clinical disease states can be useful.

Damage does not specify the disease. A positive biomarker test can be a sign of different underlying causes. For example oliguria: the decreased production of urine may be a sign of dehydration, renal failure, hypovolemic shock or urinary obstruction/urinary retention. This can produce problems for treatment, as the disease should be treated, usually not the symptoms.

Subtype classification

Biomarkers for differentiation between subtypes of a disease can be very important for treatment selection. This is usually only possible by combining several tests or with proteomics and genomics biomarker scans where there are thousands of measurements per sample.
There are two ways to come up with a novel biomarker for classification of a disease into subtypes. Research can be started by discriminating between known (clinical) subclasses of the disease and looking for biomarker differences, or by gathering data on diseased people and clustering them according to biomarker behavior.

Monitoring of disease, predicting response to treatment and prognosis

Monitoring a disease can be important in several ways. It can be important to monitor whether an intervention is needed, as in the monitoring of serum glucose levels in a diabetic. Monitoring a disease can also be important to determine whether the disease progresses, for example monitoring the size of a tumor in a cancer patient. Disease monitoring can also be used to follow the effect of a certain treatment and decide whether there should be an adaptation in this treatment. And last, it can be used to give a prognosis, for example how long it will take a patient to get better.

For the monitoring of a disease, a biomarker does not need an absolute quantitative measure to distinguish between healthy and disease, as there is serial sampling of the same individual and only relative changes are relevant. Relative changes from one sampling to the next indicate a change in disease status and can therefore indicate whether the patient gets better or worse. This also means that significant variations between patients do not inhibit the use of a biomarker for the monitoring of a disease. Even if a biomarker is not relevant for all patients, it still works when a test can specify whether it is useful in a specific patient.

Several genetic or proteomic characteristics (biomarkers) of a patient can shed light on the probable effectiveness of a certain treatment or give a prognosis for the disease progression. For example the BRCA1 gene in breast cancer can predict how aggressive the breast tumor will be.

3.2.2 General properties of clinically applicable biomarkers

Clinically applicable biomarkers are usually also applicable in research. A biomarker must have several qualities in order to be clinically applicable.
First of all, the biomarker test must be easy to perform. This means that it must be as non-invasive as possible, using external bodily fluids or blood. The biomarker test should

be performed at the bedside or with a (relatively) simple laboratory test using a rapid and reliable standardized platform.
Second, a biomarker should be highly specific for the disease and preferably be able to identify subtypes and causes of the disease (if applicable).
And last, a biomarker should be sensitive for as early detection as possible.
Preferably, the biomarker would also be able to stratify patients according to risk based on different cutoff values (Nguyen and Devarajan, 2008).

There are biomarkers which can be used in research that are not useful in the clinic. These biomarker tests are usually difficult to perform because of complicated and/or lengthy laboratory procedures. In population research, some biomarkers are not specific enough. As a result of the large number of individuals tested, a small percentage of misclassifications still leads to a large number of people are over-treated.

3.2.3 Early and late biomarkers

Early biomarkers are biomarkers which can detect renal injury within one or two days after the onset of kidney injury. Late biomarkers detect renal injury more than two days after renal injury took place.
The time point at which a biomarker test is conducted and what exactly is tested for, determines which biomarkers are relevant. In the case of kidney injury, there is a clear distinction between early and late biomarkers. If the biomarker should test for acute kidney injury, the time between onset of injury and the biomarker reaching a measurable concentration, should be minimal.
For example, biomarkers that are currently being used to detect AKI after surgery are serum creatinine and oliguria. These biomarkers are only available for testing at 3 or 4 days after the procedure. The onset of AKI is directly after the procedure. New biomarkers for early detection of AKI are needed and being researched. In case of the detection of chronic kidney injury, the body is in steady state so there is no biomarker stratification based on early and late identification.

3.2.4 Sensitivity, specificity and the predictive value of a positive and negative test

Specificity is the ability of a test to correctly identify a negative case (D / (B + D), Table 1). In case of lowered specificity, the number of false positives is increased. Sensitivity is the ability of a test to correctly identify a positive case. (A / (A + C)). In case of lowered sensitivity, the number of false negatives is increased.

Specificity:
“A detection dog used by law enforcement to track drugs may be used specifically to find drugs. If the dog begins to become less specific and starts also tracking the odor of shampoo, it will begin to lead the law enforcement agents to innocent packages. Thus, a much larger number of packages will be "picked up" as suspicious by the dog, leading to what is called false positives - test results labeled as positive (drugs) but that are really negative (shampoo). In terms of specificity, this dog doesn't miss any drugs because of its new affinity to shampoo; it just begins to track drugs and shampoo (more false positives).”
Sensitivity:
“Continuing with the example of the law enforcement tracking dog, an old dog might be retired because its nose becomes less sensitive to picking up the odor of drugs, and it begins to miss lots of drugs that it ordinarily would have sniffed out. This dog illustrates poor sensitivity, as it would give an "all clear" to not only those packages that do not

contain any drugs (true negatives), but also to some packages that do contain drugs (false negatives)." (Wikipedia, 2009)

Table 1 Sensitivity, specificity and predictive values

	Condition present	**Condition absent**	
Test positive	True positive (A)	False positive (B)	Positive predictive value
Test negative	False negative (C)	True negative (D)	Negative predictive value
	Sensitivity	Specificity	

For all biomarkers there is a trade-off between sensitivity and specificity. A good biomarker has both a high sensitivity and a high specificity. The intended use of a biomarker dictates whether more emphasis is placed on sensitivity or specificity. A clinical biomarker can be less specific than a population research biomarker, due to the fact that the number of people tested is relatively low. A false positive test result in this case leads to a small number of people receiving treatment or a follow-up test. In a population wide screening for a disease, even a small percentage of false positives lead to a high number of people needing an otherwise unnecessary follow-up examination or even going through needless treatment.
For a biomarker, the relationship between sensitivity and specificity depends on the cutoff value used for that biomarker. The cutoff value of a biomarker is the threshold (e.g. concentration) for determining whether a test is considered positive or negative. The degree to which sensitivity and specificity interact with one another is evaluated by measuring the area under the curve (AUC) of the receiver-operating characteristic (ROC) curve (a plot of sensitivity vs. 1-specificity). AUC values typically vary between 0.5 and 1.0. Tossing a coin will give an AUC of 0.5. Hence, a biomarker with an AUC of 0.5 is not considered useful. A biomarker with an AUC of 1.0 is a perfect biomarker. In reality this is almost never the case for a biomarker. Therefore, for practical purposes a biomarker is considered a good biomarker if it has an AUC of 0.75 and an excellent biomarker if it has an AUC ≥0.9

For clinical biomarkers not only sensitivity and specificity are important, but also the predictive value of a positive test (PVP) and the predictive value of a negative test (PVN). The PVP is the fraction of people with a positive test result who actually have the condition (A / (A + B), Table 1).
The PVN is the fraction of people with a negative test result which are truly free of the condition (C / (C + D). The PVP and PVN will vary depending on the prevalence of the condition in the population (e.g. the general population, people in the intensive care unit, etc.) (LaBaer, 2005;Nguyen and Devarajan, 2008).

PVP is especially important when deciding whether to give someone an expensive, invasive or risky treatment. A high PVP means that a positive test results is very likely to be correct. A high PVN is good at excluding people. A high PVN means that it is very unlikely that a negative test result is false.

3.2.5 Development of standards

In the development of biomarkers, it is very important to establish a standard for the biomarker by which it can be used in clinical practice or in research. Without a standard, a biomarker value can not indicate a positive or negative test result, as the measured

value could mean anything, because it is not known which factors have influenced it beside disease state. For a lot of biomarkers it is difficult to set such a standard, because biomarker levels change depending on different factors such as age, sex, body mass (muscle or fat), metabolism rate, etc.

Biomarkers that are used to diagnose diseases should have an absolute quantitative measure to distinguish between a negative and a positive test, between healthy and disease. An absolute quantitative measure can be compared to a reference, for example the standard biomarker value of a healthy person.

A biomarker test can be combined with a second biomarker or for example a physical examination to be more convincing or to lend more relevance to its absolute measure. This multiple testing can be done in parallel or serial. For example a biomarker test could be accompanied by a physical examination. If this then leads to the suspicion that the disease is present a biopsy could be taken or a scan could be made to confirm the diagnosis.

3.2.6 Relevance of biomarkers in kidney damage

In the clinic as well as under research conditions it is very useful to be able to measure whether there is kidney damage, what kind of damage it is and to what extend the kidney is damaged.
Routinely used (clinical) biomarkers for kidney injury are serum creatinine and BUN. These biomarkers have several disadvantages. Serum levels of creatinine may only change after about 50% of the kidney function has been lost. Altered levels of serum creatinine take time to establish and make it impossible to detect acute kidney injury early.
Baseline serum creatinine levels vary widely with age, gender, lean muscle mass, etc. This makes it difficult to determine an accurate standard for kidney morbidity based on serum creatinine.
BUN has a similar problem, as it varies with diet and state of hydration.

The kidney is one of the primary sites of drug toxicity. It is highly desirable in drug development to minimize nephrotoxicity. Not only is this desirable for the test subjects, it is also desirable for pharmaceutical companies. Early and sensitive detection of the nephrotoxicity of a component allows the component to be excluded after only a (small) preclinical study. This reduces the costs of drug development, because there are less harmful components promoted to the next developmental phase.

4. Biomarkers in kidney damage

There are several biomarkers in use to detect kidney damage both in the clinic and in research. These biomarkers have been used for a very long time, but are often not very specific. New, more specific, biomarkers are being developed. Biomarkers that can only be assessed using a biopsy will not be discussed here.

4.1 Standard biomarkers for kidney damage

Standard biomarkers for kidney damage are: glomerular filtration rate (GFR), plasma creatinine, BUN, renal blood flow and several urine qualities such as proteinuria and hematuria. These markers are described in this section. Qualities of standard biomarkers are summarized in Table 2. Most texts in section 4.1 are adaptations from (McCance and Huether, 2002b).

Table 2 Qualities of standard biomarkers

Name	Type of injury	Sample source	Technique
Plasma creatinine	C	B	Jaffe method
Blood urea nitrogen	C	B	chemical colorimetric method
Renal blood flow	A, C	B	chemical colorimetric method
Hematuria	A, C	U	Microscope
Pyuria	A, C	U	Microscope
Proteinuria	A, C	U	Dipstick, protein electrophoresis
Crystals	A, C	U	Microscope
Casts	C	U	Microscope
pH	C	U	Dipstick
Specific gravity	A, C	U	Hydrometer

Type of injury:
A: acute kidney injury
C: chronic kidney injury

Sample source:
B: blood
U: urine

4.1.1 Plasma creatinine levels

Creatinine is a break-down product of creatine phosphate, which is used as an energy resource in the muscles. Creatinine is produced by the muscles and excreted into the blood at a relatively constant rate. Creatinine is commonly used in the clinic to determine GFR in a patient.
GFR is a measurement of the functioning of the glomerulus. GFR provides a good estimate of the functioning of the renal tissue in chronic disease cases or extended acute renal injury cases. The measurement of GFR requires a substance that has a stable plasma concentration, is freely filtered and is not metabolized or actively transported by the tubules. To measure GFR a blood sample is needed in combination with a 24 hour sample of urine. The concentration of the substance is then measures in both these samples.
Creatinine is freely filtered at the glomerulus. Small amounts are secreted by the tubules, which leads to a small but acceptable overestimation of GFR. These qualities make blood creatinine levels a good measure of GFR. When the body is in steady state, the amount produced by the body approximates the amount filtered and excreted in the kidneys. The plasma concentration of creatinine changes until the amount excreted again equals the production if either the rate of production or the GFR changes. Therefore, if GFR levels decline (e.g. chronic renal failure), the plasma creatinine level increases by a reciprocal amount. The plasma levels continue to increase as the GFR

decreases, because no significant tubular adjustment occurs for creatinine. This relationship between creatinine blood concentration and renal excretion of creatinine allows plasma creatinine concentration to serve as an estimate of changing glomerular function. Plasma creatinine is measured in a plasma sample by the Jaffe method (Jaffe, 1886), which is simple and fast and is based on a reaction of creatinine with alkaline picrate.

A long term decline in GFR is reflected in the plasma creatinine concentration. Lower than normal plasma creatinine levels indicate chronic kidney injury. It takes 7 to 10 days for the plasma creatinine level to stabilize when GFR declines. Plasma creatinine levels may not change until 50% of kidney function has been lost. This makes plasma creatinine levels a suitable biomarker for chronic kidney injury and its progress, but not for acute kidney injury.

4.1.2 Blood urea nitrogen levels

Urea is a waste product of the liver and part of the urea cycle. Urea is removed from the blood by the kidneys. Urea clearance is similar to creatinine clearance but urea is both filtered and reabsorbed and urea levels vary with the state of hydration and diet. Urea clearance is therefore less than GFR, if protein intake and metabolism are constant. However, plasma levels increase as the GFR declines. If there is no tubular adaptation, urea levels change because urea is primarily excreted by glomerular filtration. BUN levels are measured by chemical colorimetric method.

The concentration of urea nitrogen in the blood reflects glomerular filtration and urine-concentrating capacity. Urea is filtered at the glomerulus and as a result, BUN levels increase as glomerular filtration drops. BUN rises in states of dehydration and acute and chronic renal failure when passage of fluids through the tubules is slowed, because urea is reabsorbed by the blood through the permeable tubules. BUN also varies as a result of changed protein intake and protein catabolism and therefore is a poor measure of GFR. BUN is used for the detection of chronic kidney injury, as BUN levels do not change until there is extensive renal damage.

4.1.3 Renal blood flow

Renal blood flow is a measure of how well the kidney is perfused and thus how well it is able to function. Renal blood flow can be measured by using a substance that is (almost) perfectly excreted into the urine by the kidney. Renal blood flow is measured by determining the clearance of a substance from the blood. Clearance is the amount of substance the kidneys can clear from the blood per unit of time. The most often used substance for this purpose is para-aminohippurate (PAH). All PAH reaching the glomerulus and tubules is secreted by the kidney. Declining blood PAH concentration can then be measured and used to determine renal blood flow. PAH concentrations are measured by colorimetric test. However, not all blood provided to the kidney reaches these structures as 10% to 15% of the renal blood travels to the supporting and non-secreting structures of the kidney. Thus, PAH clearance only measures effective renal plasma flow, which is 85% to 90% of total renal blood flow.

4.1.4 Hematuria

Hematuria is the presence of red blood cells in urine. Under normal conditions there are almost no red blood cells present in urine. Several renal diseases (e.g. severe AKI or severe inflammation) can cause the excretion of red blood cells in urine which may be

seen as red sediment or, when under alkaline conditions the red blood cells have lysed, through the presence of hemoglobin. In the case of lysed red blood cells the specific gravity will also be elevated. Hematuria is a very general marker and is only present when there is severe kidney injury.

4.1.5 Pyuria

Pyuria is the presence of white blood cells in urine. Pyuria is measured by spinning down a sample of urine and studying the pellet under the microscope. The presence of white blood cells is indicative of an infection such as urinary tract infection. The combined presence of pyuria, proteinuria, hematuria and casts is an indication of glomerulonephritis and nephritic syndrome. White blood cell casts are an indication of kidney inflammation, as casts are only formed in renal tubules.

4.1.6 Proteinuria

Proteinuria is an excess of serum proteins in urine. Proteinuria can be measured by protein electrophoresis or by dipstick test. Protein electrophoresis quantifies the total protein content of the urine, whereas the dipstick test measures mainly albumin. Proteinuria can occur as a result of glomerular disease, proximal tubule damage, or an excess of serum protein. A different measure for proteinuria is the protein/creatinine or albumin/creatinine ratio. Proteinuria only takes place after severe kidney damage and takes days to develop after kidney injury has taken place.

Albumin is the most abundant plasma protein in humans and other mammals. Albumin functions primarily as a carrier protein for steroids, fatty acids, and thyroid hormones. Albumin plays a role in stabilizing extracellular fluid volume.
Urinary albumin levels only change when there is severe kidney damage: >2/3 loss of function. Low serum albumin levels can reflect diseases in which the kidneys cannot prevent albumin from leaking from the blood into the urine, leading to high urinary albumin levels.

4.1.7 Urinary Crystals

Crystals that can be found in the urine are diverse. Urinary crystals are measured by studying the pellet of a centrifuged urine sample under a microscope. Crystals tend to form in concentrated acidic or alkaline urine and might be formed only after the urine has cooled down. Crystals can be an indication of inflammation, infection, or a metabolic disorder. Urinary crystals are a very a-specific measure of renal damage and can be influenced by diet and other factors independent of renal damage.

4.1.8 Urinary Casts

Casts are accumulations of cellular precipitates from the renal tubules, specifically the ascending limb of the distal tubule. Casts are detected by a microscope study of a centrifuged urine sample. Casts are cylindrical in shape with distinct edges because of their origin. Red cell casts indicate bleeding into the tubule, white cell casts indicate inflammation and epithelial cell casts indicate degeneration of the tubular lumen or necrosis of the renal tubules.

4.1.9 Urine pH

The kidney maintains the acid-base balance in the blood. Thus, a change in urine pH can be an indication of renal failure. Urine pH normally ranges between 5.0 and 6.5 but can vary from 4.5 to 8.0. The pH is influenced by sleep (because of intermittent

hypoventilation) and varies before and after a meal. The wide range and dependence on recent activity makes pH difficult to use as a biomarker, but continuous measurements can provide useful information.

4.1.10 Specific gravity of urine

The specific gravity of a fluid is a measurement of the solute concentration. Specific gravity is measured by comparing the weight of a specific fluid with an equal volume of distilled water and corresponds well with osmolality. Specific gravity is a measure for the ability of kidneys to concentrate and/or dilute urine. Elevated/lowered specific gravity of the urine can point to tubular injury, but can also be caused by dehydration.
Specific gravity is usually measured with a hydrometer in a volume of urine. Dipsticks are also available but are less reliable because they only give an accurate result between pH 6.0 and 7.0. Specific gravity of urine is a very general measure of kidney function.

4.2 Biomarkers currently under development

This section discusses new biomarkers. A summary of all biomarkers currently under development is shown in Appendix I. For the purpose of this review, the performance of biomarkers with ≥5 references is discussed. The selected biomarkers are highlighted in grey in Appendix I. Table 3 contains a summary of the predictive qualities of each of the selected biomarkers.

Table 3 Predictive qualities of selected biomarkers

Biomarker		Sample source	AUC	Sensitivity [%]	Specificity [%]	PVP [%]	PVN [%]
Cystatin C		U	-	-	-	-	-
		B	0.82-0.97	55-82	-	-	-
GST	Alpha-	U	0.89	-	-	-	-
	Pi-	U	0.93	-	-	-	-
IL-18		U	0.61-0.95	68-85	88-95	75	93
		B	0.84	64	92	88	86
IP10; CXCL10	Protein	U	0.68-0.84	58-71	75-95	-	-
	mRNA	U	0.73-0.90	58-100	78-85	-	-
Kim-1		U	0.61-0.80	-	-	-	-
NAG		U	0.71-1.00	50-100	64-81	33-50	78-100
NGAL		U	0.80-0.95	73-90	78-99	47	91
VEGF		U	0.73-0.90	62-85	62-84	-	-

Note: Ranges are a summary of different articles. Single values are based on one article.
Sample source: B: blood, U: urine

4.2.1 Cystatin C

Cystatin C (Cys C) is a low-molecular-weight protein of ~13 kDa which is produced at a constant rate by all nucleated cells. It is a strong inhibitor of cysteine proteinases. Cys C is freely filtered by the kidney glomerulus and reabsorbed by the tubules, where it is almost totally catabolized (Orlando et al., 2002). Non-catabolized Cys C is excreted in urine.
Urinary Cys C is thought to be a good estimate for renal tubular dysfunction, as a dysfunctional tubule will have a diminished or abolished ability to catabolyze Cys C. Serum Cys C is measured by immunoassay (Finney et al., 1997), whereas urinary Cys C is measured by ELISA (Uchida and Gotoh, 2002).

The efficacy of serum Cys C as a replacement marker for serum creatinine to estimate GFR has been extensively studied. The majority of these studies show an earlier and more accurate prediction if serum Cys C is used in stead of serum creatinine, but there are studies which show no difference or even a preference for serum creatinine. Unfortunately, it is not clear what factors influence serum Cys C. Not only specific conditions such as HIV (Collé et al., 1992), melanoma (Kos et al., 1998), thyroid dysfunction (Fricker et al., 2003), and glucocorticoid therapy (Risch et al., 2001) can influence serum Cys C, but also factors as age, gender, greater weight, height, cigarette smoking, and higher creactive protein levels (Knight et al., 2004).
Urinary Cys C was shown to be constant for age and muscle mass in a study by Uchida, *et al*. (Uchida and Gotoh, 2002).
It has also been shown that the relative proportion of serum Cys C eliminated extrarenally increases with decreasing GFR (Sjöström et al., 2005), which makes serum Cys C less suitable as a marker for GFR.

Serum Cys C may be a good biomarker for AKI, because it reacts one to two days before serum creatinine (Herget-Rosenthal et al., 2004). The R-criterion of ≥50% increase from baseline was applied to both creatinine and Cys C. Cys C showed accurate prediction of AKI with an AUC of 0.82 (sensitivity 55%) and 0.97 (sensitivity 82%) respectively on two and one days before the R-criterion was fulfilled by creatinine. Villa, *et al.* showed an even better AUC for serum Cys C in the prediction of AKI in critically ill patients. Serum Cys C had an AUC of 0.927 (95% CI: 86.1–99.4), compared to a serum creatinine AUC of 0.694 (95% CI: 54.1–84.6) (Villa et al., 2005).

Conti, *et al*. indicated that urinary Cys C is a valid biomarker to distinguish between glomerular dysfunction (GD) and tubular dysfunction (TD). There was no significant difference between urinary Cys C levels in glomerular dysfunction patients compared to controls (GD: 0.106±0.133 mg/L, range 0.01–0.64 mg/L, normal distribution, controls: 0.096±0.044 mg/L, normal distribution; t-test, not significant). Urinary Cys C concentrations were significantly higher in TD patients (4.13±3.85 mg/L, range 0.25–16 mg/L, normal distribution) compared to both GD patients ($p<0.0001$) and controls ($p<0.0001$) (Conti et al., 2006).
Urinary Cys-C to creatinine (CCR) ratio was shown to give a good indication of Cys C reabsorption in the proximal tubules, which can be seen as an indication of proximal tubule integrity. When CCR is within normal range, urinary Cys C concentration reflects glomerular filtration rate (Uchida and Gotoh, 2002).

In June 2008, the FDA approved the use of serum Cys C as a biomarker for renal damage as part of the drug review process (FDA, 2008). This is a good complement to serum creatinine and may even be a good replacement for serum creatinine, but it is no competition for other new AKI markers and will only be really relevant in chronic kidney injury cases. In these cases it can measure renal excretory function, but it can't give a differential diagnosis, which means it should be used in combination with other biomarkers.

4.2.2 Glutathione S-transferases alpha and pi

The family of glutathione S-transferases (GSTs) is a functionally diverse group of cytosolic soluble enzymes that use reduced glutathione in conjugation and detoxification reactions.
Alpha-GST is expressed in the proximal tubules, whereas pi-GST is expressed in the distal tubules and collecting duct cells (Kharbanda et al., 1991;Sundberg et al., 1994). Both enzymes are shed into the urine in case of tubular damage. This fact makes these two biomarkers very useful for distinguishing proximal and distal tubular damage. Alpha-GST and pi-GST are assayed using ELISA. If no other method than ELISA becomes available (e.g. dipstick or some similarly simple test), these markers will probably only be used as biomarkers in research.

Alpha-GST may be a useful biomarker for AKI (Boldt et al., 2003;Yavuz et al., 2009), but for pi-GST study results are not consistent (Walshe et al., 2009). Walshe, *et al* showed that urinary pi-GST is elevated in all patients with sepsis syndrome, which is not predictive of AKI as defined by the Acute Kidney Injury Network (AKIN). This urinary pi-GST elevation may be caused by a very high sensitivity of pi-GST as a renal damage biomarker, but makes pi-GST less accurate as a predictor for AKI, as not all renal damage cases develop into clinical AKI.

Acute renal impairment in intensive care can be measured by urinary alpha- and pi-GST levels. The AUC for alpha-GST level at admission was 0.893 for renal damage and for pi-GST 0.929 respectively (Westhuyzen et al., 2003).
Urinary GST-to-creatinine ratio is a sensitive early biomarker for renal injury after infrarenal abdominal aortic aneurysm repair (Cressey et al., 2002).
Alpha-GST-to-creatinine ratio showed the largest increase of the two GSTs, which occurs within 1 hour after aortic cross-clamp release. This is associated with post-operative serum creatinine levels. Elevated levels of alpha-GST may occur, even though there are no other signs of renal damage (Branten et al., 2000;Sundberg et al., 1994). Low but elevated levels of urinary GSTs can therefore be sensitive biomarkers for early renal damage and for diagnosing sub-clinical renal damage in (toxicological) research (Brüning et al., 1999a). For example: pi-GST was present in the urine of patients who had been exposed to nephrotoxicants, but conventional tests showed no kidney damage (Sundberg et al., 1994).

One important aspect in kidney transplantation is that it is very useful to be able to determine whether a donor kidney will be viable. Alpha-GST in machine perfusate before kidney transplant did not show a difference between groups of functioning and nonfunctioning of the allograft after transplantation (data post-operationally linked).
Pi-GST in machine perfusate before kidney transplant did not show a difference between functioning and nonfunctioning of the allograft after transplantation (Kievit et al., 1997).
In the urine of the donor there was a significant relationship found between elevated pi-GST level and early postoperative course ($r = 0.84$; $p<0.001$) (Sárváry et al., 2000).

Even when there is a good selection procedure before transplantation, this does not mean that rejection doesn't occur. It would be very useful to have a biomarker for the early detection of renal transplant rejection. Pi-GST was shown to be excreted in cases of acute rejection after renal transplantation. High levels of alpha-GST in the urine after renal transplant also indicate acute allograft rejection (Brüning et al., 1999a;Polak et al., 1999;Sundberg et al., 1994;Sárváry et al., 2000).

4.2.3 Interleukin-18

Interleukin-18 (IL-18) is a proinflammatory cytokine. IL-18 is primarily produced by macrophages and acts as a mediator of inflammation and ischemic tissue injury in many organs. IL-18 assay is performed using a standardized ELISA kit.

Patients with acute tubular necrosis were shown to have significantly elevated urinary IL-18 concentrations compared to healthy controls ($p<0.0001$). Patients with prerenal azotemia, UTI, chronic renal insufficiency, and nephritic syndrome showed elevated IL-18 levels, but the elevated levels were not significant. The AUC for the diagnosis of ATN by urinary IL-18 concentration was shown to be 0.95 at cutoff value of 500 pg/ml with a sensitivity of 85% and a specificity of 88%, a PVP of 75% and a PVN of 93% (Parikh et al., 2004).
urinary IL-18 predicts AKI after cardiopulmonary bypass (CPB) with peak AUC at 12h post operation (AUC was measured at three time points, at 4h the AUC was 0.61, at 12h the AUC was 0.75 and at 24h the AUC was 0.73) (Parikh et al., 2006a). Several cutoff values were tested, which did not change the AUC values. Sensitivity, specificity and PVP and PVN were optimal for a cutoff of >50 pg/ml. This cutoff is significantly lower than that used by the previous study. It is not clear whether this is due to the difference in diagnosis or a methodological difference.

Vaidya, *et al*. evaluated IL-18 as a biomarker for the diagnosis of AKI compared to healthy individuals from general population and AKI-negative individuals from the hospital (patients in intensive care or patients undergoing cardiac catheterization). The AUC for healthy individuals was estimated at 0.85 (95% CI: 0.78–0.90) with a cutoff value of 2.30 pg/ml, sensitivity 69% and specificity 92%. The AUC for non-AKI controls was estimated at 0.83 (95% CI: 0.77–0.88) with a cutoff value of 2.74 pg/ml, sensitivity 68% and specificity 95% (Vaidya et al., 2008a).
Urinary IL-18 can also be a valuable biomarker for AKI after transplantation. Urinary IL-18 was shown by Hall, *et al*. to have an AUC of 0.68 (95% CI: 0.55–0.81) at 0h post transplantation, which after day 1 increases to 0.82 (95% CI: 0.72–0.92) and after day 2 has an AUC of 0.74 (95% CI: 0.62–0.86) (Hall et al., 2009).

Serum IL-18 gene expression measured by real-time PCR can be used as a predictor for rejection in the earliest stages of graft rejection. For several windows of measurement (post operative days 5-7, 8-10 and 11-13) serum IL-18 levels in acute rejection patients were compared to patients without rejection ($p<0.01$ for all time-slots) (Simon et al., 2004). The optimal AUC was reached at 8-10 days post operation with an AUC of 0.836, sensitivity 64%, a specificity of 92%, a PVP of 88%, and a PVN of 86%.

4.2.4 IP-10/CXCL10 and CXCR3

Interferon-inducible protein-10 (IL-10 or CXCL10) is a chemokine of the CXC motif family and is a ligand for the receptor CXC chemokine receptor 3 (CXCR3). The binding of IL-10 to its receptor is important in immune reactions. It stimulates monocytes, natural killer and T-cell migration. IL-10 expression is related to allograft rejection and lupus nephritis. IP-10 and CXCR3 are often investigated in tandem, because they are functionally linked.

In surgery related AKI, urinary IP-10 protein may be a good biomarker for early detection. At post operative day (POD) 1, non-AKI urinary IP-10 levels had a mean value of 1.38 ± 0.23 versus AKI, 5.3 ± 1.2 ng/mmol of creatinine ($p=0.002$). At PODs 3 to 5, non-AKI urinary IP-10 levels had a mean value of 2.01 ± 0.33 versus AKI 5.08 ± 1.10 ng/mmol of creatinine ($p=0.01$) (Ho et al., 2009).

IP-10 and CXCR3 mRNA levels in urinary cells were shown to be good predictors of acute rejection. IP-10 demonstrated an AUC of 0.903 (95% CI: 0.824-0.982), a sensitivity of 100%, and a specificity of 78%. CXCR3 mRNA levels were less effective at predicting acute rejection with an AUC of 0.762 (95% CI: 0.644-0.881) with a sensitivity of 63%, and a specificity of 83% (Tatapudi et al., 2004). This data was supported with an immunohistological analysis of allograft biopsies during acute rejection which showed abundant expression of IP-10 and CXCR3. This expression was absent in biopsies of grafts with stable function. IP-10 urinary mRNA levels were shown to be suitable for distinction of acute rejection and three other diagnostic classes. mRNA levels were higher in acute rejection than in chronic allograft nephropathy ($p<0.01$), stable posttransplant group ($p<0.01$) or other histological findings (“other”) ($p<0.01$). Heightened urinary CXCR3 mRNA levels were suitable to distinguish acute rejection, stable posttransplant ($p<0.01$) and “other” ($p<0.01$). However no significant difference was found between acute rejection and chronic allograft nephropathy ($p>0.05$).
Levels of IP-10 mRNA and protein expression helped to identify acute rejection episodes days faster than rising serum creatinine levels. Elevated levels of urinary IP-10 protein within the first four postoperative weeks were predictive of graft function at 6 months even in the absence of acute rejection. IP-10 urinary protein expression had an optimal

AUC at 2-3 days prior to rejection with a cutoff of 185 pg/ml, AUC is 0.844 with a sensitivity of 71% and a specificity of 95% (Matz et al., 2006). In this study urinary mRNA levels were differentiated patients with different degrees of acute allograft rejection (Banff classification or borderline rejection) from non-rejecting patients (Mann-Whitney U-tests, $p<0.05$). However, the AUC for IP-10 mRNA showed very low sensitivities for all time points. Most optimal AUC was 0.731, 6-7 days prior to rejection with sensitivity of 58% and a specificity of 85%. The AUC associated with mean urinary IP-10 expression at a cutoff value of 196 pg/ml during the first month post transplant was 0.68 with sensitivity 58% and specificity 75% for the prediction of a six month GFR above or below 45 ml/min per 1.73 m^3.

In cases of LN urinary mRNA levels of IP-10 were also evaluated. Avihingsanon, *et al*. demonstrated that measurement of urinary IP-10 mRNA is able to distinguish between diffuse proliferative lupus nephritis (class IV LN) from classes II, III, V, and VI of LN. Urinary IP-10 mRNA was not detected in controls. AUC for urinary IP-10 mRNA was 0.89 (95% CI: 0.78-0.99). Conventional biomarkers scored significantly less with AUCs of 0.598 for systemic lupus erythematosus disease activity index (SLEDAI), 0.554 for creatinine clearance, 0.536 for 24h urine protein excretion, 0.589 for urinary leucocytes, and 0.741 for urinary erythrocytes. This sets apart urinary IP-10 mRNA levels from conventional markers as a biomarker for class IV LN (Avihingsanon et al., 2006). According to the same study, monthly measurements of urinary IP-10 and CXCR3 mRNA levels predict the response to treatment. Urinary IP-10 and CXCR3 mRNA levels were reduced in the responder group compared to the non-responder group ($p=0.01$ and 0.05 respectively). IP-10 and CXCR3 levels tended to increase in the non-responder group, but this was not significant ($p=0.85$ and 0.66 respectively).
The ability of IP-10 to distinguish between class IV LN and other classes is important, because repeated attacks of LN, particularly class IV, are the worst prognostic factor for the development of end-stage renal disease in systemic lupus erythematosus (El Hachmi et al., 2003).

4.2.5 Kidney injury molecule-1

Kidney injury molecule-1 (kim-1) is a very promising biomarker for kidney damage. It is structurally related to the immunoglobulin gene family (Ichimura et al., 1998). It is a type 1 transmembrane protein which is exclusively and abundantly expressed in damaged kidney cells. The Kim-1 ectodomain is shed into urine and easily detectable. Kim-1 expression is measurable within a day after the onset of kidney damage. It is a marker for acute and chronic kidney disease.

In several studies urinary Kim-1 was elevated in all participating renal disease patients. The highest levels of Kim-1 occurred in acute tubular necrosis (ATN) patients (Han et al., 2002;Vaidya et al., 2008b). Han, *et al*. quantified Kim-1 by both Western blot and ELISA. However, analysis with ELISA showed more false positive results.
Another study also showed a significant difference in urinary Kim-1 between renal patients and controls ($p<0.001$), independent of the specific type of renal disease (van Timmeren et al., 2007a). A relation was shown between Kim-1 levels and tissue Kim-1, inflammation and renal function, but not proteinuria.
Kim-1 was shown to be an acceptable biomarker for the prediction of adverse clinical outcome in AKI patients. Urinary Kim-1 had an AUC of 0.61 (95% CI: 0.53 to 0.69) for the prediction of dialysis requirement or hospital death. There was no significant difference with serum creatinine at enrollment. In combination with N-acetyl-β-D-glucosaminidase (NAG) activity the AUC was 0.71 (95% CI: 0.63 to 0.78)

and significantly different from serum creatinine at enrollment. The best predictor of adverse clinical outcome was the combination of urinary Kim-1 with Acute Physiology, Age, Chronic Health Evaluation II (APACHE II) score with an AUC of 0.80 (95% CI: 0.74 to 0.86) (Liangos et al., 2007).

In renal cell carcinoma, urinary Kim-1 normalized to urinary creatinine levels (U_{CR}), was shown to be elevated (0.39 ± 0.08 ng/mg U_{CR}; n=21) compared to both normal controls (0.05 ± 0.01 ng/mg U_{CR}; $p<0.005$; n=30) and prostate cancer patients (0.12 ± 0.03 ng/mg U_{CR}; $p<0.02$; n=10) (Han et al., 2005). There seems to be a correlation between tumor size and normalized urinary Kim-1 levels ($p<0.005$), but further study is needed to determine the value of Kim-1 as a prognostic tool.

Urinary Kim-1 has been shown to be a valid early predictor of allograft loss. A prospective study by van Timmeren, *et al.* shows that allograft loss is higher with increasing levels of Kim-1 (van Timmeren et al., 2007b), from 6.3% at 0.01–0.48 ng/24h to 35.4% at 1.15–10.04 ng/24h, with a p-value <0.001.

Because the Kim-1 biomarker can be used for early diagnosis, early clinical intervention is possible. This is in contrast with the usual biomarkers for kidney damage, creatinine, and BUN. The early detection capacity of Kim-1 may guide pharmaceutical development by providing an early indication of nephrotoxicity. In June 2008, the FDA approved the use of urinary Kim-1 as a biomarker for renal damage as part of the drug review process (FDA, 2008).

Until recently, the only method of assessing urinary Kim-1 was by Western blot, ELISA assay or microsphere-based Luminex technology. These assays take a lot of time and are (relatively) labor intensive. A new urinary Kim-1 assay, RenaStick, was developed using a dipstick (Vaidya et al., 2009). However, this is a measurement based on band intensity on the stick, so a quantitative value is not obtained.

4.2.6 N-acetyl-β-D-glucosaminidase

N-acetyl-β-D-glucosaminidase (NAG) is a lysosomal enzyme which is abundantly present in kidney tubules. It is a high molecular weight protein of ~135 kDa that prevents it from being filtrated at the glomerulus. It is excreted into the urine at a constant rate with minimal changes during the day. NAG consists of several isoenzymes. The acidic form A is present in the kidney. Basic form B is present in the liver. Both isoenzymes are present in the urine of healthy subjects with a ratio A:B of 4:1 to 10:1. In case of kidney injury, the levels of isoenzyme B in particular are elevated.
The most common assay technique for urinary NAG activity are the fluorimetric assay and spectrophotometry (enzyme substrate based colorimetry) (Csáthy and Pócsi, 1995).

Urinary NAG activity was extensively tested as a biomarker for AKI. It shows unusually high AUCs (0.71-1.00), sensitivities and specificities.
A study by Westhuyzen, *et al*. showed an AUC for NAG of 0.845 (95% CI: 0.639-0.955). The cutoff for NAG was determined in the first study (8.3 U/mmol (normalized to urinary creatinine concentration (mmol/l), U=arbitrary units of NAG activity), sensitivity 100%, specificity 81%, PVP 50%, and PVN 100%). The previously determined cutoff was then evaluated in a second study. Values for the second study were less positive: sensitivity 50%, specificity 64%, PVP 33% and PVN 78%. The test population differed from the study population by age (9.5 years older), higher plasma creatinine levels (0.13 vs. 0.08 mmol/l) and sex ratio (more men) (Westhuyzen et al., 2003).

Urinary NAG was shown to increase with APACHE II and Multiple Organ Failure (MOF) scores. Urinary NAG levels were divided into quartiles; the first quartile was compared to the other three for the association with dialysis requirement and hospital death. The second, third, and fourth quartile groups had 3.0-fold (95% CI: 1.3-7.2), 3.7-fold (95% CI: 1.6-8.8), and 9.1-fold (95% CI: 3.7-22.7) higher odds respectively. This association was still present after adjustment for APACHE II, MOF score, and the combined covariates cirrhosis, sepsis, oliguria, and mechanical ventilation. AUC for urinary NAG activity for the composite outcome of dialysis requirement or hospital death was 0.71 (95% CI: 0.63 to 0.78). This was improved by combining urinary NAG activity with APACHE II score (AUC 0.79) (Liangos et al., 2007).
Vaidya, *et al*. showed the very high AUC of 1.00 (95% CI: 0.98-1.00) for urinary NAG activity in AKI patients when compared to healthy individuals. When compared to all non-AKI individuals, this AUC declined to 0.83 (95% CI: 0.77-0.88). In the comparison to these two groups, different cutoff values were optimal. For comparison of urinary NAG activity in AKI versus healthy individuals the optimal cutoff is 0.007, for comparison with non-AKI controls the optimal cutoff is 0.015 (Vaidya et al., 2008b).
Han, *et al*. performed a cross-sectional study which showed and AUC of 0.97 (95% CI: 0.91, 1.00) for the differentiation between AKI and non-AKI patients. These AKI patients had a ≥50% increase in serum creatinine and urinary casts compared to healthy people. A second, case-control, study was then performed to study NAG activity prospectively. NAG activity was measured at different time-points. These results were less optimistic giving a maximum AUC of 0.71 (95% CI: 0.54–0.87) at 36h after cardiopulmonary bypass (Han et al., 2008).

Urinary NAG activity can be used to determine graft function after renal transplant. A study by Kuzniar, *et al.* showed elevated excretion of NAG in acute graft rejection (AGR; $p<0.005$) and acute tubular necrosis (ATN) patients compared to stable graft function (SGF; $p<0.05$) recipients. There was no significant difference between AGR and ATN patient groups (Kuźniar et al., 2006).

4.2.7 Neutrophil gelatinase-associated lipocalin

Neutrophil gelatinase-associated lipocalin (NGAL) is a 21 kDa protein of the lipocalin super family and transports low molecular weight molecules (Flower, 2000). NGAL promotes differentiation and structural organization of renal epithelial cells. NGAL is associated with various cellular responses, such as proliferation, apoptosis, and differentiation (Schmidt-Ott et al., 2006). NGAL also comprises a critical component of innate immunity to bacterial infection by sequestrating iron (Flo et al., 2004).
NGAL is one of the most robustly up regulated genes after kidney injury. NGAL is up regulated very soon after the damage takes place and is easily detected in blood and urine soon after (Mishra et al., 2003).

Both in patients who developed AKI and those who did not develop AKI, urinary NGAL was elevated directly after surgery compared to preoperative values, contrary to serum creatinine, which does not show an up regulation for 1-3 days after injury. Wagener, *et al.* showed elevated levels of urinary NGAL as soon as 1-3 hours after cardiac surgery in the case of kidney injury (Wagener et al., 2006).
However, within the first hour after surgery, urinary NGAL had increased in patients who developed AKI and decreased in patients without AKI ($p=0.002$). Peak urinary NGAL concentration was reached at 18h post operation. At this time point, the optimal cutoff is 213 ng/ml with an AUC of 0.80 (95% CI: 0.573-1.027). Sensitivity and specificity for these parameters are 73% and 78% respectively, with a PVP of 47% and a PVN of 91%.

Urinary NGAL was also tested as a biomarker for AKI in the emergency department and showed a high sensitivity and specificity (90% and 99% respectively) for the detection of AKI at a cutoff of 130 µg/g creatinine. The AUC for these parameters is 0.948 (95% CI: 0.88-1.00) (Nickolas et al., 2008).

Urinary NGAL can be a useful biomarker for delayed graft function after kidney transplant. Parikh, *et al*. showed elevated levels of urinary NGAL on day 0. This measurement, with a cutoff of 1000 ng/mg creatinine, showed an AUC of 0.9 (95% CI: 0.71-1.0) for the prediction of delayed graft function. Using this cutoff, a sensitivity of 90% and a specificity of 83% was reached (Parikh et al., 2006b). Also every 100 ng/mg creatinine increase of urinary NGAL was associated with an increased odds for developing delayed graft function of 20%.

Until now, ELISA was the best way to measure urinary NGAL concentrations. However, there is a new clinically applicable test being developed. The new test for urinary NGAL, ARCHITECT® analyzer, is being developed by Abbott Diagnostics. It is a two-step assay using chemiluminescent microparticle immunoassay technology and is intended as a standardized clinical platform for urinary NGAL determination. Bennett, *et al*. tested this assay and compared it to ELISA analysis. The ARCHITECT® assay has a functional sensitivity <2 ng/ml. The values measured by ARCHITECT® were highly correlated to the values measured by ELISA (r=0.99) (Bennett et al., 2008).

NGAL could be a very useful biomarker for the diagnosis of acute and chronic kidney injury. Further study is necessary before it can be implemented in the clinic, because previously performed studies are small and use relatively uncomplicated patient populations (only healthy controls, while in the clinic almost no individuals are healthy).

4.2.8 Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) can promote angiogenesis and permeabilization of blood vessels (Ferrara, 1999). VEGF plays a role in several pathological processes, such as diabetic complications, tumor growth, and wound healing. VEGF has five different isoforms due to splicing consisting of 121, 145, 165, 189, and 206 amino acids, respectively. The most abundantly expressed isoform is VEGF165, which is associated with several kinds of cells including leukocytes and mesangial cells (Ferrara, 1999).
Urinary VEGF can be measured using an ELISA assay, which makes it a relatively user-friendly biomarker.

Urinary VEGF was studied as a biomarker in AKI. It has a very good capability of identifying AKI in healthy individuals with an AUC of 0.90 (95% CI: 0.84–0.94), with sensitivity 77% and specificity 84% at a cutoff of 0.43 ng/mg creatinine. The diagnostic capability of urinary VEGF for AKI versus non-AKI individuals is still acceptable with an AUC of 0.73 (95% CI: 0.66–0.79), sensitivity 62% and specificity 62% at a cutoff of 0.64 ng/mg creatinine (Vaidya et al., 2008b).

In diabetes, urinary VEGF was shown to be significantly increased in normoalbuminuric, microalbuminuric, and proteinuric diabetic patients compared to non-diabetics. There is a significant rise in urinary VEGF concentrations based on severity of renal damage. The normoalbuminuric group has a higher urinary VEGF concentration compared to the control group (median VEGF/creatinine normoalbuminuria 77.6 pg/mg creatinine vs. healthy controls 27.8 pg/mg creatinine; p<0.001). Microalbuminuria patients have a

higher urinary VEGF concentration compared to normoalbuminuria patients (130.8 pg/mg creatinine vs. 77.6 pg/mg creatinine; p=0.024) and VEGF levels are higher in the proteinuria group compared to in the microalbuminuria group (245.0 pg/mg creatinine vs. 130.8 pg/mg creatinine; p= 0.021) (Kim et al., 2005).

Urinary VEGF levels were shown to be stable in the first 8 weeks after kidney transplantation in patients with stable renal function. In contrast, urinary VEGF is significantly elevated in patients with acute rejection compared to stable patients (28.57 ± 6.21 pg/µmol creatinine vs. 3.05 ± 0.45 pg/µmol creatinine (p< 0.001). Urinary VEGF levels of healthy individuals were also compared to acute rejection patients. These levels were also significantly different (28.57 ± 6.21 pg/µmol creatinine and 2.87 ± 0.35 pg/µmol creatinine respectively, (p<0.001)). For the determination of AUC for VEGF, the patients without graft rejection and the control population were pooled as it was stated there was no significant difference between the two (no p-value provided). The AUC for acute rejection versus controls was reported to be 0.871 (95% CI: 0.820 – 0.921), with a cutoff of 3.26 pg/µmol creatinine. Sensitivity was 86.6% and specificity was 71.4% at this cutoff.
Urinary VEGF is also able to distinguish between patients with stable transplant function and subclinical rejection. The optimal cutoff for this purpose is 4.69 pg/µmol creatinine. This results in an AUC of 0.819 (95% CI: 0.662 – 0.976) with a sensitivity of 84.6% and a specificity of 79.8% (Peng et al., 2007).

Elevated levels of VEGF can distinguish LN class IV and VI from the other classes of LN. However, there are other non-invasive biomarkers which can identify class VI LN. In the absence of class VI LN, the presence of elevated levels of VEGF indicates class IV LN.
VEGF has an AUC of 0.82 (95% CI: 0.68–0.96) with sensitivity 77% and specificity 76% for the prediction of LN class IV, compared to other classes of LN (Avihingsanon et al., 2006).

4.3 Combination of biomarkers

One biomarker can be very adept at diagnosing a disease, but the combined power of two or more biomarkers can be even better. In the case of the old biomarkers, a combination between one or multiple markers and a physical examination is often used. This heightens the specificity of the test result. New biomarkers can also be combined. The most promising investigated combinations are described below.

4.3.1 NGAL, HGF, kim-1, and total protein

Vaidya, *et al*. designed a model for the prediction of AKI: risk score= 2.93*(NGAL > 5.72 and HGF > 0.17) + 2.93*(PROTEIN > 0.22) −2*(KIM < 0.58). The AUC for this risk score is 0.94, which is higher than the individual biomarkers (Vaidya et al., 2008b).
Han,*et al*. determined that the combined measurement of MMP-9, NAG and Kim-1 using their individual optimal cutoffs, results in the very high AUC of 1.00 (95% CI: 0.98-1.00) for distinguishing AKI from controls (Han et al., 2008).

4.3.2 NAG, kim-1, and APACHE

Liangos, *et al*. showed that the predictive power of several combinations of biomarkers is higher than the biomarkers alone for the prediction of the composite outcome of dialysis requirement or hospital death in AKI. Optimal combinations are: APACHE II score,

urinary KIM-1 level, and NAG activity: AUC 0.83 (95% CI: 0.77-0.88), APACHE II score and urinary KIM-1 level: AUC 0.80 (95% CI: 0.74-0.86) and Cirrhosis, sepsis, oliguria, mechanical ventilation, urinary KIM-1 level, and NAG activity: AUC 0.80 (95% CI: 0.73-0.86). Single biomarker measurements result in less powerful AUCs: urinary KIM-1 level 0.61 (95% CI: 0.53-0.69) and urinary NAG activity 0.71 (95% CI: 0.63-0.78) (Liangos et al., 2007).

4.3.3 IL-18 and perforin

In graft rejection, serum perforin gene expression was found to be a useful addition to serum IL-18 gene expression. IL-18 alone gives a sensitivity 64%, specificity of 92%, PVN of 86% and a PVP of 88% at 8-10 days post operation. For double positive test results sensitivity is 55%, specificity of 100%, PVN is 84% and PVP is 100% at 8-10 days post operation (Simon et al., 2004).

5. Conclusions

Standard non-invasive biomarkers, such as serum creatinine and general urinalysis provide non-specific and late estimations of renal function. Biopsies give a more specific overview of the kind and extend of damage, but are very strenuous for the patient and take a lot of time to complete.
However, new promising biomarkers are currently being. The most prominent new biomarkers are Cys C, alpha- and pi-GST, IL-18, IP-10, Kim-1, NAG, NGAL, and VEGF. These biomarkers in particular are sensitive for the detection of renal injury and show elevation earlier in the course of disease compared to old biomarkers. Some are on the verge of being ready for clinical applications, but all of these biomarkers can already be used in research.

Serum Cys C would be a good replacement for serum creatinine as a measurement of GFR in clinical application and drug development for the detection of AKI. Serum Cys C improves GFR estimations by two days compared to serum creatinine.
Urinary Cys C can be used to distinguish between glomerular and proximal damage. This distinction is useful when treating a patient, but is also useful in both drug development and exposure research.

Alpha-GST in urine is a good predictor for both AKI and chronic renal damage in clinical application, drug development, and exposure research. It is a sensitive and early biomarker for renal injury specifically to the proximal tubule, which detects AKI as early as 1 hour after surgery.
Pi-GST is an early and a very sensitive biomarker for the detection of distal tubular injury, although it is too sensitive as a biomarker for renal damage in clinical application, because it picks up cases of renal injury which will not develop AKI. Pi-GSTs high sensitivity makes pi-GST a good biomarker for drug development and exposure research.

IL-18 in urine is a useful biomarker for AKI after surgery (e.g. CPB), because it is elevated within hours after surgery. IL-18 is also useful in drug development and exposure research to study kidney inflammation, because of the same early elevation. Serum IL-18 is not especially useful for detection of AKI in neither clinical circumstances, nor drug development, nor exposure research, because it is not faster or more sensitive at detecting AKI than serum creatinine.

IP-10 levels in urine are useful as a biomarker of acute rejection after transplantation, as IP-10 levels are elevated within days after surgery in the case of rejection. This is more specific than serum creatinine, because serum creatinine only shows the functioning of the renal allograft, not specifically whether this is due to rejection. IP-10 levels have not shown to be suitable as a biomarker in drug development and exposure research as IP-10 levels have not shown to be sensitive to minor kidney injuries.

Kim-1 in urine is a very promising biomarker for the detection of AKI, because urinary kim-1 levels are elevated within hours of AKI onset. Kim-1 is also a useful biomarker for the detection of CRF in clinics and sub-clinical kidney injury in drug development and exposure research, because it is more sensitive to renal damage than serum creatinine.

NAG in urine is useful as a biomarker for AKI as urinary NAG levels are elevated within a day after the onset of kidney injury. This quality makes NAG levels also a good biomarker in drug development. More research is needed to confirm NAG usefulness for the detection of CRF, although there is no indication of declination of NAG levels in prolonged cases of kidney injury. It is not clear whether NGAL levels are a good biomarker in exposure research, because it is not sure whether NAG is still elevated in prolonged cases.

NGAL in urine is very useful as a biomarker for the detection of AKI in clinical setting and drug development as it is elevated within three hours of AKI onset. NGAL may also be a useful biomarker in exposure research, but first it must be studied whether NGAL elevation is also present in chronic cases of kidney injury.

VEGF in urine is mostly useful as a biomarker in CRF as it shows prolonged elevation. VEGF is useful in the clinic to stratify severity of renal dysfunction and diagnose sub-clinical rejection after transplantation which would otherwise only be detectable by biopsy. In drug development this is not a very useful biomarker as its performance is similar to serum creatinine. However, it can be useful as a marker in exposure research as it is more sensitive than serum creatinine and its ability to stratify severity.

Combinations of biomarkers are also very promising, but more research needs to be done in this field to determine the most optimal panel of biomarkers and a simple way to determine patient values for these markers.

Serum Cys C, kim-1, and NAG are ready for use in clinical therapy. Serum and urinary Cys C, alpha- and pi-GST, urinary IL-18, urinary IP-10, urinary kim-1, and urinary VEGF can already be used in drug development and exposure studies.

In summary, it appears that kidney biomarkers are well under development. Some of the abovementioned biomarkers appear to be on the verge of becoming a mainstream tool for clinical therapy, drug development and exposure studies, while others still need to be studied in varying degrees.

6. List of abbreviations

Abbreviation	In full
AGR	Acute graft rejection
AKI	Acute kidney injury
AKIN	Acute kidney injury network
APACHE II	Acute physiology, age, chronic health evaluation II
ATN	Acute tubular necrosis
AUC	Area under the curve
BUN	Blood urea nitrogen
CCR	Cys-C to creatinine ration
CPB	Cardio-pulmonary bypass
CRF	Chronic renal failure
CXCL10	CXC chemokine ligand 10
CXCR3	CXC chemokine receptor 3
Cys C	Cystatin C
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and drug administration (US)
GD	Glomerular dysfunction
GFR	Glomerular filtration rate
GST	Glutathione S-transferase
HGF	Hepatocyte growth factor
IL-18	Interleukin 18
IP-10	Interferon-inducible protein 10
Kim-1	Kidney injury molecule-1
LN	Lupus nephritis
MMP-9	Matrix metalloproteinase-9
MOF	Multiple organ failure
NAG	*N*-acetyl-beta-D-glucosaminidase
NGAL	Neutrophil gelatinase-associated lipocalin
PAH	Para-aminohippurate
POD	Post-operative day
PVN	Predictive value of a negative test
PVP	Predictive value of a positive test
RIFLE	Risk Injury Failure Loss Endstage
ROC	Receiver-operating characteristic
SGF	Stable graft function
SLE	Systemic lupus erythematosus
SLEDAI	Systemic lupus erythematosus disease activity index
TD	Tubular dysfunction
UTI	Urinary tract infection
VEGF	Vascular endothelial growth factor

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Appendix I New biomarkers in kidney damage

Name	Type of injury	Location of injury	Sample source	Molecule type	Technique	Creatinine normalization	References
Aprotinin	A	Ge	U	P	SELDI-TOF-MS	N	(Nguyen et al., 2008)
Carnitine	C	Pr	U	P	Radio-enzymatic assay	Y	(Haschke et al., 2009)
CXCR3	A, C	Ge	U	R	qPCR	N	(Avihingsanon et al., 2006;Tatapudi et al., 2004)
Cystatin C	A, C	Ge, Pr	U, B	P	Urine: ELISA Serum: PENIA	General: N Proximal: Y	(Abu-Omar et al., 2005;Benöhr et al., 2006;Biancofiore et al., 2006;Conti et al., 2006;Dharnidharka et al., 2002;Herget-Rosenthal et al., 2004;Uchida and Gotoh, 2002;Villa et al., 2005;Zahran et al., 2007;Zhu et al., 2006), etc.
FABP1	A, C	Pr	U	P	ELISA	Y	(Noiri et al., 2009;Sasaki et al., 2009;Tanaka et al., 2009;Yokoyama et al., 2009)
FOXP3	A, C	Ge	U	R	PCR	N	(Aquino-Dias et al., 2008;Muthukumar et al., 2005;Tsugawa et al., 2008;Wang et al., 2009)
GSTs (alpha- and pi-)	A, C	Pr, Di	U	P	ELISA	N	(Branten et al., 2000;Brüning et al., 1999a;Cressey et al., 2002;Daemen et al., 1997;Kievit et al., 1997;Polak et al., 1999;Sundberg et al., 1994;Sárváry et al., 2000;Walshe et al., 2009;Yavuz et al., 2009), etc.
HGF	A	Ge	U	P	Microbead based assay	Y	(Szeto et al., 2006;Taman et al., 1997;Tanimoto et al., 2008;Vaidya et al., 2008b)
IL-18	A	Ge	U, B	P	ELISA	N	(Hall et al., 2009;Parikh et al., 2004;Parikh et al., 2005;Parikh et al., 2006a;Parikh et al., 2006b;Simon et al., 2004;Vaidya et al., 2008b;Washburn et al., 2008)
IP-10; CXCL10	A	Ge	U	P, R	Protein: ELISA, mRNA: qPCR	Y	(Avihingsanon et al., 2006;Hauser et al., 2005;Ho et al., 2009;Matz et al., 2006;Otto et al., 2005;Schwartz et al., 2007;Tatapudi et al., 2004;Vaidya et al., 2008b;Wolkow et al., 2008)
Kim-1	A, C	Ge	U	P	ELISA, dipstick	Y	(Han et al., 2002;Han et al., 2005;Ichimura et al., 2004;Liangos et al., 2007;Nijboer et al., 2009;Vaidya et al., 2006;Vaidya et al., 2009;Zhou et al., 2008;van Timmeren et al., 2007a;van Timmeren et al., 2007b), etc.
MMP-9	A	Ge	U	P	ELISA	Y	(Han et al., 2008)
NAG	A	Ge	U	P	Fluorimetric assay, spectrophotometry	Y	(Gibey et al., 1981;Halacova et al., 2008;Han et al., 2009;Kuźniar et al., 2006;Liangos et al., 2007;Moriguchi et al., 2009;Price, 1992;Skálová, 2005;Westhuyzen et al., 2003;Zhou et al., 2008), etc.
NGAL	A	Ge	U	P	ELISA, chemiluminescent microparticle immunoassay	Y	(Bennett et al., 2008;Bolignano et al., 2008;Devarajan, 2008;Hall et al., 2009;Mishra et al., 2003;Mishra et al., 2005;Mishra et al., 2006;Nickolas et al., 2008;Wagener et al., 2006;Zappitelli et al., 2007), etc.
NHE3	A	Ge	U	P	Semiquantitative immunoblot	N	(du Cheyron et al., 2003)
Total protein	A, C	Gl, Pr, Di	U	P	SDS-PAGE	N	(Brüning et al., 1999b)
VEGF	A	Ge	U	P	ELISA	Y	(Avihingsanon et al., 2006;Kim et al., 2005;Maroeska Te Loo et al., 2004;Paydas et al., 2007;Peng et al., 2007;Peng et al., 2008a;Peng et al., 2008b;Schips et al., 2007;Shi et al., 2009;Vaidya et al., 2008b), etc.

Name:
CXCR3: CXC chemokine receptor 3
FABP: (liver type) fatty acid binding protein
FOXP3: forkhead box P3
GST: glutathione S-transferase
HGF: hepatocyte growth factor
IL-18: interleukin 18
IP-10 / CXCL10: interferon-inducible protein 10
Kim-1: kidney injury molecule-1
MMP-9: matrix metalloproteinase-9
NAG: *N*-acetyl-beta-D-glucosaminidase
NGAL: neutrophil gelatinase-associated lipocalin
NHE3: Na+/H+ exchanger isoform 3
VEGF: vascular endothelial growth factor

Type of injury:
A: AKI, acute kidney injury following one of several incidences: contrast administration, cardiac surgery, renal transplant, chemotherapy, drug toxicity, toxin exposure, allograft rejection, etc.
C: CRF, general chronic renal failure, diabetes associated CRF, lupus nephritis, etc

Location of injury:
Di: distal damage
Gl: glomerular damage
Ge: general damage
Pr: proximal damage

Sample source:
U: urine
B: blood

Molecule type:
P: protein
R: mRNA

Technique:
ELISA: enzyme-linked immunosorbent assay
PENIA: particle-enhanced nephelometric immunoassay
SELDI-TOF: surface-enhanced laser desorption/ionization time-of-flight analysis
qPCR: quantitative PCR

Creatinine normalization:
Y: yes
N: no

References:
Etc.: more than ten articles available