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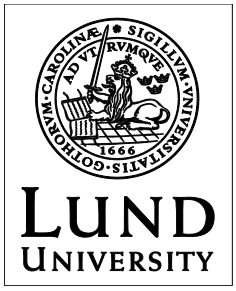

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Low molecular mass proteins as markers for renal function and dialysis efficiency

Abstract

Kidney disease is a growing problem in the whole world. It is important to find these patients in an early state of the disease because then they can be treated and dialysis treatment can be avoided. To measure the glomerular filtration rate (GFR) an invasive technique is used. In this thesis an equation to estimate GFR (eGFR) by drawing a blood sample and measure the concentration of cystatin C is presented. This equation is the first one working both for adults and children. To establish an equation working at all laboratories, primary and secondary reference preparations for cystatin C were developed. The secondary reference preparation will be used to establish an international calibrator, which can be used by the diagnostic companies to establish an uniform value of cystatin C.

Cystatin C has been proposed to be a marker of inflammation. The concentration of cystatin C was unaltered in our study of patients without any prior inflammation, who underwent elective surgery, while an increase in the concentration of CRP was seen. This result shows that cystatin C is not a marker of inflammation.

Three different types of dialysis treatments (haemodialysis, haemofiltration, haemodiafiltration) were tested for their capacity to remove low molecular mass proteins (LMMP) and thus their potential for treatment of patients with kidney failure. The LMMP have been proposed to be uraemic toxins and must therefore be removed from the circulation. The result from our study showed that cystatin C, β_2-microglobulin and β-trace protein can be used as markers for the efficiency of haemofiltration (HF) and haemodiafiltration (HDF). The elimination pattern of β-trace protein differs between HDF and HF and the free proteins might therefore be useful markers in the evaluation of different convective therapies.

Key words: GFR, eGFR, cystatin C, dialysis treatment, kidney function, low molecular mass proteins, inflammation, international calibrator

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Till mina älskade
Pär, Rasmus och Annica

Erfarenhet är inget annat än ett gott minne
Thomas Habbes

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   Scand J Clin Lab Invest 2011; 71: 145-149

Abbreviations

AKI	Acute Kidney Injury
CKD	Chronic Kidney Disease
CKD-EPI	Chronic Kidney Disease Epidermiology Collaboration
CRP	C-reactive protein
eGFR	estimated Glomerular Filtration Rate
GFR	Glomerular Filtration Rate
HD	haemodialysis
HDF	haemodiafiltration
HF	haemofiltration
IDSM	Isotope Dilution Mass Spectrometry
IFCC	International Federation of Clinical Chemistry and laboratory medicine
KDOQI	Kidney Disease Outcomes Quality Initiative
LMMP	Low Molecular Mass Protein
MDRD	Modification of Diet in Renal Disease
pI	isoelectric point
ROC	Receiver Operating Characteristic
SAA	serum amyloid A
Å	Ångström

Introduction

Kidney anatomy and filtration

The function of the kidney is to filtrate the blood and remove toxic products and small substances and excrete them into urine. The kidneys are also involved in the production of hormones, regulation of blood pressure, stimulation of production of red blood cells and activation of vitamin D. Every 24-hours period about 1500 litres of blood is passing the kidneys. When it passes the glomerulus the primary urine (180 L per 24 h) is produced. More than 99 % of the primary urine is reabsorbed in the tubulus (fig. 1).

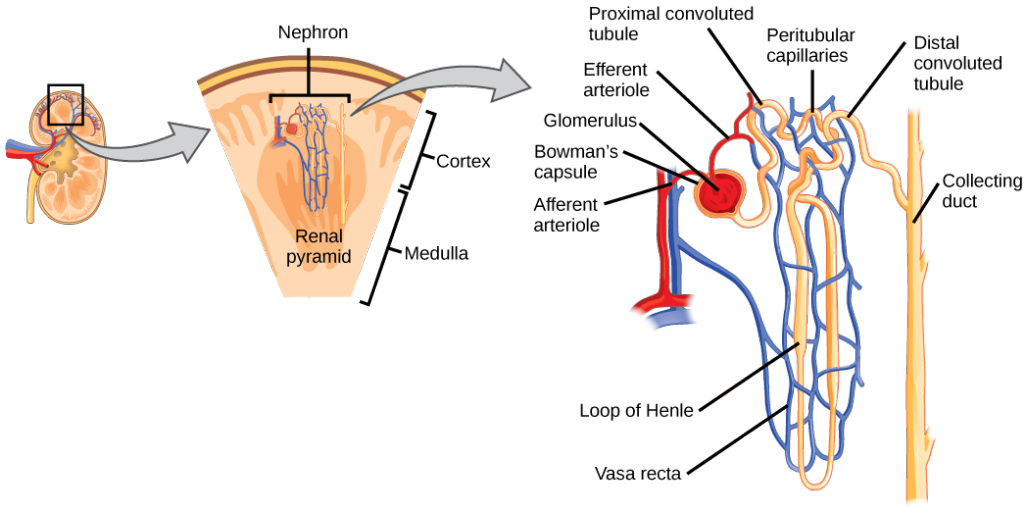


Figure 1 A kidney and one nephron.

The kidneys contain about 2 million filtering units called nephrons. Each nephron is comprised of a glomerulus with the Bowman's capsule (fig. 2) and the tubulus.

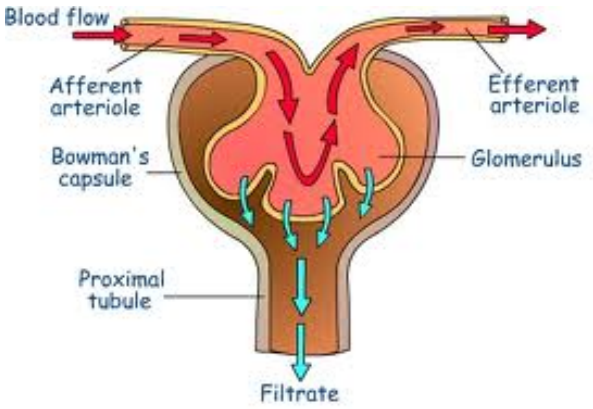


Figure 2 Bowman´s capsule and glomerulus.

The glomerular barrier contains three layers (fig. 3):

(a) the capillary endothelium that is in direct contact with the blood and contains fenestrates 50-100nm wide. It is covered with glycocalyx that is negatively charged due to sialoproteins and proteoglycans,
(b) the glomerular basement membrane (GBM) is also negatively charged and consists of many layers of inter alia collagen type IV, laminin, heparan sulphate proteoglycans. They form a cross-linked network with pores of no defined size.
(c) the podocyte foot processes (epithelial cells) which form a zipper like structure. Inside this structure there are filtration slits, about 25 to 60 nm wide.

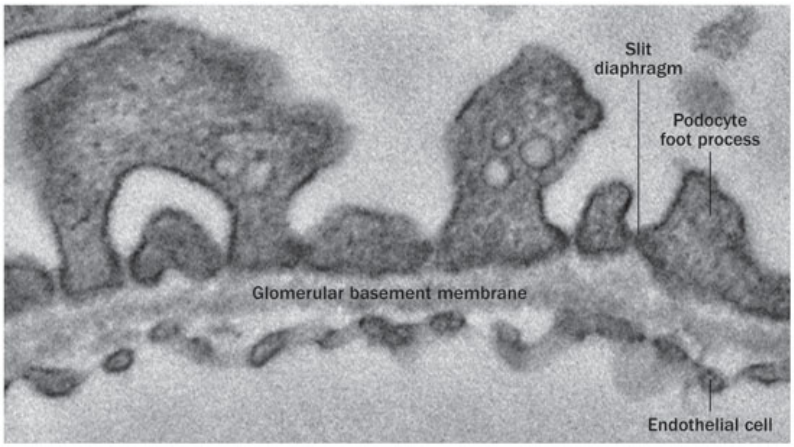


Figure 3 The glomerular barrier.

Between the glomerular basement membrane and the filtration slits there are slit diaphragms that cover the slits and consist of cell-surface proteins e.g. nephrin, podocin and CD2-associated protein [1-4]. How the size and charge selectivity of the glomerular barrier is generated is still not fully understood [5]. There are two models of how the size selectivity is produced.

I. In the slit diaphragm there are rectangular pores which are 40 x 140Å in cross-section and 70Å in length, which retain large proteins > 40kDa and allow smaller proteins, radius < 30Å, and solutes to pass [2, 6].
II. There are many small pores (46Å) and a few larger pores (80-87Å) [7].

The charge selectivity has been tested with many different substances. So far it seems that the fixed negative charges of the capillary endothelium and the glycocalyx are responsible for the charge selectivity.

The glomerular capillary walls almost exclude plasma proteins of the size of albumin or larger from the filtrate. The glomerular filtration is depending not only on the size but also on the charge, shape and deformability of the molecule**.** Positively charged proteins are more easily filtered than negatively charged proteins in the glomerular basement membrane because the membrane is negatively charged. The difference in pressure between the glomerular capillary and the luminal side of the glomerulus, the amount of nephrons and the structure of the glomerular basement membrane also affect the filtration. The primary urine continues to the tubulus where water and metabolites are reabsorbed to the small capillaries surrounding the tubulus. The low molecular mass proteins (LMMP) of the primary urine are almost completely reabsorbed in the proximal tubular cells. They are then catabolized to amino acids that are secreted to the capillaries around the tubulus or excreted in the urine [8, 9]. The concentrated urine (1.5 to 2 L in a 24-hours period) is transported via the ureters to the bladder.
Glomerular filtration rate (GFR) is defined as the total volume of glomerular filtrate from the capillaries into the Bowman's capsules per minute (mL/min). It reflects the amounts of functional nephrons in the kidneys. It is important to know the GFR to be able to detect early kidney disease, handle renal transplant patients and to correctly dose drugs or potentially nephrotoxic radiographic contrast media cleared by the kidneys to prevent further destruction of the kidneys.

Kidney disease

Acute kidney injury (AKI)

AKI is a serious condition with a rapid decrease in kidney function. In Sweden 5-20% of the patients in intensive care units and 1-2% of the patients in medicine- and surgery departments are affected. Depending on the cause of the injury, AKI can be divided in three groups.

- Prerenal, caused by some hinder in blood flow e.g. shock, heart failure, low intake of liquid, kidney thrombosis, drugs.
- Renal, caused by damage in glomeruli or tubuli. The most common is acute tubular necrosis. Contrast-induced AKI also belongs to this group. Other examples are acute interstitial nephritis and acute pyelonephritis in a single kidney.
- Postrenal, obstructions caused by e.g. kidney stone, light chains of immunoglobulins, myoglobin, hemoglobin, tumours, and prostatic hypertrophy.

The course is often very quick, within some days. The injury is often reversible in the beginning but some patients develop irreversible kidney failure and need dialysis. Therefore it is important to find these patients in an early stage and start to treat them, also because there is a certain risk of mortality. AKI also induces injury to extrarenal organs e.g. heart, lungs, liver. The definition of AKI is done according to RIFLE (risk, injury, failure, loss, end-stage renal disease) criteria. The criteria are a combination of decrease in urine production and increase in concentration of creatinine and urea. The first sign is often a decrease in urine production, which is most easy to follow. The concentration of cystatin C is increased already after 1-2 days and before the increase in creatinine, which has been used as a marker together with urea for long. Besides laboratory tests supplementary examinations are done e.g. blood pressure, heart-kidney status, palpation of prostate and anamnesis are used to diagnose AKI.

Chronic kidney disease (CKD)

CKD is a growing problem in the whole world. Between 400 and 600 million adult people are treated with dialysis or have been kidney transplanted. In Sweden there are around 8000 persons suffering from CKD who are under dialysis and every year there are 600 new patients. There are a lot of costs for both the society and the patient associated with CKD.

According to GFR, CKD is divided into 5 stages:

Stage	Description	GFR mL/min per 1,73m^2
1	Kidney damage with normal or increased GFR	≥ 90
2	Kidney damage with mildly decreased GFR	60-89
3	Moderately decreased GFR	30-59
4	Severely decreased GFR	15-29
5	Kidney failure	< 15 (or dialysis)

From: KDOQI [10]

Stage 1 to 3 is often without any symptoms. These patients are found when they are examined for increased blood pressure, diabetes or suspicion of cardio-vascular disease. In a study in Norway at a primary care unit CKD stage 1-3 was found in 5% of patients suffering from diabetes or hypertension. Moreover 10% of the healthy patients of age >55 years suffer from CKD stage 1-3 [11]. A decrease in GFR is associated with higher risk of mortality in cardiovascular disease or kidney failure [12]. Therefore it is of great importance to find the patient in an early stage to start treatment with medicine or change in lifestyle to slow down the progression of CKD and in the end dialysis or kidney transplantation. Risk factors for chronic kidney disease are e.g. diabetes, increased blood pressure, age, heredity and gender. To diagnose mild kidney damage, GFR or urine albumin/ creatinine ratio and proteinuria is used.

Glomerular filtration

Measuring GFR

There is only one “gold standard” way to measure GFR and that is to intravenously inject an exogenous substance, which is freely filtered via the glomerular system and not reabsorbed or secreted by the tubuli. Examples of such substances are inulin, ^{51}Cr-EDTA or radiographic contrast media like ^{125}I-iothalamate or iohexol. By measuring the disappearance rate in plasma of these substances and/or their appearance in the urine, the GFR can be calculated. The disadvantages of using such techniques are that you have to inject a substance, which can be a risk for the patient and that the procedures are time-consuming and expensive. Collecting urine in a correct way is not easy. You must be sure that the patient has collected all the urine produced during 24 hours. If the patient has problem to empty the bladder it must be controlled with ultrasound.

There is a variation in GFR between day and night. In a small study with both healthy persons and patients with kidney disease the authors found 21% higher GFR (inulin clearance) during daytime than in night [13].

A special case is if the patient has a single kidney. It can be due to three different scenarios:

The patient is born with a single kidney. This kidney has a bigger size and can filter up to 70-90% of normal GFR.

In a donor. The remaining kidney can compensate and open up resting nephrons. The kidney function after one year is often 70% or more. After several years this compensation may be exhausted, resulting in a decrease in GFR.

The transplanted patients new kidney is foreign for the body, which try to reject it. GFR is often not more that 50-60% of normal.

GFR markers

Creatinine

Creatinine is produced from creatine in muscle tissues. The molecular mass is 113 Da and Stokes radius is 3Å (fig 4). Plasma/serum creatinine has been used as a marker of GFR after it was discovered to be freely filtered and not reabsorbed by the tubulus in 1926 [14]. Moreover creatinine is secreted in the tubulus. Creatinine in plasma is not only dependent on kidney function but also on age, race and gender due to the muscle mass. However, creatinine has several drawbacks as a GFR-marker, since its production varies with the muscle mass of the patient and since tubular cells with a higher secretion rate, secrete creatinine when GFR is low [15-17]. When GFR < 10 mL/min/1.73 m^2 the tubular secretion of creatinine exceeds the glomerular filtration. At low levels of GFR more than 50% of the creatinine secretion occurs via tubular secretion compared to 10-20% in persons with normal GFR.

Moreover some of the creatinine is also eliminated via the bowel. When GFR is decreased this elimination contributes relatively much to the concentration of creatinine in plasma.

Meat, especially boiled, contains creatinine and after a big meal with meat the concentration can increase with 100% or more [18, 19]. Depending upon the amount of intake of meat the increase in GFR was seen after 30 minutes up to 2.5 hours after the intake [20, 21]. In persons with normal GFR creatinine concentration is normalized after 8 hours but in patients with decreased kidney function the increased level can remain longer [22].

If GFR measured with inulin clearance was performed in the same patients an increase up to 50% was seen. An explanation to this increase is the renal functional reserve. The normal kidney has capacity to work more. When the kidney is under stress after e.g. a big meal there is an increased blood flow caused by vasodilation. In more chronic state e.g. kidney donor or children with a single kidney, the kidney grows and the nephrons are getting bigger. In patients with kidney disease this renal functional reserve is smaller because before there are a decrease in GFR some of the renal reserve is used [23].

When creatine is administrated orally it forms creatinine and the creatinine concentration in plasma is increased [24, 25], which gives a decrease in estimated GFR (eGFR). A vegetarian or a person who is under low protein diet has lower concentration of creatinine and consequently increased eGFR. In both this cases GFR

measured with e.g. iohexol shows a normal value.

Plasma creatinine gives information about the function of the nephrons but as mentioned before, creatinine is also dependent of the muscle mass. If plasma creatinine is used as a single test one must therefore take into consideration the muscle mass of the patient. A person with big muscles has higher concentration of creatinine than a thin person.

Creatinine concentration can be measured in both plasma and in urine. The daily secretion is constant in urine within an individual with stabile kidney function. This is important when the concentration of proteins excreted via urine is measured. By making a ratio between the concentration of a protein e.g. albumin, IgG, protein HC, kappa- /lambda light chains and creatinine in urine a reference interval can be made which is independent on the diuresis. Urine creatinine is also measured in drug tests to check if the urine has been manipulated or if the patient has been drinking a lot of water in order to dilute the concentration of the drug.

The kidney function can be decreased up to 50% before plasma creatinine becomes abnormally high. This is called the creatinine-blind area and is when GFR is between 40 and 90 mL/min/1.73m^2 (fig. 5). It is an important area because it represents a mild to moderate decrease in GFR, which is important to find. The reason to this insensitivity is that an increase in the plasma creatinine concentration leads to an increase in the secretion of creatinine in tubuli. Therefore it has been proposed that creatinine is not a good marker of GFR.

Cystatin C

Cystatin C was called gamma-trace, post-gamma-globulin or neuroendocrine basic polypeptide when it was found in cerebrospinal fluid and urine already in 1961 [26-28]. It contains a single, 120 amino acid long, non-glycosylated polypeptide chain with a molecular mass of 13343 Da [29] and Stokes radius of 30-40Å [30] (fig. 4). The isoelectric point (pI) is 9.3. It is a cysteine protease inhibitor and belongs to the family 2 cystatins and is produced in all nucleated cells in the body with constant rate. In rats it has been demonstrated that cystatin C is removed from the circulation by the kidneys, freely filtered through the glomerular membrane and metabolized after reabsorption in the tubular cells [31].

A special mutation in the gene coding for cystatin C causes the Icelandic type of hereditary cystatin C amyloid angiopathy (HCCAA). The patients usually get their first hemorrage at a very young age (30 years) [32].

Already in 1979 Löfberg and Grubb found that the concentration of cystatin C was increased in patients with renal disease [33]. The concentration of cystatin C was measured with an enzyme-amplified single-radial-immunodiffusion (SRID) technique. This method was not suitable as a routine method because it took several days to get the result. In 1985 Grubb et al suggested that cystatin C could be used as a marker of GFR but no quick method existed [34]. When a particle-enhanced immunoturbidimetric method was developed 1994 a more rapid analysis was available in clinical chemistry laboratories [35].

The main indication of measuring cystatin C is to estimate GFR, ”kidney function”. In a recent study of the risk for patients with chronic kidney disease to develop end-stage renal disease or early death, it was shown by adding the plasma concentration of cystatin C to the plasma concentration of creatinine and urine albumin/creatinine ratio that these risk patients was found earlier [10, 36]. It has also been shown that cystatin C is an early marker of developing CKD stage 3 [37].

Studies of elderly populations without CKD have shown that if the cystatin C concentration increases there is a higher risk of death in cardiovascular causes [38], myocardial infarction and stroke [39, 40]. The risk was more pronounced if the patient was without any cardiovascular, renal insuffiency or coronary heart disease before the increase in cystatin C concentration.

An explanation to why cystatin C is a better marker for succesful aging than creatinine can be its capacity to early detect an abnormal filtration quality [41-43]. Moreover elderly persons have reduced muscle mass, which decrease the concentration of creatinine independent of the kidney function. The concentration of cystatin C is not affected by the mucle mass and the normal increase in concentration in elderly persons will therefore reflect their decrease in the kidney function.

Many studies have been performed but there are no agreements when the normal decrease in the kidney function starts. Some claim that the decrease starts already in a 20 years old person but with very slow rate (4 mL/min/1.73m^2 per 10 years) but around an age of 50 years all agree that there is a decrease. Most studies claimed that the decrease in GFR is around 10 mL/min/1.73m^2 per 10 years [44, 45].

When the risk of death in cardiovascular disease was calculated in a group of 50 years old men who was followed up after 20 years it was found that the risk increases (20-80%) for every decrease of eGFR with 10 mL/min [38].

In hospitals there are patients with contrast-induced acute kidney injury (AKI). By measuring cystatin C instead of creatinine one can find the patients who have a risk of developing AKI earlier and the patients who are not in that riskgroup can be sent home [46]. In adult patients who undergo cardiac surgery the plasma concentrations of cystatin C and NGAL (neutrophil gelatinase-associated lipocalin) can be used as early markers of AKI [47].

An explanation to an earlier detected increase of cystatin C than creatinine concentration can be the shorter half-life of cystatin C [48]. The half-life of creatinine is 3 times longer because it is distributed in the whole body water while cystatin C only is distributed in the extracellular volume [31, 49].

In kidney transplanted patients it was shown that cystatin C decreases before creatinine does, after the blood circulation started but before urine production works [50-52]. This is due to that there is a degradation of cystatin C in the tubulus, but no degradation or excretion via urine of creatinine occurs.

Cystatin C increases in the third trimester during normal pregnancy [41] but more in pre-eclampsia [53]. Moreover GFR and the renal plasma flow increases (about 40%) during the two first trimesters [43, 54, 55]. An explanation to the increase in cystatin C concentration can be that during pregnancy the glomerular pore size is getting smaller and therefore cystatin C is not filtered at the same rate. GFR measured using iohexol, which is a low molecular mass substance, is not affected. In pre-eclampsia the increase can be explained by endotheliosis, which also result in smaller pores. Pre-eclampsia is a serious condition for both the mother and the child. A decision must be taken when, or if, a caesarean section must be performed. Since the concentration of cystatin C, β_2-microglobulin, β-trace protein increases earlier than the concentrations of creatinine or urate do when GFR decreases, all three proteins can be used as markers to detect pre-eclampsia [53, 56, 57] but in practice only the concentration of cystatin C is measured.

Diabetic patients have an increased risk of developing kidney and cardiovascular disease. Therefore it is important to find an early decrease in GFR. Diabetic patients have smaller pore size in glomeruli like in pregnancy. This is reflected in the filtration quality. The plasma concentration of small substances is not affected while that of low molecular mass proteins is. It has been published that cystatin C (molecular mass 13343 Da) is better than creatinine (113 Da) or clearance of iothalamate (613 Da) to find these patients with an early decrease in GFR [58-60].

So far there are two situations when cystatin C is not reflecting the kidney function correctly. One is in patients with hyper- or hypo-thyroidism. In hyperthyroidism cystatin C is increased and in hypothyroidism it is decreased. This is probably because dysfunction in the thyroid affects the production rate of cystatin C [61-63]. If the patient with hyperthyroidism is treated, cystatin C will decrease to a normal value. When a patient with primary hypothyroidism is treated with T4, cystatin C will increase and creatinine will decrease.

The other situation when cystatin C is not reflecting the kidney function correctly is when the patient is treated with high doses of glucocorticoids. Cystatin C concentration will increase when the doses are high [64, 65]. This is because glucocorticoids cause an increase in the synthesis of cystatin C without any decrease in kidney function. Intake of glucocorticoids by oral or inhalation routes and

transdermal use of it, usually do not interfere with cystatin C as a marker of GFR because the lower levels of glucocorticoids absorbed [66-69].

Many attempts have been done to measure cystatin C production rate ($cystC_{pr}$) and the non-renal clearance ($cystC_{nr}$). The results differ because the studied groups of patients have different disorders or are healthy. Cystatin C production rate has been suggested to be between 0.099 and 0.147 mg/min/1.73m^2. The non-renal clearance has been suggested to vary between 14.1 and 29.3 mL/min/1.73 m^2 [70, 71].

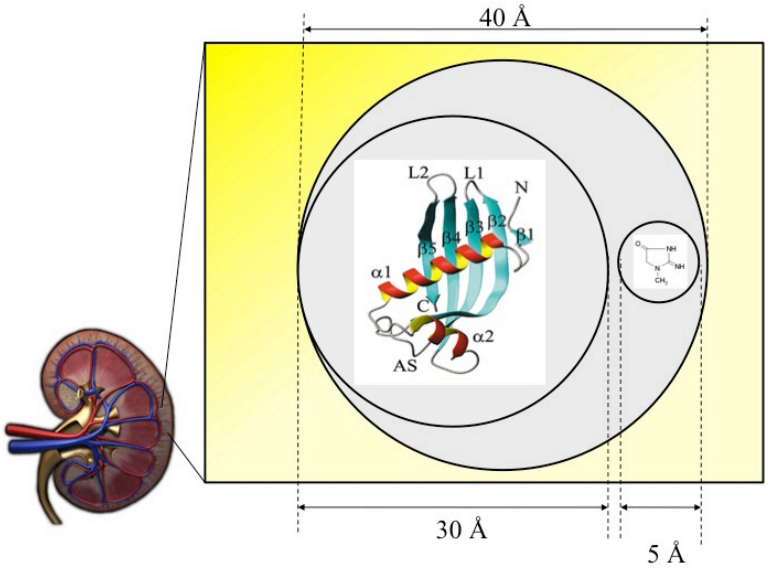


Figure 4 A comparison between the size of a pore in the kidney and the size of cystatin C and creatinine.

Plasma creatinine can also be used to estimate GFR as has been discussed before. A disadvantage to use creatinine as a marker of GFR is the so-called creatinine-blind area. It is called creatinine-blind area because when creatinine increases the tubular secretion also increase, which means that plasma creatinine only increases little and only a small decrease in eGFR is seen. The creatinine-blind area is when GFR is in the area between 40 and 90 ml/min/1.73m^2, which represent a mild to moderate decrease in GFR (fig. 5). This early decrease is important to find.

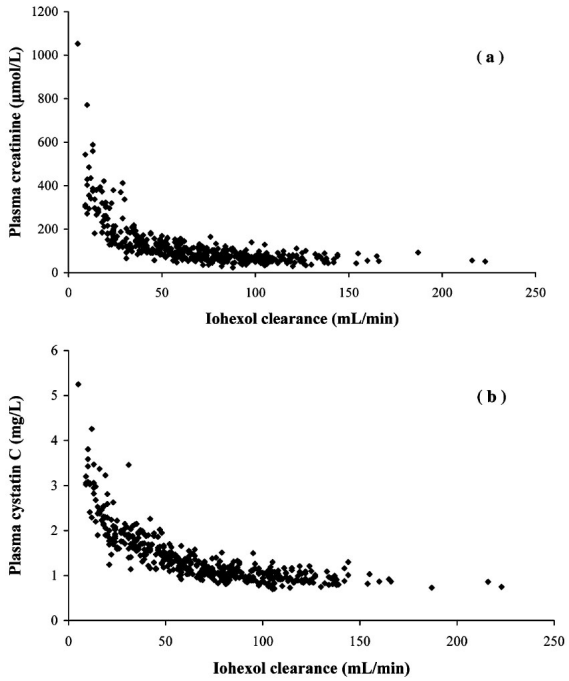


Figure 5 Creatinine-blind area. The concentration of cystatin C and creatinine as a function of GFR.

As mentioned before, creatinine is dependent upon the muscle mass. To estimate the GFR from plasma creatinine, it is therefore required to take into consideration at least the gender, age and race of the patient, which may give an estimation of the mean muscle mass of a person of a specific age, gender and race [72]. Several creatinine-based GFR-prediction equations, using, in addition to plasma creatinine, anthropometric and/or demographic data, have been suggested to estimate GFR more reliably than the creatinine level alone, since such equations partly compensate for the varying muscle mass of different individuals. Some examples of commonly used

creatinine-based GFR-prediction equations are the original MDRD equation [73], the abbreviated MDRD equation [74], the $MDRD_{IDMS}$ equation [75], the Jeliffe equation [76], the Gates equation [77], the Hull equation [78], the CKD-EPI equation [79] and a new Swedish-based equation, the LM (Lund-Malmö)-equation [80] and the revised LM-equation [81]. A drawback for these equations, with the LM-equation as the only exception [82, 83], is that they do not work for children below 18 years and that therefore specific creatinine-based GFR-prediction equations for children have to be used, e.g. the Schwartz [84] and Counahan-Barratt [85] equations.

Plasma cystatin C was suggested in 1979 [33] as an alternative to plasma creatinine as a marker of GFR. The plasma concentration of cystatin C is less influenced by the gender, age, muscle mass and race of the patient and is not secreted by the tubuli. Two equations for estimating GFR in adults were presented 2003 and 2004 [86, 87]. In 2005 the first equation working for both adults and children was published [*paper 1*]. Thereafter the concentration of cystain C has been measured in many other groups of patients and new GFR-estimating equations have been produced [88-92].

The problem so far is that there has not been any international calibrator for cystatin C. But since 2010 there is a calibrator available, ERM-DA471/ IFCC [93]. For creatinine there has been a reference method based on isotope dilution mass spectrometry (IDMS) since 2002. Most of the diagnostic companies have calibrators traceable to this calibrated creatinine.

To create all equations to estimate GFR different patient groups have been used. For example, the often-used MDRD equation is based upon adult CKD patients and the LM- equation is based upon both children and adults. A combination of a creatinine-based and a cystatin C-based equation was suggested to give the best GFR-prediction [88, 89, 94-96].

It must be observed that most of the GFR-prediction equations estimate relative GFR ($mL \cdot min^{-1} \cdot (1.73m^2)^{-1}$), which is suitable when following the kidney function in a patient. It is normalized to a certain body surface area and the reference value is not dependent on gender. For correctly dosing of drugs or contrast medium excreted via kidneys, the absolute GFR (mL/min) must be used. To calculate absolute GFR from relative GFR the DuBois and DuBois formula is often used to estimate body surface area [30, *www.egfr.se/eGFRen.htm*].

Other markers in evaluation of kidney function

Urea

The molecular mass of urea is 60 Da and its Stokes radius 3Å. Urea is synthesised in the liver and is eliminated via the kidneys. Most of the nitrogen from degradation of amino acids is eliminated via urea. It is freely filtered and partially reabsorbed in tubuli. This reabsorption is dependent on the resorption of water. A small part of urea is eliminated via perspiration or via the bowel.

The clearance of urea is used to control the efficiency of dialysis treatment. It is called Kt/V where K is the dialyser clearance of urea, t is the dialysis time and V is the distribution of urea (approximately the same as the body volume of the patient).

β_2-microglobulin

The molecular mass of β_2-microglobulin is 11.8 kDa, its Stokes radius 16Å and its pI 5.4-5.7. It consists of one 99 amino acids long polypeptide chain, which is the light chain of type 1 major histocompatibility complex. β_2-microglobulin is produced in all nucleated cells (specially those belonging to the lymphatic system) and is freely filtered through glomeruli, reabsorbed and degraded in proximal tubuli [97]. It therefore fulfils the criterion for a GFR marker but it has one disadvantage. If serum β_2-microglobulin is increased it can be due to a disease increasing its production and not reflect the kidney function. Examples of diseases with increased concentration of β_2-microglobulin but with normal GFR is patients suffering of AIDS, SLE (systemic lupus erythematosus), myelomatosis, tumours or vasculitis. Patients who undergo haemodialysis have increased concentration of β_2-microglobulin in plasma that might be accumulated in synovial fluid of joints and bone and form dialysis-related amyloidosis. The patients can develop carpal tunnel syndrome or large joint spondyloarthropathy [98, 99].

β-trace protein

β-trace protein is also called prostaglandin D-syntetase which describes its function. The molecular mass is 23-29 kDa depending of the degree of glycosylation and its pI is 5.8- 6.7. β-trace is mainly produced in the brain but also in male genital organs and heart. It is freely filtered, reabsorbed and degraded via the kidneys. Therefore, it has been proposed to be a marker of GFR. Compared to cystatin C, as a marker of GFR in the creatinine blind-area, β-trace is as good as cystatin C [100], but not better to detect a decrease in GFR [101].

In a study of children with impaired GFR, β-trace was shown to be an alternative to cystatin C [102]. Studies have been done in renal transplanted patients [103] in patients with acute heart failure [104] and in patients who are in haemodialysis [105]. In the first two studies the authors compared serum β-trace concentration with cystatin C concentration and came in the first study to the conclusion that β-trace can be used to estimate GFR especially in patients receiving steroids and in the second that both of them can be used as predictors of risk of death or heart failure hospitalization. In a later study, where cystatin C and β-trace were compared for their potential to estimate GFR, determined with inulin clearance, it was shown that the concentration of β-trace is more affected than the concentration of cystatin C in patients treated with corticosteroids [106]. In the study with haemodialysis patients, the authors conclude that the concentration of β-trace is associated to residual diuresis. The same finding was shown in a recent paper [107] where the concentration of serum β-trace in patients who undergo haemodialysis was measured. The authors found that if the patient has no residual urine the concentration of serum β-trace is higher than if the patient has some residual GFR. In a 3.3 years follow-up study in the same group of patients it was shown that higher β-trace concentration is associated with a higher risk of death.

In daily medicine the concentration of β-trace is used to control if fluid coming from nose, ear or tears originates from CNS.

Albumin

The function of albumin is to transport drugs and fatty acids and to regulate the colloid osmotic pressure. Its molecular mass is 67 kDa, its Stoke radius is 36Å and its pI 4.6. Albumin is the dominating protein in plasma according to concentration. It is produced in the liver and the production rate is regulated by the colloid osmotic pressure.

Microalbuminuria, measured as albumin-creatinine ratio in urine, has been shown to be an early sign of kidney disease and developing CKD stage 3 both in patients with and without diabetes [37, 108]. In diabetic patients microalbuminuria is due to glomerular damage and in nondiabetic patients it can also be due to endothelial and vascular dysfunction [109, 110].

Inflammatory diseases

When an inflammation occurs mainly granulocytes, macrophages and monocytes start to produce cytokines signalling to the liver to produce acute-phase proteins. The organism wants to remove pathogens, damaged cells or irritating things from the body. In the acute-phase response the protein synthesis is affected. Albumin, transferrin and transthyretin are decreased in concentration while the so-called acute-phase proteins are increased. Serum amyloid A (SAA), α_1-antitrypsin, orosomucoid, haptoglobin, α_1-antichymotrypsin, fibrinogen, complement factors C3 and C4, factor VIII (coagulation factor) and CRP (C-reactive protein) belong to the acute-phase proteins. In the daily routine work at the laboratory the concentrations of α_1-antitrypsin, orosomucoid, haptoglobin and CRP are used to diagnose the acute-phase response. CRP and SAA increase in concentration already after some hours but it takes about 1-2 days before the concentration of α_1-antitrypsin, orosomucoid and haptoglobin increase.

The inflammation can either be acute or chronic. Pain, redness, immobility, swelling and heat are the classical signs of acute inflammation on the body surface. If the inflammation is inside the body not all of these five symptoms are present. The inflammation can be in the heart (myocarditis), kidneys (nephritis), large intestine (colitis) and in blood vessels (vasculitis). Some chronic diseases e.g. rheumatoid arthritis, vasculitis, diabetes, obesity or kidney insufficiency often show residual inflammation, which can be observed as an increase in concentration of the acute-phase proteins.

Inflammatory markers

CRP

CRP (C-reactive protein) was the first (1930) described acute-phase protein [111]. It is called CRP because it binds to the C-polysaccharide in the capsule of some pneumococci. The molecular mass is 120 kDa and it has five subunits. Stokes radius is 43Å and the pI is 5-6. CRP is synthesised in the liver. It is activating the complement system when it binds to cells, which has been damaged, or to microorganisms.

The concentration of CRP can increase several hundred times during bacterial infections (already after 4-6 hours with a maximum after 48-72 hours) but not so much in chronic or viral inflammations.

In clinical medicine CRP is used to differentiate between bacterial and virus infection. The level of CRP also gives information about the degree of inflammation.

In Sweden CRP sometimes is called “snabb-sänka” (rapid erythrocyte sedimentation rate, ESR). This name is wrong because CRP is a protein and ESR is the sedimentation rate of the erythrocytes in a thin tube. This “snabb-sänka” is often used in primary care units because this test is more rapid than ESR.

During the last 10 years it has been a growing interest to measure small increases of CRP (1-3 mg/L) because it has been shown that it means a higher risk of developing diabetes, cardiovascular or hypertension diseases [112, 113]. To measure this small increase in CRP concentration a new method called high-sensitive CRP has been developed.

Serum amyloid A

SAA (serum amyloid A) is an acute-phase protein and a member of the apolipoprotein family. Its concentration increases very quickly after inflammatory stimuli. When an inflammation is present the concentration can increase up to 6000 times. The molecular mass is 11-12 kDa but serum amyloid A is bound to high-density lipoproteins and doesn´t exist in free form.

SAA can form amyloid fibrils in the kidneys in e.g. rheumatoid arthritis patients. These patients can have a slight increase in CRP but a high increase in SAA. Therefore it is recommended to follow the degree of inflammation in rheumatoid arthritis with SAA.

Haptoglobin

Haptoglobin is mainly produced in the liver. The molecule consists of four different (2α and 2β) polypeptide chains. In plasma there are three different forms, which differ in the type of α-chain. Hapto 1-1 has a molecular mass of 98 kDA but Hp 2-2 and Hp 2-1 have higher mass depending on that they form polymers due to their type of α-chain. The pI is 4.0-4.2. Haptoglobin forms complexes with hemoglobin in plasma, which are eliminated via the reticular-endothelial system. The concentration of haptoglobin in plasma is increased in inflammatory diseases and decreased when there is an increase in the turnover of hemoglobin.

Orosomucoid

Orosomucoid, also called α_1-acid-glycoprotein, belongs to the lipocalin family and is an acute-phase protein. It is produced in the liver but also in lymphocytes. The molecular weight is 45 kDa and the pI 2.7. An increased concentration can be seen in inflammation, myocardial infection, trauma and surgery but also in treatment with corticosteroids.

Summary of the metabolites and proteins measured in the paper 1 and 3

	Molecular mass (Da)	pI	Stokes radius (Å)
cystatin C	13 343	9.3	30-40
creatinine	113	-	3
urea	60	-	3
β_2-microglobulin	11 800	5.4-5.7	16
β-trace	23-29 000	5.8-6.7	
α_2-macroglobulin	720 000	5.3-6.1	88
albumin	67 000	4.6	36

Dialysis treatment

In order to treat patients with kidney failure different types of dialysis modalities have been tried (fig.6). So far there is none that is as good as the kidneys themselves [114]. Two different types of filters are used, low-flux and high-flux. They differ in pore size, low-flux has small pores and high-flux has large pores. Nowadays mostly high-flux membranes are used because they also allow filtration of larger molecules e.g. LMMP.

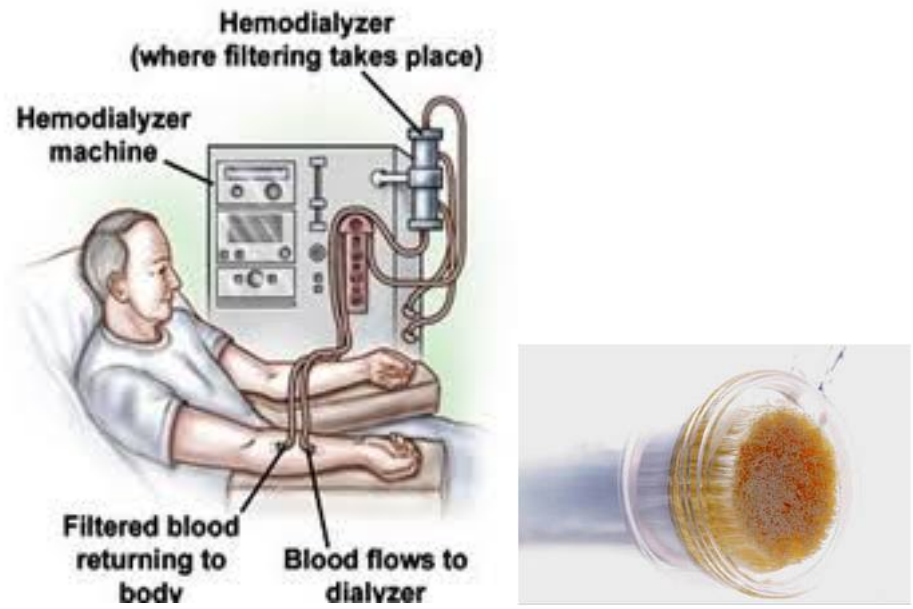


Figure 6 Haemodialysis machine and the bottom of a dialysis filter.

The dialysis membranes are made in different materials and differ in pore size, pore density, membrane thickness, surface area and ability to adsorb proteins that are important factors for the degree of elimination of solutes and proteins. The most important factor is the pore size. A small increase in size gives a big decrease in flow resistance. The pore density, which is the number of pores per surface unit, is important for the water flux and solute clearance. Both the cellulose and the synthetic membranes used today have a thickness of only about 8 μm compared to 20-40 μm previously. The surface area of a dialyzer is normally exceeding 2 m^2. Protein

adsorption takes place within the pores rather than on the surface of a membrane. The hydrophobicity of the membrane influences protein adsorption, high hydrophobicity gives higher adsorption [115]. Important factors for transport of small solutes across membranes are charge and degree of protein binding and for transport of proteins across membranes molecular mass, hydrodynamic volume and isoelectric point (charge) [116].

Haemodialysis (HD) produces only efficient clearance of water and small solutes, e.g. urea and creatinine. It is using diffusive transport across a membrane with the blood on one side and the dialysis fluid on the other side of the membrane. The dialysis fluid is pumped along the filter in the opposite direction to that of the blood and can only eliminates molecules with a mass lower than about 5 kDa.

In convective haemofiltration (HF) blood under pressure passes down on one side of a highly permeable membrane. This arrangement allows both small solutes and proteins with a molecular mass up to 20-25 kDa to pass across the membrane. The blood is diluted with a replacement solution before it enters the filter. This is because otherwise the concentration of erythrocytes and proteins will get too high.

Haemodiafiltration (HDF) combines diffusion and convection and can remove molecules with a molecular mass up to 20-25 kDa [151]. The replacement solution is added after the blood has passed the filter. It has been shown that the LMMP is removed much more efficiently if the replacement solution is added after the filter than before the filter as in HF [117-120].

It is important to remove small solutes and LMMP because they have been proposed to contain putative uremic toxins and because many of the LMMP are signalling molecules [121, 122]. β_2-microglobulin has been proposed to be the major amyloidogenic component in long-term HD and is the most popular marker to measure the capacity of a dialysis procedure to remove so called middle molecules [123]. The efficiency of dialysis treatment is usually tested by measure of urea (mw 60 Da) clearance (Kt/V).

Present work

Paper 1

Simple cystatin C-based prediction equationss for glomerular filtration rate compared with the modification of diet in renal disease prediction equation for adults and the Schwartz and the Counahan-Barratt prediction equations for children.

Clinical chemistry 2005; 51 (8):1420-31

GFR is commonly estimated by analysis of plasma/serum creatinine (molecular mass 113 Da). The disadvantage to use serum creatinine is that, in addition to the overestimation of GFR due to tubular secretion, serum creatinine is also dependent on muscle mass, age, diet, race and gender. Due to these variations many different creatinine-based GFR-prediction equations have been constructed. The Modification of Diet in Renal Disease (MDRD) equation for adults and the Schwartz and Counahan-Barratt equations for children are frequently used to estimate the relative GFR expressed in $mL \cdot min^{-1} \cdot (1.73m2)^{-1}$.

Aim:

The aim of this study was to generate a cystatin C-based GFR-prediction equation and investigate if it has as good as, or better, diagnostic performances than the traditional creatinine-based GFR-prediction equations.

Cystatin C (a non-glycosylated protein with a molecular mass of 13 343 Da) is produced in all nucleated cells in the body at a constant rate [94, 124]. It has been demonstrated in rats that cystatin C is removed from the circulation by the kidneys, freely filtered through the glomerular membrane and metabolized after reabsorption in

the tubular cells [31]. Plasma cystatin C alone was already in 1979 found to be a marker for GFR. In patients with terminal renal insufficiency the cystatin C concentration can increase more than tenfold [33]. It has also been demonstrated to be a better, or at least as good, GFR marker as serum creatinine alone [34, 35, 125-128].

In the GFR interval 60-79 $mL \cdot min^{-1} \cdot (1.73m^2)^{-1}$ cystatin C has been demonstrated to be much better than creatinine to find a reduction in GFR [129]. GFR can decrease to about 50% and creatinine still is within the reference range. Cystatin C is less dependent on muscle mass, diet, tubular secretion and gender than creatinine. One disadvantage with cystatin C as a GFR-marker is that moderate and high doses of glucocorticoids will increase the synthesis of cystatin C and therefore produce increased plasma levels of cystatin C and thus falsely indicate a reduction in GFR [64, 65, 106, 130, 131].

Materials:

536 patients (262 females and 274 males, age 0.3-96 years) where GFR have been determined by measure plasma iohexol clearance.

Methods:

An exact volume of the nonradioactive radiographic contrast medium iohexol was injected. Before the injection a blood sample was drawn. After an exact time point (usually 4 h) another blood sample was drawn. The iohexol concentration was measured by a HPLC method [132] and GFR was calculated.

Plasma creatinine was measured before the injection of iohexol in plasma to identify patients with very low GFR requiring extension of the time period before collection of the second blood sample for measurement of iohexol. The plasma concentration of creatinine was measured by an enzymatic method on a Hitachi Modular P platform (Roche, application 652).

Plasma cystatin C was measured with a particle-enhanced immunoturbidimetric method on a Hitachi Modular P analysis system [35].

Age, weight, height and gender were noted. Body surface area [133] and body mass index was calculated. Since there were no African-American patients in the group no race factor was required for the MDRD equation.

Statistical analysis:

The SPSS release 12.0.1 was used for all analysis. Linear regression could be used after log-transformation of iohexol-clearance determinations (relative GFR). Bias was

calculated by taking the median of the differences between predicted and measured GFR in percentage of measured GFR. Adjusted R^2 was defined as the correlation between predicted and measured GFR and was calculated by using the Wilcoxon signed-rank test. McNemar test was used to calculate the systematic differences between the prediction equations. ROC curve analysis was used to compare plasma cystatin C and plasma creatinine concerning their ability to differentiate GFR above and below 60 $mL \cdot min^{-1} \cdot (1.73m^2)^{-1}$.

Results:

The contribution of gender and age to cystatin C-based prediction of GFR was calculated. It showed that gender had a small, but significant, influence but not age. Since the studied creatinine-based GFR-prediction equation did not allow predictions for both adults and children, the analysed patient group was split into 2 groups, above and below 18 years of age. The adult group comprised 451 patients and the child group 85 patients.

For the adult group one cystatin C based GFR-prediction equation was constructed with a female factor and one without. When these equations were compared with the MDRD equation it turned out that MDRD overestimated GFR whereas the cystatin C-based equations did not. The MDRD equation is based on measuring plasma creatinine with a Jaffe method, which is an unspecific chromogenic method [134-136]. In addition to creatinine, it also measures so-called pseudo-creatinine chromogens. We therefore introduced a factor to, in our comparisons, compensate for the method originally used to construct the MDRD equation. Using this correction factor resulted in MDRD-GFR-predictions more close to those of the two proposed cystatin C-based equations. The cystatin C-based GFR-prediction equations containing only cystatin C or cystatin C and a factor of 0.95 for females estimated 80.0% and 82.3%, respectively, of the measured GFR +/- 30%, whereas the mathematically improved MDRD-equation estimated 79.2% of the measured GFR +/- 30% in the adults population studied. In the population of patients below 18 years of age, a cystatin C-based GFR-prediction equation using only cystatin C and a factor for children below 14 years estimated 77.6% of the measured GFR +/- 30%, whereas the Schwartz and Counahan-Barrat equation estimated only 24.7% and 62.4%, respectively, of the measured GFR +/- 30%.

ROC curve analysis of the capacity of cystatin C only and creatinine only to distinguish patients with GFR < 60 $mL \cdot min^{-1} \cdot (1.73m^2)^{-1}$ was performed. The area under the curve for cystatin C was 0.966 and for creatinine 0.943. This difference was statistically significant with $p = 0.01$.

Conclusion:

A GFR-prediction equation based upon only cystatin C levels in mg/L and a prepubertal factor might replace the simplified MDRD prediction equation for adults and the Schwartz and the Counahan-Barratt prediction equations for children.

Equation:

GFR [$mL \cdot min^{-1} \cdot (1.73m^2)^{-1}$]= 84.69 x cystatin C (mg/L) $^{-1.680}$ x 1.384 (if a child <14 years)

Paper 2

Standardization of cystatin C: Development of primary and secondary reference preparations.

Scand J Clin Lab Invest 2008; 68 (S241): 67-70

Many different equations to estimate GFR from cystatin C concentration have been published. They differ because the group of patients and the method to quantitate cystatin C differ. The diagnostic companies use different calibrators, which means that the equation to estimate GFR is only valid on a specific platform and with the calibrator, used in that study.

Aims:

The aim was to produce a primary calibrator for cystatin C and use it to make a secondary reference preparation. This preparation will, after characterization, be used by diagnostic companies to adjust their international calibrators to the same value.

Materials:

Purified lyophilized recombinant cystatin C for both the primary and the secondary reference preparation. Serum from healthy blood donors was used for the secondary reference preparation.

Methods:

Recombinant cystatin C was produced in an *E.coli* system and purified by ion exchange and size exclusion chromatography. Dry mass determination, agarose screen electrophoresis, SDS-PAGE and N-terminal sequencing were used to characterize the pure cystatin C.

Sera from 44 blood donors were frozen. After thawing the sera were tested in agarose electrophoresis for checking their protein pattern and exclude samples containing M-components. They were pooled and delipidated followed by stabilisation with inhibitors. Thereafter the pool was pH adjusted and sterile filtrated. This procedure was the same as the one used when a certified protein reference preparation was done (ERM DA470)[137].

A pilot batch was made with cystatin C-spiked serum. The tests seemed good so therefore a final big batch to be used as a secondary reference preparation was made. It was aliquoted and lyophilized in vials.

Results:

A primary cystatin C reference preparation with 5.20 g/L cystatin C was made. We were able to produce 4468 vials of the secondary reference material with a cystatin C concentration of 5-6 mg/L. The exact concentration of cystatin C in the secondary reference material will be set by using the primary cystatin C reference preparation with a know concentration. A value assignment protocol will be used to establish an exact value of the secondary reference material.

Conclusion:

It was possible to produce primary and secondary reference materials for cystatin C for international use (fig.7).

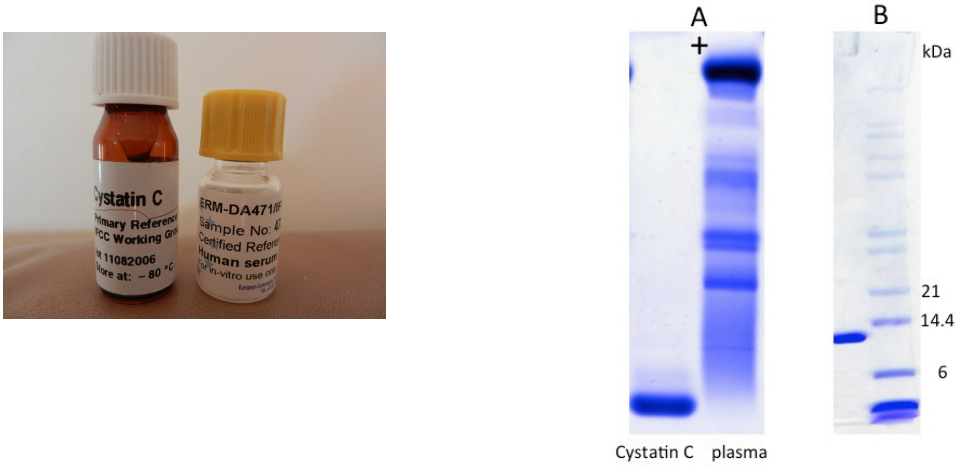


Figure 7 The primary and secondary reference preparation to the left. To the right A, agarose screen electrophoresis and B, SDS-PAGE of the primary reference preparation.

Paper 3

Different elimination patterns of β-trace protein, β_2-microglobulin and cystatin C in haemodialysis, haemodiafiltration and haemofiltration

Scand J Clin Lab Invest 2008; 68(8): 685-691

In this study we compared three different filtration modalities, haemodialysis (HD), haemodiafiltration (HDF) and haemofiltration (HF), concerning their capacities to remove proteins and solutes.

Aim:

To characterize how low molecular mass proteins are eliminated in HD, HDF and HF in order to find the best treatment for the patient.

Materials:

38 patients treated with haemodialysis for at least three months.

17 patients used standard HD with low-flux membranes, 13 patients were treated with post-dilution HDF (high-flux membrane) and 8 patients with pre-dilution HF (high-flux membrane). Blood, collected in Li-heparinate tubes, were drawn before the start, 30 minutes after the start, just before the end of dialysis and 30 minutes after the end of dialysis.

Methods:

Plasma albumin, cystatin C, creatinine and urea were measured on a Hitachi Modular P analysis system with a photometric method based on bromocresol-binding for albumin, particle-enhanced immunoturbidimetric method for cystatin C and enzyme-based methods for creatinine and urea. Plasma α_2-macroglobulin, β-trace protein and β_2-microglobulin were measured on a BNProSpec with immunonephelometric methods. Particle-enhanced methods were needed for determinations of β-trace protein and β_2-microglobulin.

In the selection of protein and solute markers, molecular mass, isoelectric point (pI) and molecular radius were considered. During the dialysis process there generally is a

haemoconcentration due to ultrafiltration of water. Albumin (67 kDa, pI 4.6) and α_2-macroglobulin (720 kDa, pI 5.3-6.1) were measured to follow haemoconcentration. The low molecular mass proteins (LMMP) β-trace protein (23-29 kDa, pI 5.8-6.7), β_2-microglobulin (11.8 kDa, pI 5.4-5.7) and cystatin C (13.3 kDa, pI 9.3) differ in molecular mass and in pI. In the kidneys they are freely filtered, reabsorbed and catabolised in the tubular cells. Creatinine (113 Da) and urea (60 Da) were analysed because of their low molecular mass. Urea clearance is commonly used to check the efficiency of dialysis treatments.

Statistical analysis:

The numbers of patients were relatively low and therefore Mann-Whitney tests were used to investigate differences between patients treated with different membranes. The median was used as central value and range to estimate dispersion.

Results:

The results showed, as expected, that the urea and creatinine concentrations in plasma decreased substantially for all different types of filter. Urea decreased in HF from start to end to 41%, in HD to 29% and in HDF to 22%. The creatinine decrease for the different filters was similar to that of urea. The increase of albumin and α_2-macroglobulin concentrations reflected the haemoconcentration. In conventional HD none of the LMMP concentrations were affected. In both HDF and HF, cystatin C and β_2-microglobulin levels were decreased to between 23 and 44%, while β-trace protein was substantially decreased (to 65%) only in HDF and remained virtually unaltered in HF (fig. 8).

Conclusion:

Cystatin C and β_2-microglobulin can be used as markers for dialysis efficiency of LMMP in the HDF and HF dialysis processes. β-trace protein was only moderately eliminated by HDF and not at all by HF and the free protein might therefore be useful for comparison of different dialysis modalities.

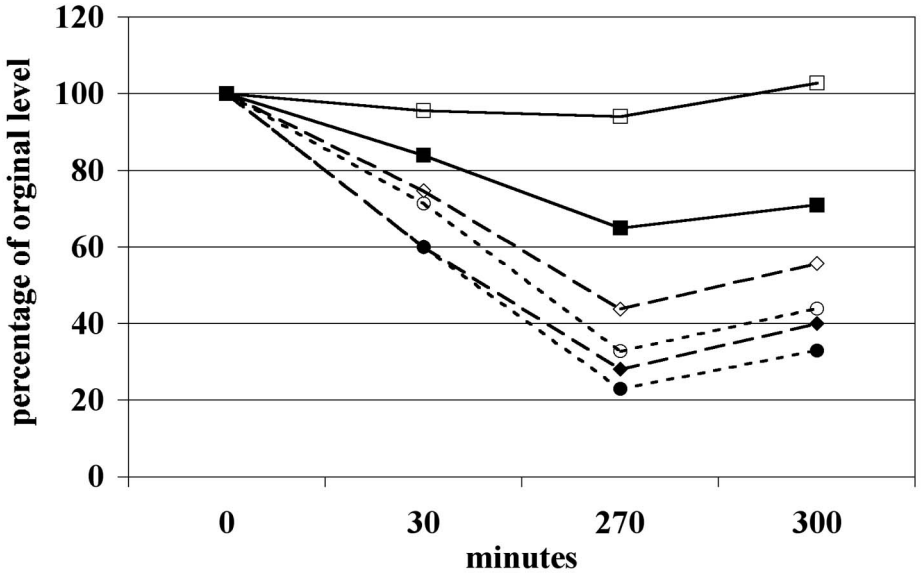


Figure 8 The resulting median levels of cystatin C, β_2-microglobulin and β-trace protein at different time points for HDF and HF treatment modalities expressed as percentages of the original levels. Cystatin C (♦), β-trace protein (■), β_2- microglobulin (●). Filled symboles are HDF treatment and open symboles are HF treatment.

Paper 4

Cystatin C, a marker for successful aging and glomerular filtration rate, is not influenced by inflammation.

Scand J Clin Lab Invest 2011; 71: 145-149

It has been proposed in some studies that the cystatin C level is raised not only as a result of low GFR but also in cases of inflammation, because an inflammatory state causes increased production of cystatin C [138-142]. In a further study on hypertensive patients an association between cystatin C and IL-6 (interleukin 6) and TNF (tumor necrosis factor) was shown, but not between cystatin C and CRP [143]. In an additional study of kidney-transplanted patients IL-2 and 6, TNF-α and cystatin C were measured before transplantation and after 30 and 180 days. No significant correlation between the inflammatory markers and cystatin C was found [144]. A cohort of orthopedic patients were studied before, and 4 and 30 days after surgery. The cystatin C level was stable, but CRP increased on day 4 but was normalized on day 30 [145]. In another study of patients, who undergo cardiac surgery, iohexolclearance was measured before and 2 days after surgery. In addition, the concentrations of cystatin C, creatinine and CRP were also measured before surgery and 1-5 days after [146]. No significant correlation between cystatin C and CRP was noted. The authors also found that after surgery $eGFR_{cystC}$, in contrast to $eGFR_{creat}$, was significantly associated with plasma clearance of iohexol. In our study we followed patients without any sign of inflammation before surgery (*i.e* at elective surgery) and then for seven days after surgery.

Aim:

To study if there is a casual relationship between cystatin C and the degree of inflammation.

Materials:

Plasma CRP concentration was measured in EDTA-plasma from thirty-five patients scheduled for elective surgery. Twenty of them had a CRP level below 1 mg/L, which is a normal value. Those twenty patients were studied further.

Methods:

Immunoturbidimetric assays on a Roche-Hitachi cobas 6000 analysis platform were used for measuring the concentrations of plasma CRP, orosomucoid and haptoglobin. The more sensitive assay, particle-enhanced immuno-turbidimetry, was used to measure the concentration of cystatin C. For SAA Siemens BN ProSpec analysis system was used employing a particle-enhanced immuno-nephelometric method. Plasma creatinine was measured with an enzymatic assay on the same Roche-Hitachi platform as described above.

CRP, orosomucoid, haptoglobin and SAA were selected because they are known as inflammatory markers. Blood samples were drawn before operation and once daily from day 1 to day 7 after the surgery.

Statistical analysis:

The SPSS release 15.0.1 was used for all analyses. Wilcoxon signed rank test was used to measure the differences between preoperative concentrations and first (CRP, SAA, creatinine and cystatin C) or second day (haptoglobin and orosomucoid) after surgery. A linear mixed model was used to test the changes in concentrations for CRP, SAA, creatinine and cystatin C on day 1 to 7 after surgery.

Results:

Our study showed that whereas the acute-phase proteins CRP and SAA increased in concentration very much already the first day after surgery, the concentration of cystatin C was unaltered for the whole study period. As expected, the acute-phase proteins orosomucoid and haptoglobin increased significantly two days after surgery. A small decrease was seen in creatinine concentration on day 3 after surgery, but the creatinine levels for all other postoperative days did not differ from the preoperative levels. These results contradict the earlier suggestions that an inflammatory process *per se* causes increased synthesis of cystatin C and thus an increased plasma level [138-142]. However, the studies suggesting a causal relationship between inflammation and cystatin C did not actually demonstrate a causal relationship but only a statistically significant correlation between the degree of inflammation, measured as the level of CRP, and the level of cystatin C. This does not automatically prove that there is a causal relationship, since the statistcally significant relationship can be due to two completely unrelated processes. For example, it is known that an increased CRP-level is a risk factor for development of atherosclerosis and thus for atherosclerosis in the renal arteries, leading to a decrease in GFR and thus to an increase in the cystatin C level in plasma.

Our results agree with those of a previous study of elective surgery in orthopedic patients.

Conclusion:

Cystatin C is not a marker of inflammation and there is no casual relationship between degree of inflammation and plasma level of cystatin C.

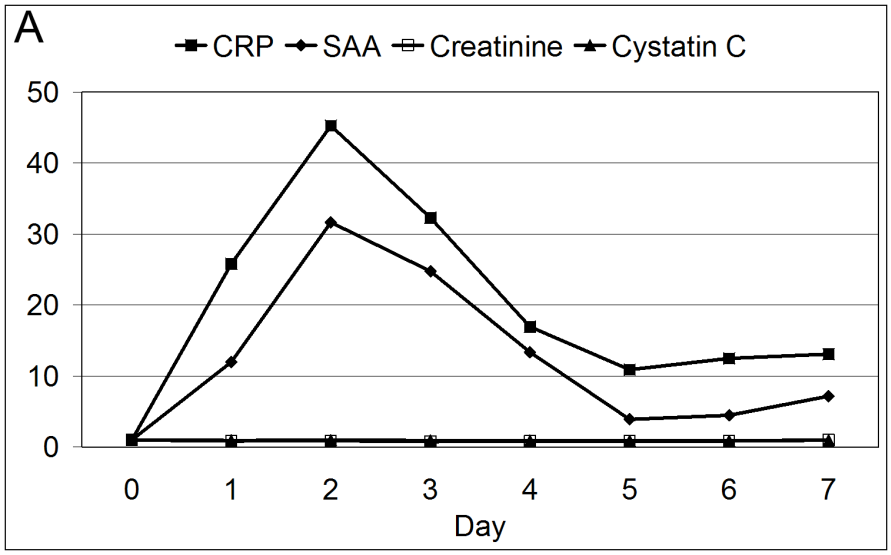


Figure 9 Changes in plasma levels of CRP, SAA, creatinine and cystatin C during seven consecutive days after elective surgery. Day zero denotes the day before surgery. The ordinates represent the median level of each analyte in multiples of the preoperative level.

What has happened after these four publications have been published?

Since publication of the first article in this thesis, proposing an equation to estimate GFR based upon cystatin C (*paper 1*), many other equations have been suggested [88, 90, 95, 147, 148]. It has been confusing with all these equations and, in particular since there is a growing interest to estimate GFR. No international reference material (calibrator) for cystatin C was available so it was not possible to standardize different methods to measure cystatin C. Each diagnostic company had their own calibrator, which did not give the same concentration of cystatin C if the samples were measured on different platforms.

In 2005 the International Federation of Clinical Chemistry and laboratory medicine (IFCC) asked Anders Grubb to form a working group (8.3.37 standardisation of cystatin C (WG-SCC)) with mission: ”a) To promote the standardisation of cystatin C measurement through definition of an international reference system, including a reference measurement procedure and primary and secondary reference materials. b) To suggest glomerular filtration rate (GFR) prediction equations based upon plasma/serum cystatin C values ”. I was one of the members in this group and the first step was to make primary and secondary reference materials (*paper 2*).

The primary reference cystatin C material was characterised with agarose electrophoresis, SDS-PAGE and N-terminal sequencing (*paper 2*). It has now been further characterised with crossed immunoelectrophoresis, Ouchterlony analysis (double radial immunodiffusion) and size exclusion chromatography. A primary cystatin C reference material, which is monomeric, highly pure, has the expected mobility in agarose electrophoresis, has the expected molecular mass, shows high homogeneity and was not truncated, was established. This preparation was used to establish a value of cystatin C in the secondary reference material. A value transfer protocol was used in which different techniques, particle enhanced immuno-turbidmetric and nephelometric methods and enzyme amplified single radial immuno-diffusion (SRID) technique, were used. Moreover these techniques were performed on different platforms (except SRID). In collaboration with the EU Institute for Reference Materials and Measurements (IRMM) the first certified reference material for cystatin C was released in 2010 [93, 149]. This international calibrator is traceable and tested

for stability and homogeneity for one year after storage in different temperatures. Moreover it resembles the patient samples. Thereafter, the diagnostic companies have performed recalibrations using this new secondary reference material. There is an ongoing process to establish an international equation to estimate GFR by measuring cystatin C. This equation includes cystatin C and age and is based upon 4000 Caucasians (Swedish and Dutch), both children (about 700) and adults, and 800 Asians (adult, Japanese). In Caucasians the method to measure GFR was plasma clearance of iohexol and for the Asians renal inulin clearance. The quantitation of cystatin C was made with seven commutable cystatin C-assays that have been adjusted to the international cystatin C calibrator (ERM-DA471/IFCC).

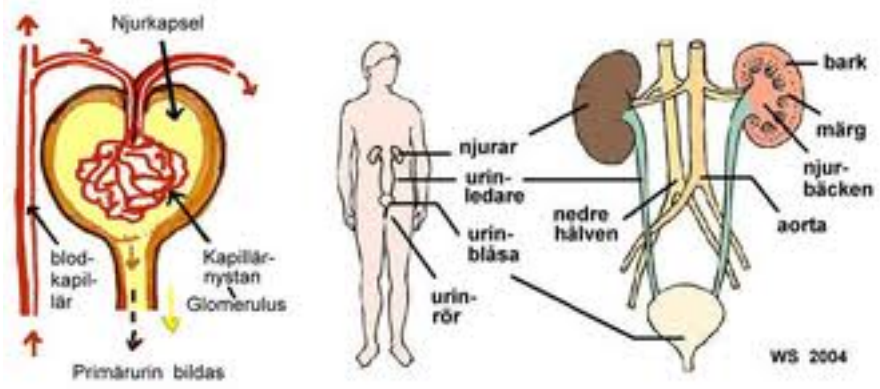
There has been a big study initiated by ¨statens beredning för medicinsk utvärdering¨ (SBU) about estimation of kidney function [150]. In that study the authors have gone through all (before 2013) published equations for estimating GFR based upon creatinine or cystatin C in serum or plasma.

The conlusions from that study is a recommendation to use the average of eGFR calculated from both cystatin C and creatinine, the ¨Lund model¨ [88, 94, 96], particulary in adult patients with low GFR. If only creatinine is used one must take into concideration the muscle mass and the intake of meat. If only cystatin C is used the intake of corticosteroids i.e the dosage must be taken into account. Moreover cystatin C based equations are often more precise and is not affected by age, gender, weight or height as creatinine based equations are.

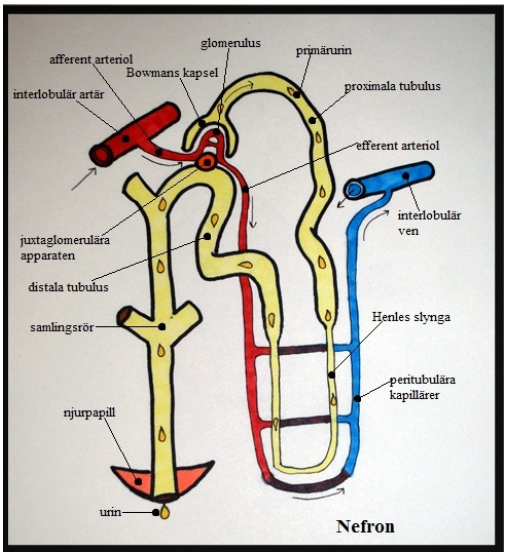
When a laboratory reports the result for cystatin C or creatinine it should also rapport the corresponding eGFRs. The accuracy of eGFR is generally acceptable up to about 90 mL/min/1.73m^2. On the web page www.egfr.se there are tools for calculating absolute and relative GFR and this page can also be helpful to evaluate estimations of GFR comparing $eGFR_{\text{cystatin C}}$ and $eGFR_{\text{creatinine}}$.

Populärvetenskaplig sammanfattning

Varje dygn passerar 1500 liter blod njuren. Varje njure innehåller ca 1 miljoner nefron som ska filtrera ut skadliga ämnen, små substanser och proteiner som har en låg molekylvikt. Nefronet består av Bowmans kapsel med ett kärlnystan, glomeruli samt av tubuli. I glomeruli finns ett system med små öppningar som inte tillåter passage av större proteiner. En primärurin på ca 180 liter per dygn filtreras genom detta system. Om det av någon anledning är färre nefron som fungerar eller om öppningarna i glomeruli har blivit större eller mindre påverkas GFR som är hur snabbt ett ämne eller ett litet protein i blodet filtreras genom glomeruli (fig. 10). Primärurinen fortsätter därefter till ett rörsystem, kallat tubulus där det mesta återresorberas till ett kapillärnätverk som ligger runt tubulus. Det är proteiner med låg vikt som bryts ner till sina byggstenar (aminosyror) och ämnen som är viktiga på något sätt som återförs till kroppen. Resterande urin med oönskade ämnen fortsätter till urinblåsan och kissas sedan ut (fig. 11).



Figur 10 Njurarnas placering och Bowman´s kapsel med glomerulus.



Figur 11 Ett nefron Källa: Bloggproffs.se

Man kan mäta hur snabbt blodet renas (GFR) genom att spruta in ett ämne t.ex. ett röntgenkontrastmedel som heter iohexol som normalt inte finns i kroppen. Efter en bestämd tid (vanligen 4 timmar) tar man ett blodprov och mäter hur mycket av det insprutade iohexolet som finns där. Man kan sedan beräkna hur snabbt reningen sker. Denna undersökningen är tidskrävande och dessutom inte helt ofarligt eftersom man sprutar in något som inte finns i kroppen normalt.

Varför behöver man då känna till denna hastigheten (GFR)? Förutom att den används för att upptäcka njurskada behöver man känna till den när man ska dosera läkemedel som utsöndras genom njuren, kontrollera njurtransplanterade patienter och för att kunna ge rätt dos av andra röntgenkontrastmedel som kan var skadliga för njuren.

Man har sedan många år tillbaka gjort en uppskattning av GFR (reningshastigheten) genom att ta ett blodprov och i det mäta koncentrationen av ett litet ämne som heter kreatinin. Genom en ekvation (det finns flera olika) kan man uppskatta GFR (eGFR). Nackdelen med kreatinin är att hur mycket man har inte bara är beroende av hur bra njuren fungerar utan också av t.ex. hur mycket muskler man har och av ålder och kön. Dessutom finns det en utsöndring av kreatinin till urinen från tubuli.

I min avhandling presenterar jag ett sätt att uppskatta GFR på genom att bara ta ett blodprov och i det mäta koncentrationen av cystatin C. Det är ett protein med låg vikt som passerar fritt genom filtret och tas upp och bryts ner i tubuli. Dessutom är cystatin C oberoende av hur mycket muskler man har och av ålder och kön. Eftersom

vi undersökt många patienter har vi kunna göra en ekvation för att kunna räkna fram eGFR med hjälp av cystatin C koncentrationen som mäts i ett blodprov. När en ny patient får tagit ett blodprov där man mäter koncentrationen av cystatin C kan man med hjälp av denna ekvationen uppskatta vilken reningshastighet njuren har.

Det finns ett intresse från många laboratorier både i Sverige och utomlands att mäta koncentrationen av cystatin C i ett blodprov och beräkna eGFR. Ett problem var att de diagnostiska företagen som säljer reagens till metoderna har olika kalibratorer. Det resulterar i många olika ekvationer som används på de enskilda laboratorierna. Dessa ekvationer fungerar endast där och om samma prov mäts på andra maskiner och med andra reagens fick man skiftande eGFR. I min avhandling redovisar jag hur vi gjorde en primär och en sekundär referenspreparation. Den sekundära referenspreparationen ska användas som en internationell kalibrator som alla företag som säljer reagens för mätning av cystatin C kan justera sin egen kalibrator mot. Eftersom denna kalibrator var tillgänglig efter alla stabilitetstester 2010 har företagen nu, eller är på god väg att skaffa, en metod som är kalibrerad mot denna internationella kalibrator.

När njuren slutar filtrera eller gör det med låg hastighet behövs dialysbehandling. Man låter då blodet passera genom ett filter som ska efterlikna njuren. Det finns olika typer av filter och dessutom kan man använda dem på olika sätt. Ett problem med behandlingen förutom att patienten måste tillbringa många timmar i veckan vid en apparat är att inget filter är så bra som en fungerande njure. Det har tidigare visats att proteiner med låg vikt kan orsaka skada på njurarna och i lederna. I min avhandling har jag testat och jämfört tre olika metoder med avseende på hur de kan filtrerar olika stora proteiner och ämnen. Slutsatsen är att alla de tre testade proteinerna med låg vikt kan användas för att testa hur effektiv behandlingen är.

När man vill använda ett protein eller någon substans som en analys är man intresserad av om det finns något förutom t.ex. njurfunktionen som påverkar vilken koncentration man mäter. För cystatin C är det tidigare visat att om man behandlas med en viss typ av medicin eller om man har fel på sköldkörtlen får man en falsk för hög koncentration. Några författare har föreslagit att inflammation påverkar koncentrationen av cystatin C i blodet precis som t. ex ett protein som heter CRP. Inflammation är ett svar från kroppen på att något främmande kommit in i kroppen t.ex. bakterier, virus, gifter mm och det måste bekämpas. Om kroppen inte hinner bekämpa i tid kan det i värsta fall sluta med döden. Inflammationen kan vara både på utsidan och inuti kroppen. Den kan dessutom vara både akut och kronisk. För att se om det är en inflammationen i kroppen finns olika laboratorietester som man kan göra. De reagerar olika snabbt beroende av var i kroppen problemet är. Det som man oftast använder först är ett protein som heter CRP som är lätt och snabbt att analysera.

På vårdcentralerna använder man CRP för att avgöra om ett det är en virus- eller en bakterieinflammation. Om det är ett virus är antibiotika verkningslöst! Man kallar testet populärt för snabbsänka men det har inget med “vanlig sänka “ att göra. Sänkan beskriver hur snabbt de röda blodkropparna sjunker i ett smalt rör medan CRP är ett protein. I min avhandling har jag undersökt cystatin C och jämfört med några proteiner som är kända för att öka i koncentration vid inflammation. Patienterna som undersöktes skulle opereras och hade ingen inflammation från början. Man vet sedan tidigare att en större operation medför en ökning i koncentrationen av t ex CRP. Resultatet från vår studie visar att koncentrationen av CRP ökade som förväntat medan koncentrationen av cystatin C inte gjorde det. Detta betyder att oavsett om patienten har inflammation eller ej och man mäter koncentrationen av cystatin C så motsvarar svaret man får hur bra njurarna fungerar.

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Första av allt vill jag nämna Anders, min handledare och mentor mm!

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