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QUANTIFICATION AND CLINICAL RELEVANCE OF CYSTATIN C

by

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The most effective way to do it, is to do it

–Amelia Earhart

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications, referred to in the text by their Roman numerals:

- I** Ristiniemi N, Qin Q-P, Lindström V, Grubb A and Pettersson K (2012) Quantification of cystatin C by time-resolved fluorometry-based immunoassays. *J Imm Methods* **378**:56-61.
- II** Ristiniemi N, Qin Q-P, Postnikov A, Grubb A and Pettersson K (2010) Dry-reagent double-monoclonal assay for cystatin C. *Clin Chem* **56**:1424-1431.
- III** Ristiniemi N, Savage C, Bruun L, Pettersson K, Lilja H and Christensson A (2012) Evaluation of a new immunoassay for cystatin C, based on a double monoclonal principle, in men with normal and impaired renal function. *Nephrol Dial Transplant* **27**:682-687.
- IV** Ristiniemi N, Lund J, Tertti R, Christensson A, Ilva T, Porela P, Pulkki K and Pettersson K (2012) Cystatin C as a predictor of all-cause mortality and myocardial infarction in patients with non-ST-elevation acute coronary syndrome. *Clin Biochem* **45**:535-540.

In addition, unpublished results are presented.

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ABBREVIATIONS

ACR, albumin-to-creatinine ratio
ACS, acute coronary syndrome
AKI, acute kidney injury
AUC, area under the curve
 β 2M, *beta*-2-microglobulin
BNP, B-type natriuretic peptide
BSA, bovine serum albumin
BUN, blood urea nitrogen
CAD, coronary artery disease
CG, Cockcroft-Gault
CI, confidence interval
CKD, chronic kidney disease
CKD-EPI, Chronic Kidney Disease Epidemiological Collaboration
CRP, C-reactive protein
cTnI, cardiac troponin I
CV, coefficient of variation
CVD, cardiovascular disease
DM, diabetes mellitus
DTPA, diethylenetriaminepentaacetic acid
EDTA, ethylenediamine tetracetic acid
eGFR, estimated glomerular filtration rate
eGFR-CG/BSA, eGFR calculated with CG formula and adjusted for body surface area
eGFR-MDRD, eGFR calculated with MDRD Study equation
ESRD, end-stage renal disease
GFR, glomerular filtration rate
HPLC, high performance/pressure liquid chromatography
HF, heart failure
HR, hazard ratio
IFCC, International Federation of Clinical Chemistry and Laboratory Medicine
IQR, interquartile range
LMW, low-molecular weight
MDRD, modification of diet in renal disease
MI, myocardial infarction
nSTE, non-ST-elevation
PENIA, particle-enhanced nephelometric immunoassay
PETIA, particle-enhanced turbidimetric immunoassay
RET, resonance energy transfer
RBP, retinol binding protein
ROC, receiver-operating characteristic
RRT, renal replacement therapy
SRID, single radial immunodiffusion
ST, ST segment of electrocardiogram tracing
TSA, tris-buffered saline with azide

ABSTRACT

An aging population and increasing rates of diabetes mellitus contribute to a high prevalence of kidney dysfunction – approximately 10 percent of adults in developed countries have chronic kidney disease (CKD). CKD is a progressive loss of kidney function and this remains permanent. Early recognition of this condition is important for prevention or impeding severe adverse cardiac and renal outcomes.

Cystatin C is a low molecular weight cysteine protease inhibitor that has emerged as a biomarker of kidney function. The special potential of plasma cystatin C in this setting is related to its independency of muscle mass, which is a remarkable limitation of the traditional marker creatinine. Cystatin C is a sensitive marker in diagnosing mild and moderate CKD, especially in small children, in the elderly and in conditions where muscle mass is affected. Cystatin C is quantified with immunoassays, mainly based on particle-enhanced nephelometry (PENIA) or turbidimetry (PETIA).

The aim of this study was to develop a rapid and reliable assay for quantification of human cystatin C in plasma or serum by utilizing time-resolved fluorescence-based immunoassay methods. This was accomplished by utilizing different antibodies, including polyclonal and 7 monoclonal antibodies against cystatin C. Different assay designs were tested and the best assay was further modified to a dry-reagent double monoclonal assay run on an automated immunanalyzer. This assay was evaluated for clinical performance in estimating reduced kidney function and in predicting risk of adverse outcomes in patients with non-ST elevation acute coronary syndrome.

Of the tested assay designs, heterogeneous non-competitive assay had the best performance and was chosen to be developed further. As an automated double monoclonal assay, this assay enabled a reliable measurement of clinically relevant cystatin C concentrations. It also showed a stronger concordance with the reference clearance method than the conventional PETIA method in patients with reduced kidney function. Risk of all-cause mortality and combined events, defined by death and myocardial infarction, increased with higher cystatin C and cystatin C remained an independent predictor of death and combined events after adjustment to non-biochemical baseline factors.

In conclusion, the developed dry-reagent double monoclonal assay allows rapid and reliable quantitative measurement of cystatin C. As measured with the developed assay, cystatin C is a potential predictor of adverse outcomes in cardiac patients.

TIIVISTELMÄ

Väestön ikääntyminen ja diabeteksen yleistymisen ovat johtaneet munuaisten vajaatoiminnan esiintyvyyden kasvuun. Nykyään arviolta kymmenyksellä kehittyneiden maiden aikuisväestöstä on krooninen munuaisten vajaatoiminta, joka tarkoittaa etenevää ja pysyvää munuaistoiminnan menetystä. Jos munuaisten vajaatoiminta havaitaan ajoissa, vakavia munuaisiin ja sydämeen liittyviä seurauksia on mahdollista estää tai hidastaa.

Kystatiini C on pienimolekyylinen kysteiiniproteaasien inhibiittori, joka on lupaava uusi munuaistoiminnan merkkiaine. Kystatiini C:n suurin tunnettu etu perinteiseen munuaismerkkiaineeseen, kreatiniiniin, nähden on, ettei lihassmassan määrä vaikuta sen pitoisuuteen veressä. Kystatiini C:n on osoitettu olevan herkkä merkkiaine lievän ja kohtalaisen munuaisten vajaatoiminnan diagnosoinnissa, erityisesti pikkulapsilla, ikääntyneillä sekä erilaisissa tiloissa, joissa lihassmassan määrä on normaalista poikkeava. Kystatiini C -pitoisuutta mitataan immunomäärityksillä, pääasiallisesti turbidimetrisillä tai nefelometrisillä menetelmillä.

Tutkimuksen tavoitteena oli kehittää nopea ja luotettava aikaeroitteeseen fluoresenssiin perustuva immunomääritys ihmisen kystatiini C -pitoisuuden mittaamiseen veriplasmasta tai -seerumista. Määrityksen kehittämisessä käytettiin polyklonaalista ja seitsemää eri monoklonaalista vasta-ainetta. Erilaisten määritystyyppien, kuten kilpailevan ja ei-kilpailevan määrityksen, soveltuvuus kystatiini C:n mittaamiseen selvitettiin. Parhaita määritystä kehitettiin edelleen ja se muokattiin kahta monoklonaalista vasta-ainetta käyttäväksi kuivakemiaan perustuvaksi automatisoiduksi määritykseksi. Lopuksi selvitettiin kehitetyn määrityksen kliininen suorituskyky munuaisten vajaatoiminnan arvioinnissa sekä sepelvaltimotautipotilaiden kuoleman ja sydänkohtauksen riskin ennustajana.

Testatuista määritystyypeistä ei-kilpaileva määritys toimi parhaiten ja valittiin jatkokehitykseen. Kehitetty automatisoitu kahta monoklonaalista vasta-ainetta käyttävä määritys soveltui kliinisesti merkittävien kystatiini C -pitoisuuksien luotettavaan mittaukseen. Sillä mitatut pitoisuudet vastasivat paremmin munuaistoiminnan tarkimpana pidetyn mittarin eli puhdistumamittauksen tuloksia kuin turbidimetrisellä menetelmällä mitatut kystatiini C -pitoisuudet, erityisesti potilailla, joiden munuaistoiminta oli heikentynyt. Kuoleman ja sydänkohtauksen riski oli suurempi korkeammilla kystatiini C -pitoisuuksilla ja kystatiini C oli näiden tapahtumien itsenäinen ennustaja myös sen jälkeen kun ei-biokemiallisten taustatekijöiden vaikutus oli huomioitu riskianalysissä.

1 INTRODUCTION

Impairment of kidney function can drastically affect the function of the whole body. If both kidneys fail, other organs do not survive as homeostasis fails and metabolic wastes and excess body fluid accumulate. Without renal replacement therapy, death follows. Chronic kidney disease (CKD) is marked by a gradual loss of kidney function over time with approximately a 10 percent prevalence in developed countries (Hallan et al., 2006a; Coresh et al., 2007) and increasing incidence in developing countries (Chen et al., 2005). Especially in the developed world, age distribution and metabolic syndrome are linked to the high prevalence of CKD.

The awareness of CKD has increased. Earlier, it was identified at later stages when it was a life-threatening condition managed by a nephrologist. Currently, it is recognized earlier and is a common condition whose detection, prevention and early management is done by a non-nephrologist physician (Levey et al., 2011). Close association of kidneys with heart is recognized and thus early detection is encouraged. By early diagnosis and treatment, kidney failure can be either prevented or postponed.

Compared to, for example, cardiac diagnostics, the advancements made in CKD testing are limited. Since the early 1900's, a single, low-cost clinical routine test of serum creatinine concentration has been a standard and simple way to get an estimate of kidney function. It has limitations and replacement diagnostics are sought – by correcting the creatinine value with known affecting factors or by searching for new biomarkers to replace or to complement creatinine.

Cystatin C, a low molecular weight protease inhibitor, was suggested as a marker of kidney function in 1985 (Simonsen et al., 1985). The clinical utility of serum cystatin C is extensively studied (Dharnidharka et al., 2002; Laterza et al., 2002; Roos et al., 2007). The advantage of cystatin C, as compared to creatinine, is in diagnosing mild CKD and complementing creatinine in situations, where creatinine measurement may be flawed.

In the following literature review, kidney structure and metabolism will be discussed, and methods for estimating kidney function and the role of cystatin C will be introduced. The potential of cystatin C as a biomarker will be reviewed in more detail. Finally, methods for the quantification of cystatin C will be covered.

2 REVIEW OF LITERATURE

2.1 KIDNEY DISEASE

2.1.1 Kidney function

The main functions of kidneys are the removal of waste products and toxins and the body's maintenance of water, electrolyte and acid-base balance. The functional unit of a kidney is a nephron, which is capable of independent production of urine. There are approximately one million nephrons in each of the two kidneys (Hayman and Johnston, 1933). The blood flow in the kidneys is approximately 20 percent of the cardiac output at rest, 1.0–1.2 Liters (L)/min, and about 10 percent of this flow is filtered and forms the primary urine (Smith, 1951). Approximately 1.5 L of urine is excreted per day in healthy adults. Glomerulus and Bowman's capsule together form a renal corpuscle, which is the initial blood-filtering component of a nephron. A glomerulus is a network of small capillary veins, through which the blood is filtered into the surrounding Bowman's capsule. Plasma components, except for proteins greater than 10–30 kDa, depending on the net charge and folding structure, are filtered into the primary urine and this plasma ultrafiltrate is then delivered to the tubule (Brenner et al., 1976). In the tubule, important substances, such as salts and water, are reabsorbed from primary urine through active or passive mechanisms. Low molecular weight (LMW) proteins are reabsorbed and then catabolized in the proximal tubular cells (Waldmann et al., 1972). The kidney thus plays a major role in the protein metabolism.

Glomerular filtration together with tubular absorption or secretion conducts vital filtering of blood in nephrons. The glomerular filtration rate (GFR) is the rate at which water and dissolved small molecular compounds capable of ultrafiltration are filtered from blood through glomeruli; i.e., filtration speed of primary urine. According to the standardized clinical practice guidelines, GFR is the primary measure of kidney function (European Best Practice Guidelines Expert Group on Hemodialysis, European Renal Association, 2002; National Kidney Foundation, 2002). GFR is usually reported as proportional to a standard body surface area (1.73 m²), thus the unit of GFR is mL per minute per 1.73 m². Normal value of GFR in healthy adults (20–30 years) is approximately 120 mL/min/1.73 m² in women and 130 mL/min/1.73 m² in men (Goldring et al., 1940).

2.1.2 Renal insufficiency

Kidney insufficiency is divided into two categories based on either acute or chronic nature. Chronic insufficiency progresses slowly to an irreversibly decreased renal function, whereas in the acute condition, loss of kidney function is rapid and usually reversible (Levey et al., 2005; Mehta et al., 2007).

2.1.2.1 Acute kidney injury

Acute kidney injury (AKI), previously acute renal failure (ARF), represents a rapid reduction in kidney function, detected as an increase in serum biomarker levels and/or

reduction in urine output within 48 hours (Mehta et al., 2007). AKI is particularly common in hospitalized patients. Incidence numbers of AKI are strongly dependent on the criteria and definitions used. One-fifth of hospitalized patients may have some degrees of acute kidney impairment during their hospital stay (Uchino et al., 2006; Wang et al., 2012). In a large multicenter, multinational, prospective, epidemiological survey of severe AKI (defined here as urine output of less than 200 mL in 12 hours and/or a blood urea nitrogen level higher than 30 mmol/L) in intensive care units, Uchino and colleagues found a 5.7 percent occurrence (Uchino et al., 2005). The rate of hospital mortality in severe AKI necessitating renal replacement therapy (RRT) is high, exceeding 50 percent (Metcalf et al., 2002; Uchino et al., 2005). The community-based incidence of AKI may be much lower; e.g., Hsu and coworkers found an incidence of 0.6 percent among members greater than or equal to 20 years of a large healthcare system in the United States (Hsu et al., 2007). The identification of AKI was mainly based on changes in inpatient serum creatinine values.

The mechanisms that lead to AKI are divided into prerenal, intrinsic renal and postrenal causes (Lameire et al., 2005b). Prerenal AKI is caused by an insufficient volume of blood, e.g., due to dehydration (Lameire et al., 2005b). Intrinsic renal causes are processes that injure renal tissue, such as diseases like glomerulonephritis and vasculitis, or acute tubular necrosis caused by ischemia or nephrotoxins (Lameire et al., 2005b). Postrenal causes include kidney stones and other causes that block the urinary tract (Lameire et al., 2005b). However, several concurrent factors may attribute to the onset of AKI. A typical cause for in-hospital AKI is renal hypoperfusion following severe systemic insult such as septic shock or surgery (causing prerenal AKI) (Uchino et al., 2005). There is bidirectional relationship of AKI and chronic kidney disease (CKD), as patients with CKD are at an increased risk of AKI, and AKI survivors are at an increased risk of CKD (Hsu et al., 2008).

2.1.2.2 Chronic kidney disease

Chronic kidney disease, previously chronic renal disease or chronic kidney injury, means a progressive permanent loss of kidney function. The prevalence of CKD is high in developed countries and up to 10–13 percent of adults over 20 years is estimated to have CKD according to studies in Norway, the USA and Taiwan (Hallan et al., 2006a; Coresh et al., 2007; Wen et al., 2008). This is also an emerging problem in the developing world (Chen et al., 2005; Singh et al., 2009). The prevalence of RRT for kidney failure was 730 per million of the population in Europe (Luijtgarden et al., 2012).

An attempt to unify the definition and classification of CKD was taken by the US National Kidney Foundation Kidney Disease Outcomes Quality Initiative (K/DOQI), which has provided an internationally recognized clinical practice guideline for patients with CKD that includes definition and classification of CKD (National Kidney Foundation, 2002). CKD is **defined** as either kidney damage *or* GFR less than 60 mL/min/1.73 m² for greater than or equal to 3 months, irrespective of diagnosis. Kidney damage is determined by pathologic abnormalities or markers of damage, such

as abnormalities in the composition of urine (manifested by, e.g., increased protein in urine or erythrocyturia) or in imaging studies (demonstrated by abnormal ultrasound). According to the definition, all individuals with GFR less than 60 mL/min/1.73 m² for 3 months or longer, with or without signs of kidney damage, are classified as having CKD. Reduction in GFR to less than 60 mL/min/1.73 m² represents loss of half or more of the adult level of kidney function. Substantial kidney damage may occur at higher GFR levels (National Kidney Foundation, 2002; Levey et al., 2005).

Furthermore, professional guidelines **classify** the severity of CKD into 5 stages that follow the level of GFR independent of the cause and the symptoms (Table 1). CKD at stage 1 is the mildest, usually without symptoms, whereas CKD stage 5, also called as kidney failure, established CKD or end-stage renal disease (ESRD) (outdated terms: chronic kidney failure, chronic renal failure, uremia), is a severe illness that leads to lower life expectancy, especially if untreated. For stages 1 and 2 (≥ 60 mL/min/1.73 m²), the presence of kidney damage is mandatory. Second and third stages represent mild and moderate kidney dysfunction. Mild anemia is typically the only symptom observed at these stages. As CKD progresses slowly, the body has time to adjust to the changes. Thus, clear symptoms do not occur until the fourth stage, where only approximately 10 percent of the nephrons are functional (Table 1). At stage 5, both kidneys are permanently damaged and the existing number of functional nephrons is not sufficient to sustain normal renal function (National Kidney Foundation, 2002).

Table 1. Stages of chronic kidney disease. Adapted from (National Kidney Foundation, 2002; Levey et al., 2005). RRT, renal replacement therapy.

| Stage | Description | GFR (mL/min/1.73 m ²) | Symptoms |
|-------|---|--------------------------------------|--|
| 1 | Kidney damage with normal or elevated GFR | ≥ 90 | No symptoms, kidneys usually manage their normal functions. |
| 2 | Kidney damage with mildly decreased GFR | 60–89 | Mild anemia (possibly). |
| 3 | Moderately decreased GFR | 30–59 | Over 50% of the nephrons may have been destroyed. Mild anemia. |
| 4 | Severely decreased GFR | 15–29 | Approximately 10% of the nephrons are functional, clear symptoms seen at this stage, such as anemia, hypertension, and tiredness. |
| 5 | Kidney failure | <15 (or dialysis) | Endocrine/metabolic derangements or disturbances in water or electrolyte balance. Changes in skin, bones, mucous membranes, heart and blood vessels. Fatal condition, RRT needed (regular dialysis or transplant). |

At the early stages of CKD, symptoms are nonspecific and uncommon to be useful in classification. In addition to the information of severity of functional impairment, as assessed by the level of GFR, and determinations of kidney damage, clinical decisions are based on the cause and pathology of kidney disease, presence or absence of complications, risk factors for progression and comorbid conditions (Stevens and Levey, 2009; Levey et al., 2011).

The five-stage classification guides the diagnosis and treatment of CKD. However, experts in the field of nephrology are not unanimous about the above classification. Individuals with mildly reduced kidney function, specifically the elderly, are considered to be at risk to be mislabelled for CKD (Bauer et al., 2008; Eckardt et al., 2009). GFR declines with age, by approximately 10 mL/min/1.73 m² per decade after the age of 30 years (Davies and Shock, 1950), and about 16 percent of persons greater than 70 years without diabetes or hypertension have an estimated GFR value less than 60 mL/min/1.73 m² (Coresh et al., 2003). Although pathological changes in kidneys of older patients include arterial medial sclerosis and global glomerular sclerosis (Kaplan et al., 1975), the clinical significance of moderate age-related decline in GFR is controversial. These clinical manifestations may reflect the presence of kidney disease or normal physiological aging (Glasscock and Winearls, 2008). A higher prevalence of common concurrent CKD-related complications: anemia; metabolic acidosis; hyperkalemia; hyperphosphatemia; hyperparathyroidism; and hypertension in older individuals with moderately reduced GFR (45–59 vs. ≥ 60 mL/min/1.73 m²) suggest clinical relevance of declined GFR in older people (Bowling et al., 2011). Physiological implications of older individuals having reduced kidney function also include dosage adjustment of drugs excreted by the kidney, and increased risk for adverse outcomes, such as mortality and developing kidney failure (Fried et al., 1998; Coresh et al., 2003; Roderick et al., 2009).

Results of longitudinal follow-up studies and meta-analyses show that the risk of mortality and kidney failure (ESRD) increased significantly by the presence of abnormal amounts of protein in urine and/or a GFR of less than 45 mL/min/1.73 m² (Keith et al., 2004; Chronic Kidney Disease Prognosis Consortium et al., 2010; Astor et al., 2011). The above definition and classification of CKD has been redefined by “Kidney Disease: Improving Global Outcomes (KDIGO) on CKD: Definition, Classification and Prognosis” working group. A collaborative meta-analysis carried out by KDIGO included over 1.5 million individuals (Levey et al., 2011). On the basis of these analyses, the current definition for CKD remained unchanged. The recommended modifications to the classification were the addition of three stages of urine abnormalities (excess albumin in urine) at all GFR stages, subdividing stage 3 at GFR of 45 mL/min/1.73 m² (3a 45–59; 3b 30–44), and emphasizing clinical diagnosis (Levey et al., 2011). Additional tools were also provided to categorize patients according to their risk for major outcomes.

Limitations of the definition of CKD are known. Especially in epidemiological studies, the clinical diagnosis is not ascertained but all people with urinary abnormalities estimated from random urine sample or estimated GFR value less than 60 mL/min/1.73

m² in single measurement are defined as having CKD, and classified according to the level of GFR (Levey et al., 2011).

2.1.3 Traditional risk factors of chronic kidney disease

Diabetes mellitus (DM) and hypertension are established and the most common risk factors for the development and the progression of kidney disease (Eknoyan et al., 2003; Bakris et al., 2009). These are also conventional risk factors for coronary artery disease (CAD). Diabetes is the leading cause of RRT in developed countries (Foley et al., 2005; Kramer et al., 2009). Microvascular complications in DM include damage to the kidneys, which is known as diabetic nephropathy, and approximately half of patients with type 1 or type 2 DM for 25 years or more have diabetic nephropathy (Hasslacher et al., 1989). Hypertension is known to be involved in the progression of diabetic nephropathy and the prevalence of hypertension in diabetic nephropathy is actually about two-fold as compared to the general population (Epstein and Sowers, 1992). Hypertension, as such, has a high prevalence – approximately 1 billion adults, worldwide, are afflicted and it is both a cause and consequence of CKD (Kearney et al., 2005; Bakris et al., 2009). The current consensus regards higher systolic rather than diastolic blood pressure as posing a greater risk for kidney disease progression (Bakris et al., 2009).

In addition to DM and hypertension, other factors linked to the risk of kidney disease include a family history of kidney disease, cardiovascular disease (CVD), older age (>60 years), male sex, physical inactivity, menopause, left ventricular hypertrophy and smoking (Bleyer et al., 2000; Sarnak et al., 2003; Meguid El Nahas and Bello, 2005). Hyperlipidemia and obesity also are risk factors for CKD (Meguid El Nahas and Bello, 2005).

2.1.4 Cardiorenal syndrome: connection between kidney and heart disease

Cardiorenal syndrome (CRS) refers to interactions between the cardiovascular system and the kidneys. Declining kidney function in heart failure (HF) was considered to be a direct result of primary changes in cardiac function, such as impaired renal blood flow due to depressed left ventricular systolic function (Bock and Gottlieb, 2010). Also, the prevalence of CKD among patients with acute coronary syndromes (ACSs) is high. Fox et al found a 30 percent prevalence in ST-elevation myocardial infarction (STEMI) and 40 percent prevalence in non-STEMI (Fox et al., 2010). However, outcomes of CKD include increased risk of CVD (Drey et al., 2003; Sarnak et al., 2003) and the increase in this risk is progressive according to the level of CKD (Manjunath et al., 2003; Levey et al., 2011). This is partly explained by factors associated with decreasing kidney function, including anemia, disturbances in calcium-phosphate balance, inflammatory factors and oxidative stress, as these are further associated with accelerated atherosclerosis and adverse cardiac remodeling – potentially acting through hyperactivation of the renin-angiotensin system (McCullough, 2003; Shlipak et al., 2003; Muntner et al., 2004).

Increasing evidence supports the contribution of several pathophysiological mechanisms to a complex syndrome linking the heart and the kidneys (Figure 1). The

nature of pathophysiological interactions between the (failing) heart and kidneys is recognized to be bi-directional (Stevenson et al., 2005; Ronco et al., 2008). Potential contributors to CRS include anemia, central venous congestion, neurohormonal elaboration, oxidative stress, and renal sympathetic activity (Bock and Gottlieb, 2010).

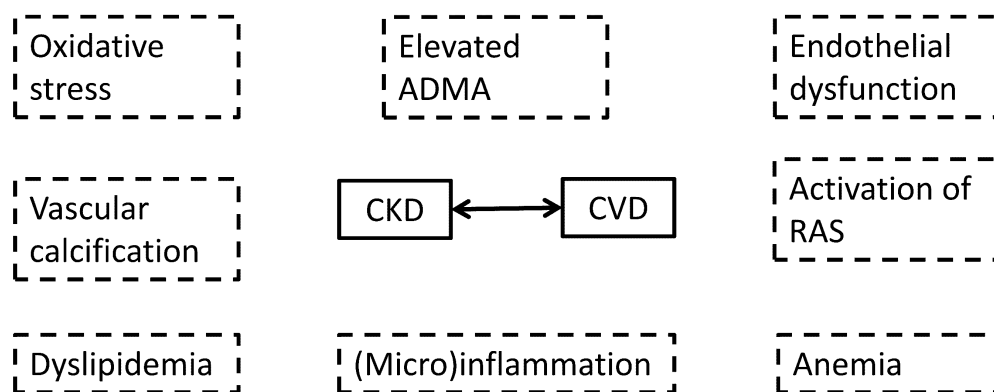


Figure 1. Pathophysiological mechanisms in the association of chronic kidney disease (CKD) and cardiovascular disease (CVD). ADMA, asymmetric dimethyl-L-arginine; RAS, renin-angiotensin system.

CRS is generally defined as “a pathophysiological disorder of the heart and the kidneys in which acute or chronic dysfunction in one organ may induce acute or chronic dysfunction in the other organ,” and it is divided into five subtypes, described in Table 2 (Ronco et al., 2008; Ronco et al., 2010). The categories are not fixed. When the disease progresses, patients may change to another subtype of CRS (Ronco et al., 2010).

Table 2. Classification of cardiorenal syndrome. Adaptation from Ronco et al. (Ronco et al., 2008; Ronco et al., 2010).

| Cardiorenal syndrome | |
|----------------------|---|
| Type I | <i>Acute cardiorenal syndrome</i> Abrupt worsening of cardiac function (e.g., acute cardiogenic shock or acutely decompensated congestive heart failure) leading to acute kidney injury |
| Type II | <i>Chronic cardiorenal syndrome</i> Chronic abnormalities in cardiac function (e.g., chronic congestive heart failure) causing progressive and potentially permanent chronic kidney disease |
| Type III | <i>Acute renocardiac syndrome</i> Abrupt worsening of renal function (e.g., acute kidney ischaemia or glomerulonephritis) causing acute cardiac disorder (e.g., heart failure, arrhythmia, ischaemia) |
| Type IV | <i>Chronic renocardiac syndrome</i> Chronic kidney disease (e.g., chronic glomerular or interstitial disease) contributing to decreased cardiac function, cardiac hypertrophy and/or increased risk of adverse cardiovascular events |
| Type V | <i>Secondary cardiorenal syndrome</i> Systemic condition (e.g., diabetes mellitus, sepsis) causing both cardiac and renal dysfunction |

2.1.5 Outcome of chronic kidney disease

Major outcomes of CKD include kidney failure, complications of decreased kidney function and increased risk for CVD (Sarnak et al., 2003). The risk of AKI also is higher in patients with CKD (Hsu et al., 2008). Although CKD means progressive loss of kidney function, patients with CKD are more likely to die than to develop kidney failure and CVD is a common cause of death in individuals with CKD (Drey et al., 2003; Sarnak et al., 2003; Go et al., 2004). In a longitudinal study of nearly 28 000 CKD patients, 24.9 percent of those with CKD stage 2–4 died within 5.5 years while only 3.1 percent progressed to ESRD requiring RRT (Keith et al., 2004). In a cross-sectional health survey in a general Norwegian population of greater than or equal to 20 years, 1 percent of patients with CKD stage 3 progressed to ESRD during a 8-year follow-up period, whereas death, including cardiovascular mortality, was a much more typical endpoint than ESRD (Hallan et al., 2006b). However, age and factors that coexist with CKD may affect the outcome, as e.g., in relatively healthy persons (mean 49.9 years, standard deviation (SD) \pm 12.9 years) with nondiabetic CKD stage 2–4, ESRD was almost four times more likely outcome than death (Menon et al., 2008). Moreover, in elderly subjects (\geq 65 years) with CKD, there was almost twofold difference in the incidence ratio of RRT vs. death in diabetic or non-diabetic subjects, 1:6 and 1:11, respectively (Foley et al., 2005). O'Hare and colleagues determined cutoff GFR values below which the risk of ESRD requiring treatment exceeded the risk of death for persons representing different age groups: the cutoff ranged from 45 mL/min/1.73 m² (18 to 44 years) to 15 mL/min/1.73 m² (65 to 84 years) (O'Hare et al., 2007). Once in RRT/ESRD, mortality of patients in dialysis is approximately 20 percent per year and over half of the deaths are related to CVDs (Sarnak et al., 2003; Kramer et al., 2009). In young people (25–34 y) with ESRD, the risk of cardiovascular mortality can be 500-fold higher than in age-matched controls with normal kidney function (Sarnak et al., 2003).

As described in section 2.1.4, complications of decreased kidney function include anemia and several other metabolic disturbances, such as hyperparathyroidism, hyperphosphatemia, and metabolic acidosis that worsen with progressive decrease in kidney function (Inker et al., 2011a). Consequently, changes in coagulation and fibrinolysis, endothelial dysfunction, and other abnormalities develop, many of which have the potential to cause vascular damage (Shlipak et al., 2003; Bonello et al., 2008). Common complications in elderly patients with decreased GFR ($<$ 30 mL/min/1.73 m²) are hypertension, malnutrition, neuropathy, and decreased quality of life (National Kidney Foundation, 2002). Furthermore, cognitive impairment and frailty in elderly subjects with CKD exist (Shlipak et al., 2004; Kurella et al., 2005).

Due to the slowly progressive and silent nature of CKD, many patients are diagnosed with CKD in later stages, when there are fewer opportunities to prevent adverse outcomes. By early diagnosis and treatment, complications and progression of CKD can be prevented or delayed. Management of hyperlipidemia, strict blood pressure control, control of blood glucose in diabetic patients, decreasing proteinuria, cessation of smoking and limitation of potentially nephrotoxic drugs are central in prevention

and treatment of CKD (Luft, 2000; El Nahas and Bello, 2005). Nathan et al. reported that intensive diabetes treatment significantly reduced cardiovascular risk and prevalence of early stages of CKD in patients with type 1 DM (Nathan et al., 2005). Treatment with angiotensin converting enzyme (ACE) inhibitors delays the progression of kidney disease in diabetic and nondiabetic persons (Lewis et al., 1993; HOPE study investigators, 2000; Mann et al., 2001). Interference of the renin-angiotensin system can alternatively be achieved with angiotensin receptor blockers (Lewis et al., 2001).

Many of the renoprotective efforts are also cardioprotective. Treatment of CKD reduces cardiovascular risk, whereas a rapid decline in GFR, on the contrary, increases risks (Shlipak et al., 2009b). Successful strategies to lower the risk of adverse cardiovascular events in patients with CKD include optimal management of blood pressure and dyslipidemia (HOPE study investigators, 2000; Strippoli et al., 2008). Despite significant progress in ACS treatment and use of optimal medication and revascularization, the cardiovascular prognosis is worse in patients with CKD than in those with normal kidney function (Anavekar et al., 2004; Bonello et al., 2008). One explanation for poor outcomes of subjects with CKD is that they do not receive adequate treatment (Mann et al., 2001; Shlipak et al., 2002; Fox et al., 2010). The term “renalism” or “therapeutic nihilism” has been used to describe the low rates of cardioprotective therapies in patients with CKD (McCullough, 2003; Chertow et al., 2004). Differential use of cardiovascular medication may contribute to increased risk of cardiovascular events of patients with both CAD and CKD (Bansal et al., 2011), although not all studies have found clear differences in medication and suggest other factors to be responsible for the increased risk (Winkelmayer et al., 2008). Other explanations for increased numbers of adverse outcomes after cardiovascular events in CKD include numerous comorbidities in patients with CKD, potential toxicity of therapies used and the pathobiology of CKD, which contributes to a worsened cardiovascular state (Freeman et al., 2003; McCullough, 2003). As the prevalence of CKD increases with age, comorbidities are common.

2.2 BIOCHEMICAL ASSESSMENT OF KIDNEY FUNCTION

2.2.1 Glomerular filtration rate as the basis for assessment of kidney function

The level of GFR, i.e., the filtration speed [mL/min] of primary urine in glomeruli, is generally accepted as the best overall measure of kidney function (European Best Practice Guidelines Expert Group on Hemodialysis, European Renal Association, 2002; National Kidney Foundation, 2002). However, GFR cannot be measured directly. A related quantity, *renal clearance*, is defined and expressed as the volume of plasma that can be completely cleared of a particular substance in a unit of time [mL/min], presuming that the concentration of the substance in circulation is stable (Smith, 1951). As its definition is based on the axiom that the substance is excreted into the urine at an equal rate to its removal from plasma by the kidney, the renal clearance of an ideal filtration marker is equal to GFR (Smith, 1951).

Properties of an ideal marker include that it should be: metabolically inert and non-toxic; in a stable level in the blood; a free molecule not bound to proteins or blood cells; freely filtered by the glomeruli; and eliminated from the blood only by the kidneys. It should not alter the renal function and should not be reabsorbed from the filtrate, or secreted, synthesized or metabolized within the kidney (Smith, 1951; Odlind et al., 1985; Swan, 1997). Although no perfect filtration marker is known, the GFR is traditionally *measured* as the renal clearance of a near-ideal substance (Swan, 1997). Renal clearance of a substance *s* (C_s) is calculated from its steady-state plasma concentration (P) and urinary concentration (U) in a timed volume of urine (V [mL/min]) according to the following equation: $C_s(mL/min)=UV/P$ (Smith, 1951). This formula assumes that the substance is not reabsorbed and/or catabolized in renal tubules after filtration (Smith, 1951). As the GFR varies according to age, gender and body size, it is usually reported as adjusted to a standard body surface area, which is 1.73 m^2 for adults (McIntosh et al., 1928).

For practical reasons, several indirect methods for *estimating* kidney function (GFR) exist. Basically, generation, renal excretion, and extrarenal elimination determine plasma concentrations of endogenous filtration markers (Stevens and Levey, 2009). When kidney function (GFR) decreases, clearance of these substances is slowed down and their blood levels increase. The inverse relationship of GFR and the plasma concentrations of these markers are used to estimate the level of kidney function (GFR).

While the methods approximating GFR give a rough estimate of the number of functioning nephrons, i.e., the overall status of kidney function, increased protein in urine is an indication of kidney damage (National Kidney Foundation, 2002). These different approaches for assessment of kidney function from plasma and urine measurements are shown in Figure 2 and explained in more detail in the following chapters.

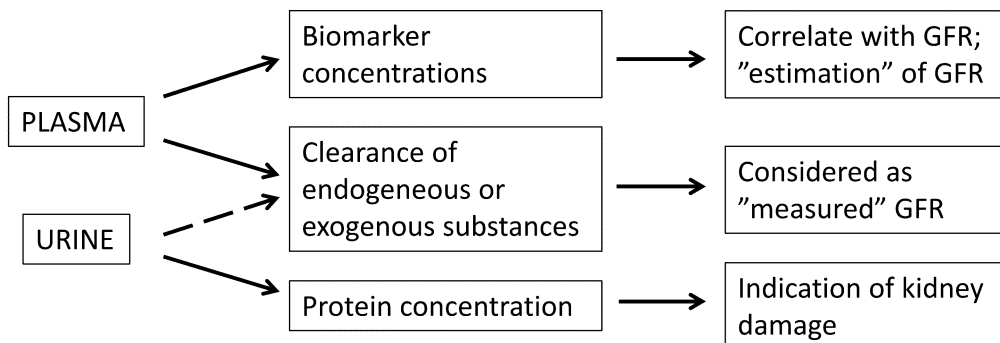


Figure 2. Methods for assessment of kidney function from measurements in plasma and/or urine.

GFR has a remarkable role in the guidance of treatment strategies. It is primarily approximated to evaluate the state of and to detect changes in kidney function. This

information is further used to detect an early reduction in kidney function and to prevent further deterioration, to define the need and frequency for renal replacement therapy, to avoid drug toxicity by adjusting the dosage of drugs cleared by the kidneys, such as aminoglycosides, digoxin and chemotherapy medication, according to GFR, and to identify individuals who should not receive potentially nephrotoxic contrast media (Grubb et al., 2010).

2.2.2 Single serum/plasma measurements of biomarkers

2.2.2.1 Urea and creatinine

Urea, $(\text{NH}_2)_2\text{CO}$ (60 Da), measured from plasma or urine with urease- or diacetylmonoxime-based methods, was the first marker of kidney function (Smith, 1951; Swan, 1997; Francis et al., 2002). It is freely filtered in glomeruli and excreted in urine, being the main waste nitrogen compound excreted by humans (Francis et al., 2002). Urea forms in the liver as the end product of nitrogen metabolism. Circulating urea concentrations are affected by a variety of extrarenal factors, including diet, the body's balance of nitrogen, and liver dysfunction (Smith, 1951). Passive adsorption of urea occurs in renal tubule, and the state of diuresis affects the amount of urea excreted (Smith, 1951). Due to these factors, the results are difficult to interpret and urea is insensitive in detecting kidney disease – it is generally recognized to be a poor marker of kidney function (Swan, 1997). Also guidelines do not recommend urea measurement alone (European Best Practice Guidelines Expert Group on Hemodialysis, European Renal Association, 2002; National Kidney Foundation, 2002). Still, urea or blood urea nitrogen (BUN), possibly as a ratio to creatinine, is used to assess kidney function (Ferguson and Waikar, 2012).

Plasma or serum creatinine (113 Da) is the typically used marker to estimate kidney function (GFR) (Ferguson and Waikar, 2012). Creatinine is produced in the muscle as a metabolic by-product from the nonenzymatic conversion of creatine and phosphocreatine (Borsook and Dubnoff, 1947). Creatinine circulates unbound to plasma proteins, and owing to its small size, it is freely filtered in the glomeruli (Shemesh et al., 1985). Creatinine has relatively minor absorption, is physiologically inert and not metabolized by the kidney. However, tubular secretion of creatinine occurs that is not constant but varies within and between individuals and increases with decreasing kidney function (Berlyne et al., 1964; Carrie et al., 1980; Shemesh et al., 1985). Tubular secretion may lead to overestimation of kidney function determined by serum creatinine. Tubular reabsorption does not normally occur except in aged kidneys (Musso et al., 2009) and with low urine volumes (Chesley, 1938).

As such, plasma concentration of creatinine reflects muscle mass and, due to constant removal by kidneys, varies minimally daily. However, the dependence on muscle mass leads to non-GFR related variation in creatinine levels across age, gender, race, diet, physical activity and body composition (Cockcroft and Gault, 1976; James et al., 1988; Preiss et al., 2007; Baxmann et al., 2008). Thus, the accuracy of creatinine in measuring kidney function is limited especially in older patients due to the combined

effect of decreasing muscle mass (=lower creatinine) and reduced GFR (=higher creatinine). These may compensate, and thus serum creatinine concentration may theoretically remain unchanged despite worsening renal function. A term “creatinine-blind range” refers to the insensitivity of creatinine and creatinine levels may remain in the normal range even until approximately 50 percent of functional nephrons are lost (Swan, 1997). Specifically, this means that creatinine is insensitive to the decrease of GFR at CKD stages 1–3. This may be a limitation of a single (random) creatinine measurement as repeated measurements reflect decreases in GFR (Dalton, 2010). Careful interpretation is, however, needed and creatinine concentration is not recommended to be reported without adjustment to muscle mass-related factors (European Best Practice Guidelines Expert Group on Hemodialysis, European Renal Association, 2002; National Kidney Foundation, 2002). In addition, serum creatinine is elevated in patients with hypothyroidism, and decreased in hyperthyroidism (Verhelst et al., 1997).

Creatinine is routinely measured by modifications of colorimetric alkaline picrate assay first described by Jaffe in 1886 and introduced by Folin in 1914, or by enzymatic or partially enzymatic assays (Myers et al., 2006). The reaction of creatinine with picrate ion under alkaline conditions forms a red-orange complex that is easily detected (Myers et al., 2006). The major analytical limitation of this “Jaffe method” is high vulnerability to sample interference (Myers et al., 2006). Interfering substances either reduce alkaline picrate (e.g., ascorbate, glucose) or react with it to form colored complexes (e.g., ketoacids, proteins) (Weber and van Zanten, 1991; Perrone et al., 1992). The error caused by sample protein may account for 15–25 percent overestimation of serum creatinine (Wuyts et al., 2003; Myers et al., 2006). A kinetic version of alkaline picrate method utilizes different rate of color development of creatinine versus non-creatinine chromogens and allows for a rate-dependent separation of these compounds and reduces positive interferences caused by non-creatinine chromogens (Fabiny and Ertingshausen, 1971). Compensated Jaffe creatinine methods mathematically correct for non-specific reaction caused by pseudo-creatinine chromogens, mainly due to proteins (Boutten et al., 2013). However, bilirubin causes negative interference also in kinetic Jaffe methods (Lolekha et al., 2001).

Enzymatic methods for quantification of creatinine include a reaction of creatinine with creatinine iminohydrolase to form N-methylhydantoin and ammonia – the latter is quantified by reaction with bromophenol blue (Toffaletti et al., 1983). Another “coupled-enzyme” assay is based on a reaction of creatinine with creatinine amidohydrolase and further with creatinine kinase, linked with reactions catalyzed by pyruvate kinase and lactate dehydrogenase – a decrease at 340 nm caused by utilization of NADH is measured (Moss et al., 1975; Jaynes et al., 1982). A third method utilizes creatininase, creatinase and sarcosine oxidase and one of the endproducts, hydrogen peroxidase, is quantified by reaction with 2,4,6-tribromo-3-hydroxybenzoic acid, 4-aminoantipyrine, and peroxidase to yield a purple quinone dye (Fossati et al., 1983). Enzymatic assays are sensitive and specific to creatinine and less affected by

endogenous non-creatinine substances than assays based on the Jaffe reaction (Apple et al., 1986). Due to less analytical interference and variability, enzymatic assays are often recommended over Jaffe methods (Drion et al., 2012; Boutten et al., 2013). However, enzymatic methods are also vulnerable to negative interference from bilirubin (Owen and Keevil, 2007; Greenberg et al., 2012). Furthermore, while in uncompensated Jaffe-based methods, protein error may compensate tubular secretion, in enzymatic methods the absence of protein error may lead to overestimation of GFR, especially in children (Apple et al., 1986; Wuyts et al., 2003).

High inter-laboratory variation in serum creatinine results drove a standardization project started by the National Kidney Disease Education Program's Laboratory Working Group in collaboration with the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) and the European Federation of Clinical Chemistry and Laboratory Medicine (Myers et al., 2006). An international calibrator for standardization of creatinine measurement has been available since 2007 from the National Institute of Standards and Technology: a serum-based Secondary Reference Material (SRM 967) for establishment of calibration traceability to an Isotope Dilution Mass Spectrometry (IDMS) reference measurement procedure (Dodder et al., 2007). Despite the significant efforts put on global restandardization on SRM 967, results among laboratories are not equivalent, which is explained by calibration differences (Delanghe et al., 2008) and by the Jaffe method's non-specificity problems (Drion et al., 2012).

2.2.2.2 Low molecular weight proteins

Low molecular weight proteins such as *beta*-2-microglobulin, *beta*-trace protein, cystatin C and retinol binding protein are potential alternative markers of GFR (Grubb et al., 1985; Hoffmann et al., 1997; Priem et al., 1999; Filler et al., 2002; Donadio, 2010). These proteins are freely filtered in the glomerulus, and subsequently resorbed and degraded by tubular cells. Increased serum concentrations of these proteins are indicative of kidney dysfunction (Maack et al., 1979). There are commercial, mainly nephelometric or turbidimetric, immunoassays available for these LMW proteins (Filler et al., 2002; Donadio, 2010; Astor et al., 2012).

Beta-2-microglobulin (β 2M; 11.8 kDa) is a component of the MHC class I molecule, which is expressed on all nucleated cells (Grey et al., 1973). It may be equal or superior to serum creatinine as a marker of GFR (Bianchi et al., 2001; Donadio, 2010). However, β 2M increases in several malignancies and autoimmune- and inflammatory diseases, which limits its use as a GFR marker (Evrin and Wibell, 1973; Bataille et al., 1984; Filler et al., 2002). Corticosteroids may also affect its concentration (Bokenkamp et al., 2002).

Beta-trace protein (β TP), also known as lipocalin-type prostaglandin D2 synthase, is a 23–29 kDa glycoprotein that is mainly produced in the central nervous system (Nagai et al., 2000). Diagnostic performance of serum β TP is similar or even superior to creatinine in diabetic patients (Priem et al., 1999), children with renal disease (Filler et

al., 2002), renal transplant patients (Poge et al., 2005; Gerhardt et al., 2011), and in non-diabetic and diabetic CKD (Spanaus et al., 2010). However, certain inflammatory states may increase its expression (Hokari et al., 2011) and corticosteroid therapy appears to decrease circulating concentrations of β TP (Abbink et al., 2008). Short-term, intra-person variability of β TP may be higher than that of creatinine and β 2M (Selvin et al., 2013).

Retinol binding protein (RBP) is an approximately 21 kDa protein, mainly secreted by the liver (Ronne et al., 1983), and it has minimal tubular excretion (Kanai et al., 1968; Peterson and Berggard, 1971). RBP forms polymers and binds to other proteins, which limit its accuracy to reflect GFR (Donaldson et al., 1990). The production rate of RBP also decreases due to acute phase response and diet affects its circulating concentrations (Donaldson et al., 1990; Aeberli et al., 2007). RBP is significantly less accurate than serum creatinine and other LMW proteins in CKD patients (Donadio, 2010).

Cystatin C will be thoroughly covered in other chapters of this thesis.

2.2.3 Clearance measurements as determinants of GFR

Clearance of *endogenous* substances, mainly creatinine clearance and urea clearance involving timed urine collection (usually 24 hours) and blood sampling during the collection period are typically assessed to approximate GFR and provide greater accuracy than single measurements of serum creatinine or urea (Smith, 1951; Swan, 1997). The clearance measurements actually preceded the single serum measurements of these endogenous substances (Moller et al., 1928; Miller and Winkler, 1938; Smith, 1951). However, uncertainties with urine collection, especially incomplete collection of the specimen may limit the reliability of these measurements, in addition to the already discussed limitations of urea and creatinine. Despite the known fact that creatinine clearance overestimates GFR by 10–40 percent due to tubular secretion (Berlyne et al., 1964; National Kidney Foundation, 2002) and secretion by small bowel, endogenous creatinine clearance is widely used as a measure of glomerular function (Wuyts et al., 2003). Creatinine methods affected by protein error lead to an underestimation of creatinine clearance due to a remarkable difference in protein concentration of serum and urine. However, protein error may compensate for the overestimated clearance (Apple et al., 1986; Wuyts et al., 2003). To avoid the limitations of each of the markers alone, the European Renal Association recommends using an average of urea and creatinine clearance to determine the level of kidney function in advanced CKD (European Best Practice Guidelines Expert Group on Hemodialysis, European Renal Association, 2002).

Clearance of certain *exogenous* substances is measured by administration of an inert compound not endogenously present in body, either as an intravenous or subcutaneous bolus injection or as a constant infusion, and following the elimination of the substance due to renal clearance in timed urine and/or blood samplings (Gaspari et al., 1995). Inulin (5.2 kDa) is one of the first exogenous clearance markers (Shannon and Smith,

1935). It fulfils nearly all of the criteria for an ideal marker and its urinary clearance using continuous infusion is considered as the gold standard measure of GFR (Breckenridge and Metcalfe-Gibson, 1965).

Isotope-labelled substances, such as ^{125}I -iothalamate (Elwood and Sigman, 1967), chromium ethylenediamine tetracetic acid (^{51}Cr -EDTA) (Chantler et al., 1969), and diethylenetriaminepentaacetic acid ($^{99\text{m}}\text{Tc}$ -DTPA) (Hilson et al., 1976) were more convenient to measure and they subsequently replaced inulin until the introduction of an automated enzymatic assay for inulin (Summerfield et al., 1993). However, the requirements of continuous intravenous infusion, bedrest and sometimes catheterization make urinary inulin clearance impractical for clinical practice. Single injection methods of radioactive markers, where either urinary or plasma clearance is measured, are used (Perrone et al., 1990; Gaspari et al., 1995). The introduction of sensitive high-pressure liquid chromatography (HPLC) methods for detection of nonradioactive compounds enabled X-ray contrast media, such as iothalamate and iohexol, to be used in GFR determination, which avoids the use of radioactive substances (Krutzen et al., 1984; Gaspari et al., 1991). Plasma clearance of iohexol is an increasingly popular method, along with ^{51}Cr -EDTA in Europe (not available in the USA) and ^{125}I -iothalamate in the USA (Brandstrom et al., 1998; Stevens et al., 2008).

Although used as reference procedures for determination of kidney function (GFR), there are several limitations for the use of exogenous substances to estimate clearance. These methods are invasive, as they require intravenous administration and may require radiation exposure. They are also expensive, labor-intensive, time consuming and involve special specimen handling and relatively difficult analytical methods (Swan, 1997; Coll et al., 2000; Huber and Risch, 2005). In addition to known practical limitations, even standard methods show considerable variation. Despite apparently high agreement between the exogenous clearance methods (Elwood and Sigman, 1967; Perrone et al., 1990; Sterner et al., 2008), in stable kidney function, if the clearance of an exogenous substance is performed twice within a short period of time, not all of the second determinations will be within ± 30 percent of the first (Grubb, 2010). For example, in persons with reduced CKD, 8 percent of the second ^{125}I -iothalamate measurements (after 62 days) were reported to be outside ± 30 percent of the first (Kwong et al., 2010). Another concern is tubular secretion of iothalamate (Odlind et al., 1985) – a marker that is used as a reference in high-impact studies, such as Modification of Diet in Renal Disease (MDRD) Study (Levey et al., 1999).

2.2.4 Estimated GFR from formulae

Due to the limitations of creatinine, and to overcome the practical problems encountered with the measurement of clearance, mathematical equations exist, which estimate kidney function based on single serum creatinine concentration measurement, anthropomorphic and demographic data. The most common formulae are Cockcroft-Gault (CG) (Cockcroft and Gault, 1976), and the MDRD Study equation (Levey et al., 1999), usually known in its four-parameter version (National Kidney Foundation, 2002) (Figure 3). The CG formula corrects for age, sex and weight – variables that

estimate muscle mass and creatinine production (Cockcroft and Gault, 1976). As it was generated to estimate the creatinine clearance, it overestimates GFR due to creatinine secretion (Shemesh et al., 1985). Often the value is further adjusted to body surface area calculated by e.g., the DuBois equation (DuBois and DuBois, 1916). The MDRD Study equation corrects for age, sex and race (African-American only) (Levey et al., 1999; National Kidney Foundation, 2002). The original MDRD Study equation has a factor 186, whereas Isotope Dilution Mass Spectrometry calibrated assays should use factor 175 instead (Levey et al., 2006). The same four variables are found in the newer CKD Epidemiology Collaboration (CKD-EPI) equation (Figure 3), which uses a different mathematical model than the MDRD Study equation for estimation of GFR for standardized creatinine (Levey et al., 2009).

MDRD Study equation, four-parameter version:

$$eGFR = 186 \times (SCr(\frac{\mu mol}{L}) \div 88.4)^{-1.154} \times Age^{-0.203} \times (0.742 \text{ if female}) \times (1.212 \text{ if African-American})$$

Cockcroft-Gault equation:

$$CrCl(\frac{mL}{min}) = \frac{(140 - Age) \times Mass(\text{in kg}) \times 1.23 \times (0.85 \text{ if female})}{SCr(\text{in } \frac{\mu mol}{L})}$$

CKD-EPI equation:

$$eGFR = 141 \times \min(SCr/\kappa, 1)^\alpha \times \max(SCr/\kappa, 1)^{-1.209} \times 0.993^{Age} \times 1.018(\text{if female}) \times 1.159(\text{if black}), \text{ where}$$

$\kappa = 0.7 \text{ if female}, 0.9 \text{ if male}$

$\alpha = -0.329 \text{ if female}, -0.411 \text{ if male}$

$min = \text{the minimum of } SCr/\kappa \text{ or } 1$

$max = \text{the maximum of } SCr/\kappa \text{ or } 1$

Figure 3. Equations for estimated glomerular filtration rate (eGFR) based on single serum creatinine measurement in adults. MDRD, Modification of Diet in Renal Disease; eGFR, estimated glomerular filtration rate; SCr, serum creatinine, CrCl, creatinine clearance; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration. Adapted from (Cockcroft and Gault, 1976; Levey et al., 1999; National Kidney Foundation, 2002; Levey et al., 2009).

As creatinine has a strong dependence on age and body composition, these equations are valid for adults only, whereas for children, there are different equations, such as the Schwartz formula (Schwartz et al., 1976), updated after the standardization of creatinine measurements (Schwartz et al., 2009), and the Counahan-Barratt formula (Counahan et al., 1976). Certain conditions, such as pregnancy and extremes of body size preclude the use of any estimating equations. The use of estimating equations also presumes a stable concentration of the marker in serum – in acute kidney injury (AKI), estimated GFR (eGFR) equations perform poorly (Bragadottir et al., 2013).

Although these formulae are better estimates for GFR than serum creatinine alone, concern has been expressed about the uncertainty of eGFR values (Froissart et al., 2005). The CG formula was developed in predominantly hospitalized male patients although widely applied in heterogeneous populations, particularly for drug dosing (Cockcroft and Gault, 1976; Rule, 2010). The MDRD Study equation was developed in CKD patients with high risk of ESRD (mean GFR 40 mL/min/1.73 m²) and its

reliability is known to be limited especially in low-risk patients with GFR greater than 60 mL/min/1.73 m² where a 29 percent underestimation of GFR is reported (Rule et al., 2004). In the original validation of the MDRD Study equation, 91 percent of the eGFR values were within \pm 30 percent of the GFR values measured by a reference clearance method (P₃₀) (Levey et al., 1999). In a larger, cross-sectional analysis, with a more diverse population (n=5504), the MDRD Study equation showed relatively low bias for eGFR less than 60 mL/min/1.73 m². However, for eGFR levels between 60 and 119 mL/min/1.73 m², the MDRD Study equation underestimated and for eGFR greater than 120 mL/min/1.73 m² it overestimated the ¹²⁵I-iothalamate GFR levels, with an overall P₃₀ of 83 percent (Stevens et al., 2007). Considerable overestimation (10.6 mL/min/1.73 m²) was especially observed in younger (<40 years) individuals with higher GFR values. Because of limited accuracy at greater than 60 mL/min/1.73 m², reporting of eGFR-MDRD values is recommended only when less than 60 mL/min/1.73 m² (Levey et al., 2006).

The CKD-EPI equation was developed in mixed and diverse population (n=8254), including more individuals without CKD (mean GFR 68 mL/min/1.73 m²) than the MDRD Study (Levey et al., 1999; Levey et al., 2009). The CKD-EPI equation was externally validated with pooled data from 16 studies (n=3896) (Levey et al., 2009). Less bias was observed at higher GFR values as compared to the MDRD Study equation (median difference between iothalamate GFR and eGFR was 2.5 vs. 5.5 mL/min/1.73 m²) and precision was improved resulting in greater accuracy, P₃₀ 84 percent vs. 81 percent (Levey et al., 2009). Similar results regarding bias have been reported (Schold et al., 2011), and a meta-analysis of direct comparisons of the MDRD Study equation and CKD-EPI equation in adult European-ancestry populations (n>100) concluded that the CKD-EPI equation is superior to the MDRD Study equation in GFR greater than 60 mL/min per 1.73 m², whereas the MDRD performs better than CKD-EPI at lower GFR levels (Earley et al., 2012).

When prevalence of CKD was re-estimated by using the CKD-EPI equation, it decreased from 13.1 percent (MDRD) to 11.5 percent (Levey et al., 2009). Decreased CKD classification was also reported in outpatients of a large healthcare system (Schold et al., 2011) and in the general population (White et al., 2010). Specifically, this decline only occurred in younger patients (<60 years) (Schold et al., 2011). The CKD-EPI development and validation groups only had 12–15 percent of individuals greater than or equal to 65 years (Levey et al., 2009). In elderly population (range: 74–97 years; the median GFR was 53 mL/min/1.73 m²), equivalent performance of the two equations as compared to iothalamate GFR was observed and the accuracy (P₃₀) for the MDRD Study and CKD-EPI equations were 81 percent and 83 percent, respectively (Kilbride et al., 2013).

None of the validated eGFR equations are optimal for all GFR ranges and all populations (Froissart et al., 2005; Poggio et al., 2005; Earley et al., 2012) and the use of estimating equations is scrutinized (Kallner et al., 2008). Although aimed to be more generalizable than the MDRD Study equation, the CKD-EPI authors stated that “a

single equation is unlikely to work equally well in all populations” (Levey et al., 2009). Imprecision of exogenous clearance measurements, differences in creatinine methods and non-GFR determinants of creatinine all contribute to diminished accuracy (Grubb, 2010; Drion et al., 2012).

2.2.5 Proteinuria

Excretion of abnormally high amounts of protein to urine, proteinuria, is a common feature of chronic nephropathies (Lamb et al., 2009). Leakage of proteins to urine may occur by several different mechanisms in a diseased kidney. For example, inflammation in glomeruli may increase the permeability to higher molecular mass proteins, or diseases affecting tubuli may prevent reabsorption of LMW proteins. Various definitions of proteinuria exist. It is measured, for example, by 24-hour excretion (≥ 150 to ≥ 300 mg/day – defining clinical proteinuria), spot urine dipstick (≥ 300 mg/L) or spot urine protein-to-creatinine ratio (≥ 200 mg/g) (National Kidney Foundation, 2002; Lameire et al., 2005a). Proteinuria is often determined by automated methods, such as turbidimetric assays using benzethonium chloride or by dye-binding assays using e.g., pyrogallol red molybdate or pyrocatechol violet dye (Dube et al., 2005), whereas dipstick methods are usually based on dye-binding using bromophenol blue. Urine protein results vary among methods because the specificity of used dyes, and the complexity of proteins makes standardization difficult (Dube et al., 2005). The threshold levels that define clinically significant proteinuria vary (Lamb et al., 2009). High intra-individual variability, exercise, posture, diurnal variation and urinary tract infection are factors that affect urine protein concentration (Mogensen et al., 1995). Collections at 24 hours reduce the possible fluctuation in protein excretion during the day and it is considered as the confirming test for proteinuria (Lameire et al., 2005a). However, in clinical practice, a remarkable number of collections are excluded from analysis because of poor compliance in sampling (Shaw et al., 1983; Price et al., 2005; Gaspari et al., 2006). Measurement of protein or protein-to-creatinine ratio in spot urine specimen is intensively studied. Price et al. reviewed protein-to-creatinine ratio on a random urine specimen and concluded that it can be used to rule out the presence of significant proteinuria (Price et al., 2005). A review by Gaspari and coworkers concluded that the first morning urine specimen would be the most convenient choice (Gaspari et al., 2006). However, some prefer the 24-hour collection (Shidham and Hebert, 2006).

Proteinuria usually refers to loss of total protein, whereas excess excretion of albumin (66.5 kDa) to urine is called albuminuria (Mogensen et al., 1995). The normal mean values for urine albumin and total protein excretion are approximately 10 mg and 50 mg per day, respectively. In addition to albumin and LMW proteins filtered from blood, proteins derived from the urinary tract are major constituents of normal urine. In most types of CKD, albumin is the most abundant protein in urine and albuminuria is an early and sensitive marker of kidney damage (Lamb et al., 2009). With sensitive laboratory methods, predominantly turbidimetric immunoassays, even trace amounts of albumin levels in urine (excretion rate 30–300 mg/d or concentration 30–300 mg/L in a single-voided early morning specimen) can be measured. This is referred to as

microalbuminuria (Mogensen et al., 1995). Albuminuria is however not present in all individuals even with GFR less than 30 mL/min/1.73 m². In US National Health and Nutrition Examination Surveys (NHANES) 1988–1994 and 1999–2004 studies, 63–66 percent of individuals with GFR 15–29 had albuminuria. However, 6–8 percent of individuals with GFR greater than or equal to 90 mL/min/1.73 m² had albuminuria (Coresh et al., 2007).

Urine albumin methods are more robust and uniform than urine total protein methods. A reference system to standardize urine albumin is in development by Working Group of the National Kidney Disease Education Program (NKDEP) and the IFCC (Miller et al., 2009; Lieske et al., 2013). There are, however, several factors affecting the results. Diagnostic methods, especially the antibodies used, sample handling, and timing of the sampling (first morning void; 24-hour collection) affect the results. To overcome some of these challenges, albumin-to-creatinine ratio (ACR) has been suggested, especially as a routine measurement for patients with diabetes (Peralta et al., 2011b). Both urine albumin and urine creatinine demonstrate high within-individual variation (>30%). However, ACR decreases this remarkably (11%) (Selvin et al., 2013). ACR greater than 2.5 mg/mmol in women, greater than 3.5 mg/mmol in men (or 30 mg/g in US) is considered as evidence of (micro)albuminuria, (Coresh et al., 2007; Lamb et al., 2009). While albumin is the main excreted protein in diabetic nephropathy, there is caution about utilizing ACR for management of CKD in non-diabetic patients (National Kidney Foundation, 2002; Methven et al., 2010). Uncertainty about whether total protein-to-creatinine ratio or ACR is a more sensitive screening test to predict clinically relevant proteinuria exists (Methven et al., 2010), especially in those with known or suspected renal disease (Atkins et al., 2003). The diagnostic performance of both proteinuria, predicted with total protein-to-creatinine ratio, and ACR may vary considerably with age and gender. This necessitates careful interpretation of the results (Methven et al., 2010). In the USA, National Kidney Foundation recommends measurement of either ACR or total protein-to-creatinine ratio in single random urine samples for assessment of proteinuria. Persistent proteinuria is considered an indication of CKD stage 1 and 2 (National Kidney Foundation, 2002).

2.3 CYSTATIN C AND ITS USE AS A BIOMARKER

2.3.1 Structure, expression and role of cystatin C

Cystatin C (also cystatin 3; formerly *gamma* trace, post-*gamma* globulin, neuroendocrine basic polypeptide) is a monomeric 13.3 kDa globular, non-glycosylated protein. It has a high isoelectric point (pI=9.3) and in all bodily fluids, is positively charged. The third amino acid residue, proline, is hydroxylated to hydroxyproline in approximately every second cystatin C molecules. No other post-translational modifications occur, physiologically (Grubb and Lofberg, 1982).

Human cystatin C is encoded by the *cst3* gene, which is located in the chromosome 20 (Abrahamson et al., 1989). The transcript contains an N-terminal hydrophobic signal sequence of 26 amino acids that is cleaved before secretion of the 120 amino acid

polypeptide out of cells (Abrahamson et al., 1987). The *cst3*-gene is considered as a housekeeping gene (Olafsson, 1995) and cystatin C is thus produced at a nearly constant rate by all cells with a nucleus and it can be found in virtually all tissues and bodily fluids (Abrahamson et al., 1986; Abrahamson et al., 1990). Its concentration is the highest of all known low-molecular-weight cystatins in most of the extracellular fluids in human, approximately 1–10 mg/L (Poulik et al., 1983; Abrahamson et al., 1986). The concentration of cystatin C in urine is remarkably lower, approximately 0.1 mg/L (Lofberg and Grubb, 1979; Poulik et al., 1983).

Cystatin C belongs to the cystatin gene family, which is divided, based on sequence homology and the presence and position of intradomain disulphide bridges, into type 1 (mainly intracellular), type 2 (extracellular) and type 3 (intravascular; multidomain proteins) cystatins and some unclassified cystatins (Barrett et al., 1986). For type 2 cystatins, such as cystatin C, two stabilizing intramolecular disulfide bridges are typical (Barrett et al., 1986). As typical for monomeric proteins in cystatin family, cystatin C has a long *alpha* 1 helix, which is perpendicular to the five-strand antiparallel *beta* sheet (Janowski et al., 2001).

Cystatins are reversibly binding, non-covalent, competitive inhibitors of cysteine proteinases/peptidases of the papain family (C1), and some – like cystatin C – also inhibit legumain family proteases (C13) (Barrett et al., 1984; Chen et al., 1997). The primary biological function of cystatin C is to inhibit cysteine proteinases of host and microbial origin present in extracellular fluids (Barrett et al., 1984; Abrahamson et al., 1987). In humans, cystatin C is a predominant cysteine protease inhibitor in cerebrospinal fluid (~8 mg/L), milk (~3 mg/L) and seminal plasma (~50 mg/L) (Poulik et al., 1983; Abrahamson et al., 1986). In plasma, kininogen and *alpha*-2-macroglobulin are more abundant cysteine protease inhibitors (Abrahamson et al., 1986).

Cysteine proteases are enzymes responsible for many crucial physiological processes, such as intracellular protein degradation, apoptosis, MHC class II immune responses, prohormone processing and bone remodeling (Chapman et al., 1997). By inhibiting the function of several cysteine proteases, cystatin C participates in the regulation of the balance of catabolism and modulates many of these normal body processes (Abrahamson et al., 1987). Other roles for cystatin C are discussed in literature and they include a role in the atherosclerotic process (Shi et al., 1999), in antigen presentation (Pierre and Mellman, 1998), in defense against bacteria and viruses (Bjorck et al., 1989; Bjorck et al., 1990) and as a growth factor for neural stem cells (Taupin et al., 2000). Cystatin C knock-out mice (*cst3* deletion) were reported to have increased resistance against the metastatic spread of certain cancers (Huh et al., 1999).

Cystatin C is active as a monomer. It may, however, undergo dimerization through domain swapping, and the formed dimer is inactive (Ekiel and Abrahamson, 1996; Janowski et al., 2001). During intracellular trafficking, prior to secretion from the endoplasmic reticulum in a monomeric form, the precursor of cystatin C is transiently dimerized and becomes inactive (Merz et al., 1997).

The role of cystatin C as a biomarker can be divided into three categories based on current knowledge. First of all, cystatin C is a biomarker of kidney function. Secondly, it may play a role in prediction of cardiovascular disease and, thirdly, in neurological disorders. These roles of cystatin C as a biomarker will be covered in more detail in the following sections 2.3.2–2.3.4.

2.3.2 Biomarker of kidney function

2.3.2.1 Catabolism and factors affecting circulating cystatin C

Cystatin C is mainly removed from bloodstream by the kidneys (Abrahamson et al., 1990; Tenstad et al., 1996). As cystatin C is *per se* produced at a constant level, its concentration in the circulation remains nearly stable when kidney function is normal. Consequently, the rate at which cystatin C is filtered at the glomerulus is the primary determinate of blood cystatin C level. Suggested as a marker of GFR in 1985 (Simonsen et al., 1985), serum or plasma cystatin C (later: “cystatin C”) fulfills many criteria that are set for an ideal endogenous biomarker of kidney function. As a low-molecular-weight globular protein with a high isoelectric point, cystatin C is almost freely filtered through the glomerular membrane, and subsequently completely reabsorbed and catabolized by the proximal tubular cells (Grubb et al., 1985; Jacobsson et al., 1995; Tenstad et al., 1996). Plasma clearance of cystatin C is 94 percent to that of ⁵¹Cr-EDTA in rats (Tenstad et al., 1996). No secretion or reabsorption in circulation occurs (Tenstad et al., 1996). Estimates of non-renal clearance of cystatin C are 15 percent in rats (Tenstad et al., 1996) or approximately 20 mL/min/1.73 m² in humans (Sjostrom et al., 2005).

Cystatin C concentration decreases rapidly from birth to 1 year of age (Bokenkamp et al., 1998a), after which it remains reasonably constant up to age of 50 years (Norlund et al., 1997; Finney et al., 2000a; Kottgen et al., 2008). After the age of 50, an increase in cystatin C follows the age-dependent decline in GFR (Norlund et al., 1997; Finney et al., 2000a; Kottgen et al., 2008). The biological variation of cystatin C in healthy subjects is low, approximately 5–7 percent (Toffaletti and McDonnell, 2008; Selvin et al., 2013).

Stevens et al. reported that factors other than GFR, such as DM, measures of body size, and inflammation, affect serum cystatin C levels (Stevens et al., 2009). Although a significant correlation between circulating cystatin C and the degree of inflammation is a common finding (Knight et al., 2004; Koenig et al., 2005; Keller et al., 2007), no causal relationship has been found between cystatin C and inflammation (Grubb et al., 2011). Men have (mildly) higher average cystatin C than women (Finney et al., 2000a; Knight et al., 2004; Groesbeck et al., 2008; Kottgen et al., 2008; White et al., 2009). Ethnicity may also affect the level of cystatin C (Groesbeck et al., 2008; Kottgen et al., 2008). Recently, obesity (defined by body to mass index) has been found to be independently associated with elevated cystatin C concentrations in persons without CKD (Muntner et al., 2008). In another study, eGFR determined by cystatin C was associated with visceral and subcutaneous adipocyte tissue (Young et al., 2008),

perhaps linked to a role of cystatin in adipogenesis (Taleb et al., 2006). However, studies are unanimous that cystatin C is less dependent on factors related to body composition than creatinine (Vinge et al., 1999; Baxmann et al., 2008; Groesbeck et al., 2008; Kottgen et al., 2008; Stevens et al., 2009; White et al., 2009).

There are, however, known factors (other than age) that affect circulating cystatin C concentrations. These include thyroid disorders and corticosteroid use (Risch et al., 2001; Fricker et al., 2003). Cystatin C concentrations are decreased in the hypothyroid and increased in the hyperthyroid state (Fricker et al., 2003; Wiesli et al., 2003). Glucocorticoid therapy increases production of cystatin C and this increase is related to the dose of the glucocorticoid used (Bjarnadottir et al., 1995; Risch et al., 2001). Up to an 80 percent increase of cystatin C secretion by HeLa cells following dexamethasone exposure occurs (Bjarnadottir et al., 1995). Continuous low-dose therapy may not increase the level of cystatin C significantly (Bokenkamp et al., 2002; White et al., 2009), although underestimations of GFR occur in some studies, e.g., with steroid-dependent asthmatic patients (Cimerman et al., 2000). Moderate and high dose glucocorticoids limit the usefulness of cystatin C, e.g., early after kidney transplantation (Poge et al., 2004).

2.3.2.2 Estimated GFR based on cystatin C

While calculation of eGFR from single creatinine measurement and demographic data has become the suggested method to estimate kidney function (GFR), also eGFR formulae for cystatin C exist to aid result interpretation and comparison. The dosing of drugs that are eliminated *via* kidneys is based on GFR. Due to an inverse relationship between cystatin C and GFR, prediction equations usually are of the type $GFR = A \times \text{cystatin C}^{-B}$ (Grubb et al., 2005). Until standardized, equations used are method and laboratory (calibration) specific (Larsson et al., 2004). There are also different equations developed for certain patient groups, such as children or kidney transplant receivers (Le Bricon et al., 2000; Filler and Lepage, 2003; Rule et al., 2006). The first formulae for adults from a single cystatin C measurement (n of development population ≥ 100) are presented in Figure 4.

Early evaluations of the formulae conclude that there is a better performance of the cystatin C-based compared to creatinine-based formulae (Hoek et al., 2003; Grubb et al., 2005; Rule et al., 2006; Flodin et al., 2007). Later studies have judged these formulae by their bias, accuracy and precision. When cystatin C-based formulae are evaluated, typical P₃₀ values are within 80–89 percent (White et al., 2005; Flodin et al., 2007; Nyman et al., 2009) with the goal for accuracy being over 90 percent (National Kidney Foundation, 2002).

PENIA

Hoek equation (Hoek *et al.*, 2003):

$$(GFR, mL/min/1.73m^2) = \frac{80.35}{cystatin\ C} - 4.32$$

Larsson equation (Larsson *et al.*, 2004):

$$(GFR, mL/min) = 77.24 \times (cystatin\ C)^{-1.2623}$$

Rule equation (Rule *et al.*, 2006):

$$(GFR, mL/min/1.73m^2) = 66.8 \times (cystatin\ C)^{-1.30}$$

PETIA

Larsson equation (Larsson *et al.*, 2004):

$$(GFR, mL/min) = 99.43 \times (cystatin\ C)^{-1.5837}$$

Grubb equation (Grubb *et al.*, 2005):

$$(GFR, mL/min/1.73m^2) = 84.69 \times (cystatin\ C)^{-1.680}$$

Flodin equation (Flodin *et al.*, 2007):

$$(GFR, mL/min/1.73m^2) = 79.901 \times (cystatin\ C)^{-1.4389}$$

Figure 4. Equations for estimation of GFR from a single cystatin C measurement in adults. PENIA, particle-enhanced nephelometric immunoassay; PETIA, particle-enhanced turbidimetric immunoassay.

In the general population, cystatin C-based eGFR formulae were reported not to improve estimation of GFR over the abbreviated MDRD Study or CKD-EPI-creatinine formulae (Eriksen *et al.*, 2010). However, by using the same population, the same group modeled eGFR based on cystatin C, creatinine or both and concluded that the combination of creatinine and cystatin C can improve estimation of GFR in normal range compared to the creatinine alone formula (Eriksen *et al.*, 2012). In 2008, the CKD-EPI had provided three equations incorporating cystatin C that were developed utilizing pooled data set of 3 studies (n=1935) (Figure 5) (Stevens *et al.*, 2008). In their internal and external validation data sets, the Levey equation 3 combining cystatin C, creatinine and demographic coefficients, namely age, gender and race was the best in terms of bias (GFR, 0.1 mL/min/1.73 m²), precision (inter-quartile range, IQR, 9.2 mL/min/1.73 m²) and accuracy (P₃₀, 89%) (Stevens *et al.*, 2008).

Levey equation 1:

$$eGFR = 76.7 \times (cystatin\ C)^{-1.19}$$

Levey equation 2:

$$eGFR = 127.7 \times (cystatin\ C)^{-1.17} \times age^{-0.13} \times (0.91\ if\ female) \times (1.06\ if\ black)$$

Levey equation 3:

$$eGFR = 177.6 \times SCr^{-0.65} \times (cystatin\ C)^{-0.57} \times age^{-0.20} \times (0.82\ if\ female) \times (1.11\ if\ black)$$

Figure 5. Chronic Kidney Disease Epidemiological Collaboration (CKD-EPI) formulae for estimation of GFR (in mL/min/1.73 m²). eGFR, estimated GFR; SCr, serum creatinine. Adapted from (Stevens *et al.*, 2008).

The same CKD-EPI formulae have later been re-expressed with standardized cystatin C measurement (Inker *et al.*, 2011b) and, furthermore, the CKD-EPI Investigators published new equations in 2012, presented in Figure 6 (Inker *et al.*, 2012a). The new equations were developed and validated with pooled data sets including participants with diverse clinical characteristics, and thus, the new equations were stated to provide greater clinical applicability than the previous equations from the same group. The

performance of the CKD-EPI 2012 equations in two separate external validation data sets is shown in Tables 3 (adults) and 4 (elderly).

CKD-EPI cystatin C (2012):

$$eGFR = 133 \times \min(\text{cystatin C}/0.8, 1)^{-0.499} \times \max(\text{cystatin C}/0.8, 1)^{-1.328} \times 0.966^{age} \times (0.932 \text{ if female}), \text{ where}$$

$\min(\text{cystatin C}/0.8, 1)$ = the minimum of (cystatin C/0.8) or 1

$\max(\text{cystatin C}/0.8, 1)$ = the maximum of (cystatin C/0.8) or 1

CKD-EPI creatinine-cystatin C (2012):

$$eGFR = 135 \times \min(\text{SCr}/\kappa, 1)^\alpha \times \max(\text{SCr}/\kappa, 1)^{-0.601} \times \min(\text{cystatin C}/0.8, 1)^{-0.375} \times \max(\text{cystatin C}/0.8, 1)^{-0.711} \times 0.995^{age} \times (0.969 \text{ if female}) \times (1.08 \text{ if black}), \text{ where}$$

$\kappa = 0.7$ if female, 0.9 if male

$\alpha = -0.248$ if female, -0.207 if male

$\min(\text{SCr}/\kappa, 1)$ = the minimum of (SCr/ κ) or 1

$\max(\text{SCr}/\kappa, 1)$ = the maximum of (SCr/ κ) or 1

Figure 6. Chronic Kidney Disease Epidemiological Collaboration (CKD-EPI) formulae (2012) for estimation of GFR (in mL/min/1.73 m²) based on cystatin C or cystatin C and creatinine. eGFR, estimated GFR; SCr, serum creatinine. Adapted from (Inker et al., 2012a).

Table 3. Performance of Chronic Kidney Disease Epidemiological Collaboration (CKD-EPI) equations with standardized creatinine and cystatin C measurements in external validation data set (n=1119). P₃₀, estimated GFR values within ± 30% of measured GFR. Modified from (Inker et al., 2012a).

| | Bias (95% CI) (mL/min/1.73 m ²) | Precision (95% CI) (mL/min/1.73 m ²) | Accuracy P ₃₀ (%) |
|--------------------------------------|--|---|------------------------------|
| Creatinine (2009) | 3.7 (2.8–4.6) | 15.4 (14.3–16.5) | 87.2 (85.3–89.1) |
| Cystatin C (2012) | 3.4 (2.3–4.4) | 16.4 (14.8–17.8) | 85.9 (83.8–87.8) |
| Creatinine-cystatin C (2012) | 3.9 (3.2–4.5) | 13.4 (12.3–14.5) | 91.5 (89.8–93.0) |
| Average of creatinine and cystatin C | 3.5 (2.8–4.1) | 13.9 (12.9–14.7) | 91.8 (90.1–93.3) |

Table 4. Performance of Chronic Kidney Disease Epidemiological Collaboration (CKD-EPI) equations in elderly (74–97 years) (n=394). P₃₀, estimated GFR values within ± 30% of measured GFR. Modified from (Kilbride et al., 2013).

| | Bias (95% CI) (mL/min/1.73 m ²) | Precision (95% CI) (mL/min/1.73 m ²) | Accuracy P ₃₀ (%) |
|------------------------------|--|---|------------------------------|
| Creatinine (2009) | 1.7 (0.3–3.2) | 13.1 (11.7–14.6) | 83 (79–87) |
| Cystatin C (2012) | -1.2 (-2.2–0.0) | 14.2 (12.5–15.9) | 86 (82–89) |
| Creatinine-cystatin C (2012) | 0.8 (-0.4–1.9) | 12.7 (11.5–13.9) | 86 (82–90) |

In contrast to using a combined formula, one proposed strategy, the “so-called” Lund-model for estimation of GFR, is to use both cystatin C-based and creatinine-based eGFR equations (Grubb, 2010; Grubb et al., 2012). In situations where the two eGFR values are in agreement, the arithmetic mean should be used. Otherwise, clinical data are evaluated to find a reason for the disagreement, such as reduced muscle mass that might distort the result of the creatinine-based eGFR, or thyroid dysfunction that has

influence upon cystatin C. If an unambiguous reason is found, the eGFR value from the more appropriate equation is used, however, if no obvious exclusion criterion exists, GFR should be measured by the gold-standard clearance procedures (Grubb, 2010; Grubb et al., 2012). The arithmetic mean of cystatin C-based and creatinine-based formula performs equally well as some more complex equations combining cystatin C and creatinine (Nyman et al., 2009). Recently, Schwartz et al. suggested a similar approach to estimate GFR in children: univariate equations (height/SCr and cystatin C) are compared and eGFR is determined as explained above (Schwartz et al., 2012).

2.3.2.3 Overview of clinical utility

Early studies in the mid-1980's demonstrated that serum cystatin C was a potential marker for GFR compared to creatinine and a better marker than other LMW proteins including β 2M, RBP and complement factor D (Grubb et al., 1985; Simonsen et al., 1985). Since then, numerous studies have evaluated the usefulness of cystatin C as a marker of GFR, mostly compared to creatinine. The main differences of the two markers are summarized in Table 5. Notably, due to the strong relationship of muscle mass to creatinine concentrations, cystatin C could be a more reliable marker in persons with extremes of muscle mass *per se*, including children, the elderly and in clinical conditions that negatively affect muscle mass (Bokenkamp et al., 1998b; Fliser and Ritz, 2001; Thomassen et al., 2002).

Table 5. Comparison of cystatin C and creatinine as markers of GFR.

| | Cystatin C | Creatinine |
|------------------------------------|--|--|
| Site of production | All nucleated cells (direct gene product) | Muscle |
| Main route of elimination | Glomerular filtration, catabolized in proximal tubules | Glomerular filtration, tubular secretion |
| Known factors affecting production | Thyroid function, glucocorticoids | Muscle mass, thyroid function |
| Other sources | - | Diet: e.g., cooked meat |
| Reference ranges | Different reference ranges outside 1–50 years | Changes over age; various different reference ranges |
| Measurement | Immunoassay | Colorimetric or enzymatic assay |

A meta-analysis in the early 2000's demonstrated that cystatin C was superior to creatinine as a marker for the detection of reduced kidney function with standard clearance methods used for reference (Dharnidharka et al., 2002). This was based on analysis of 46 studies and 8 other data sets. The reciprocal of serum cystatin C ($1/\text{cystatin C}$) had a higher average correlation coefficient, 0.816 vs. 0.742 ($P < 0.001$), and a higher mean Receiver Operating Characteristic (ROC) plot Area under the Curve (AUC) value, 0.926 vs. 0.837 ($P < 0.001$), compared to the reciprocal of serum creatinine, respectively, indicating a greater identity of $1/\text{cystatin C}$ with GFR measured by the reference clearance methods (Dharnidharka et al., 2002). Similarly, Laterza et al. reviewed studies that had compared cystatin C and creatinine as markers of GFR and concluded that cystatin C performs at least equally to serum creatinine and may be superior to creatinine in specific patient populations (Laterza et al., 2002). They listed 24 studies that had examined clinical utility. Fifteen of these studies

concluded that cystatin C is superior to serum creatinine and 9 concluded that cystatin C is not superior. When studies providing sensitivity and specificity data for detecting impaired GFR were combined, the AUCs under the summary ROC curves were 0.95 and 0.91 ($P=0.003$) for cystatin C and creatinine, respectively (Laterza et al., 2002). A recent meta-analysis of diagnostic accuracy in 27 study population groups by Roos et al. showed higher pooled sensitivity (81%) but similar specificity (88%) of cystatin C compared to creatinine (sensitivity 69%, specificity 88%) in predicting reduced GFR (Roos et al., 2007). The diagnostic odds ratio was higher for cystatin C, indicating better discrimination, but the confidence intervals (CI) were overlapping, 54.001 (95% CI 30.175–96.641) vs. 16.297 (8.348–31.785) for cystatin C and creatinine, respectively (Roos et al., 2007).

Although comparisons between cystatin C and creatinine concentrations are criticized for not accounting for the various reference limits of creatinine for different age and gender groups (Deinum and Derkx, 2000), comparisons of sensitivity have concluded better (Perkins et al., 2005; Pucci et al., 2007) or equal (Hoek et al., 2003) performance of cystatin C against creatinine-based eGFRs that correct for age and gender. Another potential limitation of comparisons is that the cut-off GFR value for renal dysfunction varies especially in the early studies exploring clinical utility of cystatin C, being mostly in the range from 60 to 90 mL/min/1.73 m² (Laterza et al., 2002). The introduction of CKD classification by the US National Kidney Foundation has mostly unified the used limit internationally to 60 mL/min/1.73 m² (National Kidney Foundation, 2002).

Due to inexpensive measurement of serum creatinine, cystatin C is often useful in specific populations where creatinine concentrations or creatinine-based eGFR values might obviously lead to erroneous results and these are presented below. Moreover, cystatin C is recommended for use as a confirmatory test for CKD in those with creatinine-based eGFR 45–59 mL/min/1.73 m² (Inker et al., 2012a).

2.3.2.4 Clinical utility in different patient groups

Plasma cystatin C reflects the maturation of the kidneys below one year of age, after which it stays nearly constant, whereas the concentration of plasma creatinine is affected by an increase in muscle mass (Figure 7) (Finney et al., 2000b). Increases in muscle mass is often compensated by correcting creatinine levels for height in children (Counahan et al., 1976; Schwartz et al., 1976; Schwartz et al., 2009). Use of methods for measuring true GFR in children is limited due to their invasive nature. Cystatin C-based eGFR can accurately estimate GFR (^{99m}Tc-DTPA) in children with high risk of kidney disease (Nehus et al., 2013). Several studies have found cystatin C to be diagnostically (e.g., bias, accuracy, precision, sensitivity) superior to creatinine in pediatric populations with various conditions (Bokenkamp et al., 1998b; Filler et al., 2002; Filler and Lepage, 2003; Grubb et al., 2005), although some have not observed a superiority over creatinine (Willems et al., 2003) or suggest estimation of GFR by combining the information of both markers (Bouvet et al., 2006). Cystatin C is also a potential marker of kidney function in neonates. Cystatin C does not cross the placental

barrier and maternal and neonatal serum cystatin C concentrations do not correlate (Cataldi et al., 1999).

In the elderly, loss of muscle mass (sarcopenia) and poor nutrition may mask the increase of creatinine due to declining GFR. As a result, a normal creatinine level cannot exclude significant kidney insufficiency (O'Riordan et al., 2003). Drug-dosage errors are a notable consequence of unrecognized kidney impairment (O'Riordan et al., 2003; Hallberg et al., 2004). Creatinine is usually used for this purpose, but even eGFR, based on creatinine, may differ significantly when different equations are used (Gill et al., 2007). Cystatin C can potentially estimate a steeper decline of GFR than creatinine and thus be a more sensitive marker of GFR (Shlipak et al., 2009a). Many studies comparing cystatin C to creatinine or creatinine-based eGFR in the elderly suggest a higher sensitivity of cystatin C (Finney et al., 1999; Fliser and Ritz, 2001; O'Riordan et al., 2003).

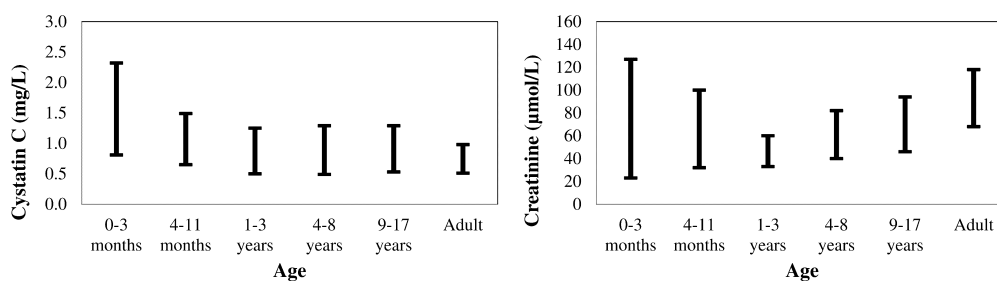


Figure 7. Examples of 95% reference intervals for cystatin C and creatinine in children. Adult reference ranges are included for comparison. Reference ranges from (Finney et al., 2000b).

In patients with spinal cord injury, cystatin C was better than creatinine or eGFR calculated by the MDRD Study equation when compared to ^{51}Cr -EDTA clearance in two separate studies, but no significant difference was found between cystatin C and 24-hour creatinine clearance (Thomassen et al., 2002; Erlandsen et al., 2012). According to recent studies, in patients with HIV infection, cystatin C-based CKD-EPI formula does not offer a more accurate estimation of GFR than CKD-EPI-creatinine (Inker et al., 2012b; Gagneux-Brunon et al., 2013). Other conditions where cystatin C may be a better estimate for GFR include spina bifida in children (Pham-Huy et al., 2003; Morgan et al., 2008), Duchenne muscular dystrophy (Viollet et al., 2009) and certain other chronic diseases such as heart failure (Damman et al., 2012).

Serum creatinine is known to overestimate GFR in patients with liver cirrhosis, which is a condition relatively often accompanied by progressive deterioration in kidney function (Sherman et al., 2003). Cystatin C has higher sensitivity than creatinine for detecting reduced GFR in cirrhotic patients (Woitak et al., 2000; Demirtas et al., 2001; Orlando et al., 2002). However, when eGFRs based on cystatin C or creatinine were evaluated in cirrhosis patients, results were not unanimous – cystatin C had less bias and better precision in one study (Poge et al., 2006a) but poor agreement with ^{51}Cr -EDTA clearance and equal performance with eGFR-MDRD in another study (Xirouchakis et al., 2011). Mindikoglu et al. found that the CKD-EPI equation,

combining creatinine and cystatin C, was superior to other studied eGFR equations, including CKD-EPI formulae with creatinine or cystatin C alone, but the performance was worse than reported in non-cirrhotic subjects (Mindikoglu et al., 2013).

Diabetes causes a high risk for CKD. Estimation of GFR with creatinine in this group is challenging due to variations in body weight according to the type of DM, modifications in nutrition and loss of lean mass with age (Rigalleau et al., 2011). Early changes in diabetic nephropathy include elevated capillary volume causing hyperfiltration (Osterby et al., 1988). eGFR, calculated from creatinine in children, is affected by hyperfiltration, unlike eGFR based on cystatin C (Huang et al., 2011). Cystatin C accurately detects early decline in renal function in DM compared to enzymatically measured creatinine (Christensson et al., 2004) or to creatinine-based estimates of GFR (Perkins et al., 2005; Pucci et al., 2007). Cystatin C followed the decline in renal function of DM patients as measured by iothalamate clearance ($r=0.77$), whereas eGFRs based on creatinine did not ($r<0.35$) (Perkins et al., 2005). Separate studies reported cystatin C to parallel changes in GFR better than eGFR-CG in type 2 DM (Hoek et al., 2003) and in type 1 DM (Premaratne et al., 2008). Cystatin C was also reported to improve estimation of GFR and the diagnosis and stratification of CKD in diabetic patients compared to eGFR calculated by using CG and MDRD Study equations (Rigalleau et al., 2008). Many studies have concluded better diagnostic performance of cystatin C in DM patients using standard clearance methods as the reference (Mussap et al., 2002; Tan et al., 2002; MacIsaac et al., 2007), although cystatin C was not always more sensitive than creatinine or β 2M for detecting mild kidney impairment (Odozo et al., 2001).

In contrast to CKD, reliable detection of rapid changes of kidney function is needed in AKI. Several studies show that serum cystatin C is an early and reliable marker of AKI in patients in intensive care units. This enables the diagnosis of AKI even 1–2 days earlier than with serum creatinine (Delanaye et al., 2004; Herget-Rosenthal et al., 2004; Nejat et al., 2010). Shorter half-lives and smaller volume of distribution favor improved accuracy of cystatin C as a marker of rapidly changing GFR compared to creatinine (Sjostrom et al., 2005; Inker and Okparavero, 2011). There are, however, new markers, such as urinary neutrophil gelatinase-associated lipocalin and kidney injury molecule-1 that may provide significantly earlier and cause-specific diagnosis of AKI (Nickolas et al., 2012).

During uncomplicated pregnancy, GFR progressively increases to over 40 percent higher values than in nonpregnant women (Davison, 1983). Serum levels of cystatin C and other 10–30 kDa molecules are not decreased in the first and second trimester, unlike creatinine, and their concentration increases in the third trimester, possibly reflecting glomerular changes caused by endotheliosis (Strevens et al., 2002; Strevens et al., 2003; Kristensen et al., 2007). The significant association of cystatin C level with structural renal changes may explain the reported superior diagnostic accuracy of cystatin C compared to creatinine for preeclampsia – a complication in pregnancy that

could lead to kidney impairment and is associated with fetal and maternal morbidity and mortality (Strevens et al., 2001; Strevens et al., 2003).

In renal transplant patients, early detection of decreased renal function is crucial so that measures to prevent further decrease of graft function can be taken. During the early post-transplantation period, serum cystatin C decreases more rapidly than creatinine (Le Bricon et al., 1999; Christensson et al., 2003). Immunosuppressive therapy, especially glucocorticoids, increase cystatin C concentrations and may lead to underestimation of GFR (Bjarnadottir et al., 1995; Risch et al., 2001). However, in stable renal graft recipients, despite low-dose immunosuppressive therapy, cystatin C correlates strongly with GFR measurement and detects GFR impairment earlier than creatinine or creatinine-based eGFR (Le Bricon et al., 1999; Risch et al., 1999; Christensson et al., 2003; White et al., 2005; Poge et al., 2006b; White et al., 2007; Maillard et al., 2008). Recently, CKD-EPI formulae were compared for their accuracy of estimating GFR, determined by the gold standard – inulin clearance, in adult kidney transplant recipients (n=670) with stable kidney function (Masson et al., 2013). This multicenter study used centralized, standardized assays for cystatin C and creatinine. Despite immunosuppressive treatment, formulae based on cystatin C and the combination of cystatin C and creatinine were less biased, more accurate and precise than the CKD-EPI-creatinine formula (Masson et al., 2013).

Cystatin C equations seem to perform well also after liver transplantation (Wagner et al., 2012) but not necessarily better than the best creatinine-based formulae (Boudville et al., 2009). In a study of liver-transplanted pediatric patients (n=48), Berding et al. concluded that if radiotracer methods cannot be used, cystatin C is a better choice than creatinine (Schwartz eGFR) for screening GFR (Berding et al., 2010).

2.3.3 Role in assessment of risk for adverse cardiac events and mortality

In recent years, cystatin C has extensively been studied as a potential risk predictor of adverse cardiac outcomes and mortality. The association of cystatin C to these events in different subpopulations is described below.

2.3.3.1 Risk assessment in different subpopulations

Patients with diagnosed cardiovascular disease:

Early risk stratification of the heterogeneous group of patients with non-ST-elevation acute coronary syndrome (nSTE-ACS) may benefit from biochemical markers not evolving from myocardial damage. Cystatin C is one of these potential biochemical indicators. In patients hospitalized with suspected or confirmed nSTE-ACS (n=726), elevated cystatin C was independently associated with death (median follow-up: 40 months) but not with subsequent myocardial infarction (MI) (follow-up: 6 months) (Jernberg et al., 2004). Elevated cystatin C concentrations were associated with increased mortality rate. After adjustment for well-known predictors of outcome, patients in the highest cystatin C quartile (≥ 1.25 mg/L) were at the higher risk for death (Hazard ratio (HR), 4.28; 95% CI, 1.64–11.2) but not for subsequent MI. When

compared to creatinine and eGFR-CG, cystatin C discriminated more precisely between survivors and nonsurvivors (Jernberg et al., 2004). In patients from the Mediterranean, who had nSTE-ACS (n=525), high cystatin C was independently associated with the composite endpoint of cardiac death, non-fatal MI and unstable angina at 1-year follow-up (highest quartile adjusted HR, 1.57; 95% CI, 1.04–2.49), whereas creatinine and eGFR-MDRD were not found to predict the composite endpoint (Taglieri et al., 2010). In a Dutch Invasive versus Conservative Treatment in Unstable Coronary Syndromes (ICTUS) trial, high concentrations of cystatin C were found to independently predict increased risk of 4-year mortality (highest tertile HR, 2.04; 95% CI, 1.02–4.10; $P=0.04$) and recurrent spontaneous MI at 3 years (HR, 1.95; 95% CI, 1.05–3.63; $P=0.04$) in nSTE-ACS patients with elevated cardiac troponin T (Windhausen et al., 2009). Åkerblom et al confirmed that cystatin C independently predicted cardiovascular death or MI in a larger study population (n=8053) with nSTE-ACS (HR per increase of one SD in cystatin C level, 1.12; 95% CI, 1.04–1.20) (Åkerblom et al., 2012).

In a more heterogeneous population of 1033 patients with CAD (aged 30–70 years), elevated cystatin C was associated with a combined endpoint of fatal and nonfatal secondary CVD events at a mean follow-up of 33.5 months (Koenig et al., 2005). Creatinine and eGFR-CG were not associated with the study endpoint. In multivariate analysis, patients in the highest cystatin C quintile had a significantly increased risk for CVD events (HR, 2.27; 95% CI, 1.05–4.91), which was not weakened after adjustment for eGFR-CG (Koenig et al., 2005). Later, the same group published a comparison of creatinine and cystatin C based eGFR formulae in the same setting (mean follow-up: 63.4 months). Cystatin C-based eGFRs were associated with subsequent CVD events after adjustment for multiple covariates, whereas creatinine-based eGFRs were not (Zhang et al., 2010). In ambulatory persons with CAD (82% of male gender), individuals in the highest cystatin C quartile were at 3.6-fold increased risk (95% CI, 1.8–7.0) of all-cause mortality compared to persons in the lowest quartile (Ix et al., 2007). Similarly, a 2-fold risk of CVD events (95% CI, 1.0–3.8) and 2.6-fold risk of incident HF (95% CI 1.0–6.9) were reported after adjustment for traditional cardiovascular risk factors (Ix et al., 2007). In the highest cystatin C quartile, the risk for these adverse outcomes was similar among persons with eGFR-MDRD less than or equal to 60 mL/min/1.73 m² or higher than 60 mL/min/1.73 m² and among persons with or without microalbuminuria (Ix et al., 2007).

In patients (n=1827, 81% males) with stable CAD or ACS and normal or mildly reduced kidney function (eGFR-MDRD >60 mL/min/1.73 m²), (standardized) cystatin C, unlike creatinine, was associated with cardiovascular death during a median follow-up of 3.7 years (adjusted HR, 1.55; 95% CI, 1.25–1.92) (Keller et al., 2009). Sex-specific quartiles of the markers were used to consider the effect of gender for creatinine levels. Keller and coworkers noticed a potential threshold effect as cystatin C concentrations in the highest quartile clearly had the highest number of endpoints compared to lower quartiles – the risk of cardiovascular mortality was 3.87-fold (95% CI, 2.33–6.42) in the highest quartile compared to the pooled lower quartiles; this

association was slightly attenuated in the fully adjusted [age, sex, body to mass index, smoking, DM, hypertension, LDL/HDL ratio, C-reactive protein (CRP), N-terminal proBNP (NT-proBNP)] model (HR, 2.91; 95% CI, 1.67–5.05) (Keller et al., 2009).

Arimoto et al. found increased serum cystatin C levels in patients with HF (n=140) (Arimoto et al., 2005). The cardiac event rate was reported to be markedly higher in those with an elevated cystatin C concentration (>1.0 mg/L) than in those with a normal level. This was also observed in a subgroup of patients (n=91) with normal creatinine levels. A change of 1 SD in cystatin C concentration remained an independent predictor for cardiac events (HR, 1.94; 95% CI, 1.29–6.64, $P=0.0099$) in multivariate analysis (Arimoto et al., 2005). Lassus and coworkers found an increased mortality risk in acute HF patients with elevated cystatin C and normal plasma creatinine levels (Lassus et al., 2007). The 12-month risk in this group was 40.4 percent and it was significantly higher ($P<0.0001$) than the 12.6 percent risk in those with normal cystatin C and creatinine. Overall, there was a significant increase in mortality rate for each tertile of cystatin C, and categorization of patients into nine groups according to combined cystatin C and NT-proBNP tertiles further improved the risk stratification (Lassus et al., 2007). The evidence for the superiority of cystatin C compared to creatinine and eGFR-MDRD and complementary prognostic information of cystatin C and NTpro-BNP in acute HF was strengthened by Manzano-Fernandez et al. (Manzano-Fernandez et al., 2009). They also included cardiac troponin T (cTnT) in the analysis – all of these three biomarkers provided independent prognostic information.

Among patients hospitalized with acute decompensated HF (n=220), cystatin C, unlike creatinine, eGFR-MDRD and BUN, was a significant predictor of mortality and/or HF hospitalization (HR, 1.50; 95% CI, 1.13–2.01; $P=0.006$) during follow-up period with a median of 500 days (Manzano-Fernandez et al., 2011). Also β Tp was a significant predictor of study endpoints. Even in patients with an eGFR-MDRD >60 mL/min/1.73 m², elevated levels of cystatin C (>1.05 mg/L) and β Tp (>0.96 mg/L) were associated with significantly increased risk of adverse outcomes (log-rank test $P<0.001$ and $P=0.021$, respectively) (Manzano-Fernandez et al., 2011).

Older adults:

Comparison of cystatin C and creatinine as risk factors for adverse outcomes in a community-based cohort of older adults (≥ 65 years; n=4637) of Cardiovascular Health Study (CHS) showed different associations of the markers with death from all causes. Cystatin C was directly associated, whereas creatinine and eGFR-MDRD had a more “J-shaped” association with the mortality risk (Shlipak et al., 2005). Within each quintile of creatinine, higher cystatin C levels were associated with increased mortality (Shlipak et al., 2005). Another report from the same investigators showed that quintiles of cystatin C predicted a stepwise increased risk for HF in older adults without previous HF (n=4384) (Sarnak et al., 2005). Serum creatinine and eGFR-MDRD were not independent risk factors for HF; hazard ratios (HRs) in fifth vs. first quintile (95%

CI) were 2.16 (1.61–2.91), 1.14 (0.87–1.49) and 1.26 (0.99–1.61) for cystatin C, creatinine and eGFR, respectively, in the fully adjusted model (Sarnak et al., 2005).

Several other reports from the CHS investigators exist. Fried et al. found that quartiles of cystatin C were associated with non-cardiovascular death – mortality rates increased from 1.7 percent per year in the lowest quartile to 5 percent per year in the highest quartile (Fried et al., 2005). After adjustment for demographic factors such as CRP, hemoglobin, and the presence of CVD or DM, HR in fourth vs. first quartile was 1.69 (95% CI, 1.33–2.15) (Fried et al., 2005). Among those without clinical CKD (defined as eGFR-MDRD ≥ 60 mL/min/1.73 m²; n=3659), cystatin C was a prognostic biomarker of risk for death and CVD, after adjustment for a wide range of risk factors, during a median follow-up of 9.3 years, whereas creatinine level only predicted cardiovascular mortality (Shlipak et al., 2006a). Furthermore, patients, who progressed to CKD, had a significantly higher risk of death, cardiovascular death and HF. The CHS investigators concluded that cystatin C identifies a state of preclinical kidney disease, which is associated with adverse outcomes and not detected with creatinine or eGFR-MDRD (Shlipak et al., 2006a). Rifkin et al. reported that without clinical CKD (eGFR-MDRD >60 mL/min/1.73 m²), abnormal levels of cystatin C were also associated with risk of incident HF in addition to risk of all-cause and cardiovascular mortality within 8.3 years median follow-up (Rifkin et al., 2010). They used samples (n=3291) from the 1996–1997 annual visit of CHS participants and included microalbuminuria, measured as ACR, in the analysis. Abnormal cystatin C and microalbuminuria identified different subsets of the study population and they were concluded to have independent roles in risk prediction (Rifkin et al., 2010).

In diabetic, elderly persons (n=691) of the CHS study, eGFR-cystatin C was more strongly associated with all-cause mortality than eGFR-MDRD (de Boer et al., 2009). Interestingly, a within-individual change in eGFR-cystatin C over time was not a stronger predictor of mortality than a single baseline cystatin C measurement, HR 1.73 (95% CI, 1.37–2.18) vs. 1.57 (1.22–2.01) for eGFR less than 60 mL/min per 1.73 m² and greater than or equal to 3 mL/min per 1.73 m²/year, respectively, suggesting that a single baseline measurement of cystatin C carries prognostic information on its own, regardless of prior context (de Boer et al., 2009).

In octogenarians of the CHS study (n=1053), the lowest eGFR-cystatin C quintile (<52 mL/min/1.73 m²) calculated with one-variable Levey formula (CKD-EPI; Levey equation 1 in Figure 5) was associated with increased all-cause mortality (HR, 2.04; 95% CI, 1.12–3.71) compared to the highest quintile (Shastri et al., 2012). eGFR calculated with CKD-EPI-creatinine had a U-shaped association with mortality as the lowest and the highest quintiles were independently associated with the endpoint (Shastri et al., 2012).

In well-functioning ambulatory elderly of the Health, Aging, and Body Composition Study (n=3075; aged 70 to 79 years; follow-up: 6 years) cystatin C was independently associated with all-cause and cardiovascular mortality (Shlipak et al., 2006b). The

mortality rates increased from 1.7 percent to 5.4 percent per year from the lowest to the highest quintile of cystatin C. After multivariate adjustment [demographic risk factors, comorbid health conditions, and inflammatory biomarkers: CRP, interleukin 6, tumor necrosis factor α], HRs (95% CIs) for all-cause mortality were 1.74 (1.21–2.50), 1.51 (1.05–2.18), 1.49 (1.04–2.13) and 2.18 (1.53–3.10) for cystatin C quintiles 2 to 5, respectively, with quintile 1 as referent (Shlipak et al., 2006b).

In elderly, primary care patients with symptoms of HF (n=464), the highest quartile of cystatin C levels had increased risk of cardiovascular mortality as compared to others (HR, 2.92; 95% CI, 1.23–4.90) (Alehagen et al., 2009). Combining the quartiles of cystatin C and NT-proBNP markedly improved risk stratification – patients with both cystatin C and NT-proBNP levels within the highest quartile had over 13-fold risk for cardiovascular mortality during the 10 year follow-up period as compared to those with the levels within the lowest quartiles (Alehagen et al., 2009).

Established CKD:

Menon et al. evaluated the prognostic role of cystatin C for long-term outcomes in 825 non-diabetic adults (aged 18 to 70) with established CKD at stage 3 or 4 from the MDRD Study equation. In these patients, the reciprocal of cystatin C level was associated with all-cause and CVD mortality equal to GFR measured as iothalamate clearance (Menon et al., 2007). The multivariate-adjusted risks for all-cause mortality were 1.27 (95% CI, 1.08–1.49), and 1.41 (1.18–1.67), for one SD decreases in iothalamate GFR and 1/cystatin C, respectively, and 1.28 (1.04–1.59), and 1.64 (1.28–2.08) for CVD death, respectively. The reciprocal of creatinine predicted outcome similarly to iothalamate GFR (Menon et al., 2007). Peralta et al. found that one-variable Levey cystatin C formula (CKD-EPI) and Levey creatinine formula (CKD-EPI 2009) differently classified individuals as having CKD (Peralta et al., 2011a). The risk of mortality, CVD and HF was not significant in those having CKD by creatinine-based formula only, whereas those having CKD either by cystatin C-based formula or by both formulae had increased adjusted risk compared to those without CKD (Peralta et al., 2011a).

General population:

In a nested, case-control study within the Prospective Epidemiological Study of Myocardial Infarction (PRIME Study) including 9758 adult men, aged 50 to 59 years without CAD on entry, plasma cystatin C was associated with the occurrence of first ischemic coronary event within 5-years follow-up (Luc et al., 2006). The association remained significant after adjustment for traditional risk factors [age, DM, smoking, hypertension, body to mass index, triglycerides, LDL- and HDL-cholesterol], but not after inclusion of CRP in the model. The investigators concluded CRP to be more discriminative in predicting coronary risk than cystatin C, but suggested that cystatin C may participate in the inflammatory process in atherosclerotic lesions due to its strong correlations with inflammatory markers (Luc et al., 2006).

Astor and coworkers first reported better performance in prediction of mortality if eGFR formula based on cystatin C alone was used compared to an equation combining cystatin C and creatinine in the general population (Astor et al., 2009). Equations based on creatinine alone showed weaker associations with increased risk for all-cause and cardiovascular mortality. At higher levels of eGFR, the investigators observed a reverse association between creatinine-based eGFR and all-cause mortality (Astor et al., 2009).

Peralta et al. evaluated the combination of creatinine (CKD-EPI 2009 formula), cystatin C (one-variable Levey CKD-EPI formula) and ACR for improvement of identification of risks associated with CKD compared to creatinine alone in a large cohort (n=26643) of US adults participating in the Reasons for Geographic and Racial Differences in Stroke study (Peralta et al., 2011b). Adding cystatin C to the combination of creatinine and albuminuria improved the identification of persons with increased risk for all-cause mortality (Peralta et al., 2011b). Furthermore, Astor et al. compared cystatin C, *beta*-trace protein and β 2M to eGFR-creatinine (CKD-EPI 2009) as predictors of mortality, CAD, and HF in 9988 participants of the Atherosclerosis Risk in Communities Study, a prospective, observational cohort of individuals aged 45–64 years (Astor et al., 2012). For all outcomes, the LMW proteins predicted study endpoints better than eGFR-creatinine, and they significantly improved risk prediction when added to models including eGFR-creatinine and all covariates, such as ACR.

A recent meta-analysis of 11 general population studies (n=90750) compared the association of eGFR-creatinine, eGFR-cystatin C, and eGFR. The analysis combined creatinine and cystatin C (all calculated by the newest CKD-EPI formulae utilizing standardized values) with the risk of all-cause mortality, mortality from CVD causes and kidney failure (ESRD) (Shlipak et al., 2013). The eGFR-cystatin C yielded notably a higher prevalence of eGFR less than 60 mL/min/1.73 m² than eGFR-creatinine (13.7% vs. 9.7%). The reclassification of the eGFR with the measurement of cystatin C, as compared to creatinine, improved the prediction of risks for mortality and kidney failure (Shlipak et al., 2013).

2.3.3.2 Factors accounting for the improved risk assessment

As described above, compared to other markers of diminished kidney function, primarily creatinine or creatinine-based eGFR, cystatin C has shown superior performance for prediction of all-cause mortality, CVD events and incident HF. The association of cystatin C was extended to normal range of creatinine or eGFR-MDRD. Increased mortality or CVD events in persons with normal serum creatinine or eGFR based on creatinine but increased cystatin C has been found in several studies (Arimoto et al., 2005; Shlipak et al., 2005; Shlipak et al., 2006a; Lassus et al., 2007; Rifkin et al., 2010).

Several potential explanations can be found in the literature for the improved risk prediction of cystatin C compared to creatinine or creatinine-based estimates of GFR. Cystatin C potentially estimates true GFR more precisely, especially in the near-normal

range and thus improves early risk stratification (Dharnidharka et al., 2002; Kuan et al., 2005; Shlipak et al., 2006a). The CHS investigators concluded that cystatin C identified a state of preclinical kidney disease, which is associated with adverse outcomes and not detected with creatinine or eGFR-MDRD (Shlipak et al., 2006a). As this has not been seen in all studies/populations, there might be factors other than GFR that contribute to the improved prediction of outcome, but these are not completely understood (Menon et al., 2007; Eriksen et al., 2010; Rule et al., 2013). One such explanation is that cystatin C is affected by systemic inflammation (Knight et al., 2004). Cystatin C is involved in the atherosclerotic process, by inhibiting elastolytic proteases (Liu et al., 2004), and increased concentrations of cystatin C associate with increased left ventricular mass and its concentricity (Patel et al., 2009). Differential effects of non-GFR determinants on concentrations of creatinine and cystatin C may explain the difference. Stevens et al. showed an opposite direction of the relationships of known risk factors for mortality [DM, higher CRP, higher white blood cell count, lower serum albumin] to the levels of creatinine and cystatin C. These risk factors were associated with higher cystatin C levels but lower creatinine levels (Stevens et al., 2009). Although studies of risk prediction have been adjusted for many of these factors, residual effects might have been present due to errors in measurement (Stevens et al., 2009).

One common limitation related to practical reasons is that most of the studies lack GFR measured with the reference clearance methods. The lack of information on albuminuria, which is known to be a strong risk factor for CVD and mortality, questions the definitions of “pre-CKD” in several studies. However, in studies incorporating albuminuria, it has either not limited the predictive potential of the highest cystatin C concentrations (Ix et al., 2007) or albuminuria and cystatin C were concluded to have independent roles in risk prediction (Rifkin et al., 2010; Peralta et al., 2011b; Astor et al., 2012).

2.3.4 Cystatin C in cerebral disorders

Mutation Leu68Gln in cystatin C gene is known to result in cystatin C amyloid deposition in various sites of body whereas the cystatin C concentration in the cerebrospinal fluid is abnormally low (Grubb et al., 1984; Ghiso et al., 1986). Accumulation of these insoluble aggregates in the cerebral vessels (arterioles) causes cerebral amyloid angiopathy, cerebral hemorrhage and premature stroke (Ghiso et al., 1986). This dominantly inherited fatal disorder, amyloidosis type 6, exists in the Icelandic population and is thus known as Icelandic type of hereditary cerebral hemorrhage with amyloidosis or cerebral amyloid angiopathy (Grubb et al., 1984; Levy et al., 1989). Mutations in cystatin C are associated with age-related macular degeneration type 11 (Zurdel et al., 2002).

Elevated serum cystatin C is a risk factor for cognitive impairment, which is common in CKD (Sarnak et al., 2008; Yaffe et al., 2008). Although Alzheimer’s disease (AD) is a common cause of dementia (Small et al., 1997), high serum cystatin C may protect for the development of AD (Sundelof et al., 2008), suggesting different mechanisms

for these associations. Cystatin C may provide a strong protective role in neurodegenerative disorders, such as AD, by preventing accumulation of toxic forms of proteins, e.g., oligomers of amyloid-*beta*, and thus protecting neuronal cells from toxicity of these oligomers (Kaeser et al., 2007; Mi et al., 2007; Tizon et al., 2010). Increasing evidence exists showing that cystatin C levels in cerebrospinal fluid are decreased in neurodegenerative disorders, such as multiple sclerosis, amyotrophic lateral sclerosis and AD (Nagai et al., 2000; Wilson et al., 2010; Zhong et al., 2013). Thus, the cystatin C concentration in cerebrospinal fluid might prove useful in diagnosis and follow-up of treatment of neurodegenerative disorders.

2.4 QUANTIFICATION OF CYSTATIN C BY IMMUNOASSAYS

Cystatin C, normally around 1.0 mg/L, only adds about 0.001 percent to circulating proteins. The low concentration makes demands on the analytical sensitivity and specificity of cystatin C assays (Finney et al., 1997). However, specific antibodies against cystatin C exist (Olafsson et al., 1988) and are utilized in detection methods to meet these demands. These methods are explained in more detail in the following pages.

2.4.1 First quantitative methods: multistep assay based on detection of label

The first definite quantitative immunoassays for measuring cystatin C in human biological fluids were an enzyme amplified single radial immunodiffusion (SRID) and an enzyme immunoassay (EIA) developed at the end of 1970's (Lofberg and Grubb, 1979). Sensitivity of the EIA allowed quantitative measurement of cystatin C from all investigated bodily fluids, which was a clear improvement of immunoprecipitation methods widely used in the 1970's. These assays were followed by a competitive radioimmunoassay (RIA) method, which was developed with both a monoclonal and a polyclonal antibody raised against endogenous cystatin C purified from human urine (Poulik et al., 1983). The assay used ¹²⁵I-labeled cystatin C and the sensitivity of this assay was better than the sensitivity of EIA (1.3 µg/L vs. 30 µg/L), but the assay took approximately 16 hours to complete (Poulik et al., 1983).

In the late 1980's and early 1990's, several non-competitive assays, mostly enzyme-linked immunosorbent assays (ELISAs), were developed (Olafsson et al., 1988; Ishiguro et al., 1989; Colle et al., 1992; Pergande and Jung, 1993). In these assays, a polyclonal antibody was used for capture, and bound cystatin C was recognized either by a monoclonal antibody, which was further detected by an enzyme-labeled secondary antibody (Olafsson et al., 1988; Ishiguro et al., 1989), a biotin-labelled polyclonal detection antibody that was bound by enzyme-labelled streptavidin (Colle et al., 1992) or by an enzyme-conjugated polyclonal antibody (Pergande and Jung, 1993). The latest of these assays was the first to utilize commercially available antibodies (Pergande and Jung, 1993). With a 2-hour turn-around time and several steps, the assay was, however, time-consuming and inconvenient to use in daily routine.

2.4.2 Automated, separation-free assays

In the early 1990's, the existing assays for cystatin C were slow and laborious (Kyhse-Andersen et al., 1994). The fact that cystatin C was a potential marker of diminished renal function and thus competed with creatinine – the leading kidney analyte with high-capacity routine assays – there was a need for automated and rapid assays for cystatin C (Newman et al., 1995; Finney et al., 1997). From 1994 to 1997, three homogeneous, fully automated assays usable on large-scale basis were developed for the measurement of cystatin C (Kyhse-Andersen et al., 1994; Newman et al., 1995; Finney et al., 1997).

The first particle-enhanced turbidimetric immunoassay (PETIA) for rapid and automated measurement of cystatin C in undiluted serum and EDTA-plasma was published in 1994 (Kyhse-Andersen et al., 1994). At that time, PETIA methods already existed for other analytes such as CRP (Price et al., 1987) and β 2M (Medcalf et al., 1990). The assay was developed in Cobas Fara analyzer (Roche Diagnostics, Basel, Switzerland), and it used carboxylate-modified latex particles, 38 nm in diameter, covalently coupled to a rabbit polyclonal antibody against human cystatin C (Dako, Copenhagen, Denmark), and recombinant cystatin C as the standard material (Abrahamson et al., 1988; Dalboge et al., 1989). The reaction between cystatin C and immunoparticles resulted in the formation of agglutinates. The increase in absorbance due to formation of these antigen-antibody complexes (at 37 °C) was measured at 340 nm. The assay took 7 minutes for one sample and the throughput of the method was 90 samples per hour. The detection limit was 0.15 mg/L and the range 0.4–14.1 mg/L. The within- and between-run imprecision was reported to be 0.9 percent and 2.2 percent, respectively (Kyhse-Andersen et al., 1994).

The second PETIA method for the measurement of serum cystatin C was reported simultaneously (Newman et al., 1995). The aim of the group was to develop a rapid method that would be effective for automated high-capacity routine measurement and could improve the competitiveness of cystatin C compared to creatinine. The assay resembled the first PETIA; rabbit anti-human cystatin C (Dako) coated 77 nm latex particles formed complexes in the presence of cystatin C (at 37°C) and a change in absorbance was detected after a fixed time at 340 nm. Solutions of purified recombinant cystatin C (Abrahamson et al., 1988) prepared in horse serum (Sigma, Poole, Dorset, UK) were used as calibrators. The assay was fully automatic and it was developed on a centrifugal analyzer (Monarch 2000; Instrumentation Laboratory, Warrington, UK) that was used routinely for the measurement of creatinine. Differences to the first PETIA included smaller sample volume, 5 μ L vs. 20 μ L, a lower detection limit (0.027 mg/L vs. 0.15 mg/L) and shorter incubation time, 5 min. However, within and between-run precision were somewhat higher, less than 3 percent and less than 5 percent, but they were reported across the assay range. Also, slight underrecovery was observed, 93 percent \pm 3.8 percent. Results from the assay correlated well with SRID results: $r = 0.98$; $PETIA = 0.074 + 0.93 \times SRID$ ($n=100$). Costs of this revised PETIA were estimated to be equal to creatinine methods. (Newman et al., 1995).

The third fully automatic assay for cystatin C was a particle-enhanced nephelometric assay (PENIA), a separation free assay like PETIA, but where scattered light caused by antigen-antibody complexes is measured instead of decrease in light going through the sample and is directly proportional to the concentration of cystatin C in sample (Finney et al., 1997). The PENIA used a rabbit polyclonal anti-cystatin C coated on latex particles and endogenous cystatin C purified from human urine. The assay was developed by Behringwerke Diagnostica (Marburg, Germany; currently Siemens Healthcare Diagnostics, Deerfield, IL, USA) and it was performed at room temperature on their Behring nephelometer 100 system. The assay range was 0.23–7.25 mg/L (undiluted sample), the within- and between-run imprecision were less than 3.3 percent and 4.5 percent, respectively, assay time was 6 min per sample and capacity 75 samples per hour. Samples were prediluted 100-fold in two stages (1:5 followed by 1:20) before being analyzed. The sample volume needed (80 μ L) was higher than that needed for the PETIAs (Finney et al., 1997). The assay was compared to an in-house PETIA (Newman et al., 1995) and a commercial PETIA (Dako) performed on a Cobas Bio centrifugal analyzer (Roche Diagnostics). Results agreed, but with a notable mean difference in cystatin C values: 1.13 ± 0.86 mg/L (vs. Dako) and 1.73 ± 2.10 mg/L (vs. in-house PETIA). Calibrators of the PETIA assays yielded lower cystatin C concentrations in PENIA assay revealing differences in immunoreactivity or quantification of the calibrators. (Finney et al., 1997).

Nephelometric N Latex Cystatin C assay (Dade Behring Diagnostics, Milan, Italy; nowadays Siemens Healthcare Diagnostics) was evaluated on a Behring Nephelometer Analyzer (BNA) (Mussap et al., 1998). The described method was essentially identical with the assay reported by Finney et al. (Finney et al., 1997). The assay had a range of 0.2–6.8 mg/L, and the linear range was slightly narrower, 0.7–6.8 mg/L. Reference range for adults was determined to be 0.37–1.22 mg/L. The intra- and interassay coefficients of variation were 1.6–3.8 percent and 5.6–11.5 percent, respectively. The assay was compared to Dako PETIA performed on Cobas Fara II (Roche Diagnostics). PENIA values were lower with the mean difference between the two methods being -0.536 mg/L. (Mussap et al., 1998).

Concurrently, the N Latex Cystatin C assay was evaluated employing the Behring Nephelometer II (BN II) (Erlandsen et al., 1999), where the assay is performed at stabilized temperature (37°C) instead of at ambient temperature on BNA (Mussap et al., 1998). The assay time and throughput were similar as in BNA, the measuring range was wider, 0.25–7.90 mg/L, and within- and between-run imprecision were remarkably lower, 1.8 percent in the concentration range 0.87–4.63 mg/L. Cystatin C measured in heparin plasma and EDTA-plasma agreed well with cystatin C concentrations measured in serum. When compared to Dako PETIA run on Cobas Mira (n=50), the results were remarkably closer to the PETIA than in the comparisons done by Finney et al. (Finney et al., 1997) and by Mussap et al. (Mussap et al., 1998): $y=1.105x-0.340$ (Passing-Bablok); average bias was reported to be 0.25 mg/L. (Erlandsen et al., 1999).

Although reported to have been applied on several instruments (Finney et al., 1997; Erlandsen et al., 1999), evaluations of Dako turbidimetric assay were not published until recently (Bargnoux et al., 2011b).

2.4.3 Current assays

At present, PETIA and PENIA are still the two most widely-used methods for measurement of cystatin C. Siemens Healthcare Diagnostics (formerly Dade Behring) produces cystatin C reagents dedicated for their nephelometry instruments (Flodin et al., 2006), in contrast to reagents intended for turbidimetric applications that are sold as kits applicable to several different routine clinical chemistry analyzers thus making them less restricted with respect to instrumentation (Li et al., 2010).

Reagents intended for turbidimetric cystatin C applications are provided by several companies, including Dako (Bargnoux et al., 2011b), Gentian (Moss, Norway) (Flodin et al., 2007; Sunde et al., 2007), Roche Diagnostics (Conde-Sanchez et al., 2010; Erlandsen and Randers, 2010), Genzyme (Cambridge, MA, USA) (Al-Turkmani et al., 2008), Abbott (Abbott Laboratories, Abbott Park, IL, USA) (Flodin and Larsson, 2009), Binding Site (Birmingham, UK) (Bargnoux et al., 2011a), and Sentinel Diagnostics (Milano, Italy) (Bargnoux et al., 2012). Most of the assays use rabbit polyclonal antibodies, whereas Binding Site's assay uses sheep polyclonal antibodies (Bargnoux et al., 2011a). The Gentian reagent consists of chicken egg yolk IgY antibodies bound to latex particles (Flodin et al., 2007) to avoid interference caused by rheumatoid factors and human anti-mouse IgG (Larsson et al., 1991; Hansson et al., 2008). The significance of this has, however, been questioned by a study that did not find any interference caused by rheumatoid factors to assays using either avian or rabbit antibodies (Delanaye et al., 2008). Performance characteristics of three widely-marketed cystatin C assays are shown in Table 6. (Li et al., 2010).

Table 6. Performance characteristics of three widely-marketed cystatin C assays. Adapted from (Li et al., 2010).

| Provider, assay (autoanalyzer) | Method | Particle reagent ¹ | Sample volume (µL) | Reaction time (min) | LoD (mg/L) | Quoted normal range (mg/L) |
|-----------------------------------|--------|--|--------------------|---------------------|------------|----------------------------|
| Siemens N Latex Cystatin C (BNII) | PENIA | Polystyrene particles coated with rabbit antibodies | 30 | 6 | 0.05 | 0.53–0.95 |
| Roche Tina-quant (Cobas c501) | PETIA | Polystyrene particles coated with rabbit antibodies | 2 | 10 | ≤0.4 | 0.47–1.09 |
| Genzyme Cystatin C (Cobas c501) | PETIA | Colloidal gold particles coated with rabbit antibodies | 2 | 10 | 0.05 | 0.61–1.17 |

¹according to (Erlandsen et al., 1999; Al-Turkmani et al., 2008; Erlandsen and Randers, 2010)

In addition to nephelometric and turbidimetric methods, there are also producers of cystatin C ELISA kits, such as BioVendor (Brno, Czech Republic) (Hossain et al., 2009). Those, however, have several steps and longer turnaround times (Flodin et al., 2006; Hossain et al., 2009). A two-site immunometric assay from TOSOH Bioscience (Tokyo, Japan) utilizing magnetic particles and fluorescence, however, is a rapid assay (10 minutes reaction time) that requires a specific immunoassay instrument (Li et al., 2010).

2.4.4 Interference and limitations

The methods based on detection of scattered light or turbidimetry are potentially vulnerable to interferences – especially those caused by particles and chromogens in the sample matrix (e.g., resulting from lipemia, hemolysis, and bilirubinemia) (Lin et al., 2013). A summary of studied interfering factors in different assays is presented in Table 7. The first PETIA methods were subject to interference from high triglyceride concentrations (>10 mmol/L) (Newman et al., 1995) and remarkably elevated bilirubin (150–300 $\mu\text{mol/L}$) (Kyhse-Andersen et al., 1994). Later, triglycerides greater than 4 mmol/L or greater than 5.7 mmol/L interfere with the cystatin C assay based on Dako reagents (Stowe et al., 2001; van Rossum et al., 2006). Also high hemoglobin interferes with PETIA assays (Kyhse-Andersen et al., 1994; Sunde et al., 2007; Conde-Sanchez et al., 2010). Turbidity was later speculated to have caused artificially increased measured biological variation in a study by Keevil and colleagues (Keevil et al., 1998; Filler et al., 2005). This speculation was strengthened by an observation that turbidity caused remarkable problems for the cystatin C kit (Dako PET) that was used by Keevil and colleagues, especially with frozen samples (Tanaka et al., 2004).

The first PENIA was reported to be unaffected by high concentrations of potentially interfering substances. There were up to 5.1 percent differences in cystatin C concentrations caused by these substances – however, the clinical significance was estimated to be negligible (Finney et al., 1997). The high predilution of sample (1:100) resulting in a low sample fraction in the assay (0.38%) was speculated to reduce vulnerability to interferences compared to the first two PETIA assays with sample fractions of 1.19 percent and 3.57 percent (Finney et al., 1997).

Rheumatoid factor is a potential cause of interfered results due to binding to Fc region of IgG molecules resulting in non-specific agglutination – especially in nephelometric detection, where earlier stages of an agglutination reaction are followed – however, latex-particle based assays seem to be resistant to this interference (Newman, 2003; Delanaye et al., 2008). Recoveries of rheumatoid factor positive samples were good (92–115%) with PETIA assays from Dako and Gentian, and PENIA from Siemens (Delanaye et al., 2008). The concentrations of rheumatoid factors investigated in interference studies have varied, though (Table 7), and e.g., Dako cystatin C PETIA has been mentioned to be interfered by rheumatoid factors (Sjostrom et al., 2005).

The reasons for the lack of incorporation of cystatin C into clinical routine include its higher reagent cost. In the USA, the limited availability of cystatin C reagents effective

for standard laboratory equipment delayed its widespread use. Siemens PENIA was the first assay to be cleared by the US Food and Drug Administration (FDA) and it requires a dedicated nephelometer, which is not a standard instrument in all laboratories. Currently, several assays for measurement of human cystatin C in blood plasma/serum have acquired FDA clearance (510k). (Shlipak et al., 2011)

Table 7. Interference data for cystatin C assays. Concentrations of interfering factors denote levels where no interference has been reported.

| Method (producer ¹) | Rheumatoid factors (IU/mL) | Bilirubin (μmol/L) | Hb (g/L) | Triglycerides (mmol/L) | Reference |
|---------------------------------|-----------------------------|--------------------|----------|----------------------------------|-----------------------------|
| PENIA | <2000 | <488 | <8.0 | <23 | Finney et al., 1997 |
| PENIA | ≤ 1116 IU/L | ≤418 | ≤12 | ≤10.5 | Mussap et al., 1998 |
| PENIA | NT | 512 | 16.1 | 20 g/L ² | Erlandsen et al., 1999 |
| PENIA (Siemens) | <884 | 1160 | ≤8 | ≤15.6 | Delanaye et al., 2008 |
| PETIA | ≤323 | <150 | ≤1.2 | ≤8.5 | Kyhse-Andersen et al., 1994 |
| PETIA | concentrations not reported | 700 | 1.0 | <10 ² | Newman et al., 1995 |
| PETIA (Gentian) | NT | ≤660 | ≤16.1 | ≤ 16.2 ² | Flodin et al., 2007 |
| PETIA (Gentian) | NT | <718 | <7 | <14 g/L; <11 g/L ² | Sunde et al., 2007 |
| PETIA (Genzyme) | ≤1000 | ≤513 | ≤8 | ≤10 g/L | Al-Turkmani et al., 2008 |
| PETIA (Dako, Gentian) | <884 | 1160 | ≤8 | ≤ 15.56 | Delanaye et al., 2008 |
| PETIA (Abbott, Gentian) | NT | 437 | 23.4 | 21 | Flodin and Larsson 2009 |
| PETIA (Roche Tina-quant) | NT | <330 | <14.5 | <20 g/L ² | Erlandsen and Randers 2010 |
| PETIA (Roche) | NT | ≤240000 | ≤6 | ≤27 | Conde-Sanchez et al., 2010 |

Criteria for “no interference” vary, i.e., less than 10% change in cystatin C concentration (Al-Turkmani et al., 2008). Myeloma paraprotein was tested only by Finney et al. (1997) in a PENIA assay and less than 41 g/L caused 5.0% deviation. Hb, hemoglobin; NT, not tested; PENIA, particle-enhanced nephelometric immunoassay; PETIA, particle-enhanced turbidimetric immunoassay. ¹ Where known; ² Intralipid.

Reported problems with the assays include lack of stable calibration with Siemens PENIA (Larsson et al., 2011; Shlipak et al., 2011), and nonlinearity, large interlaboratory variation and lower analytical precision for the Dako cystatin C reagent implemented on several instruments (Flodin et al., 2006; Delanaye et al., 2008). With the Dako reagent, changes in assay protocol were reported to result in even 50 percent change of eGFR calculated from cystatin C concentration (Flodin et al., 2006). Also imprecision in the Genzyme assay and overrecovery at low cystatin C levels by Roche’s Tina-quant assay have been noticed (Li et al., 2010). Potentially related to the 19 percent decrease in cystatin C values from 2000 to 2010 reported for the Siemens method (Larsson et al., 2011), also described as systematic shifts in PENIA assay between 2006 and 2010 (Maahs et al., 2011), a bias between Siemens and Gentian assays has recently been noted (Voskoboev et al., 2012).

2.4.5 Standardization of methods measuring cystatin C

Need for standardization was emphasized with presentation of cystatin C-based GFR prediction equations. Due to assay-specific differences in patient sample results, variation in calibrators and the use of non-harmonized methods, a large number of assay-specific equations have been required (Grubb et al., 2010). Potential variation between the methods may result from: the use of recombinant (Abrahamson et al., 1988; Dalboge et al., 1989) versus native (endogenous) cystatin C molecule as the calibrator; differences in antibodies and other reagents; and different detection technologies (Finney et al., 1997; Flodin et al., 2006; Delanaye et al., 2008).

An international certified reference material for cystatin in human serum (ERM-DA471/IFCC) was produced and characterized by the IFCC Working Group on Standardisation of Cystatin C together with the Institute for Reference Materials and Measurements (Blirup-Jensen et al., 2008; Grubb et al., 2010). The reference material is serum that is spiked with recombinant cystatin C and lyophilized in aliquots. It is intended to be used for the calibration of immunoassays and to control the measurement of cystatin C (Grubb et al., 2010).

Following the release of the reference material, cystatin C assays are or are in the process of being calibrated to international cystatin C reference material (Shlipak et al., 2011). During the international standardizations, considerable differences between assays has been observed, e.g., Gentian PETIA traceable to the reference material had a positive bias (25%) compared to Siemens PENIA that had not been standardized (Voskoboev et al., 2012).

Regarding the estimation of GFR using prediction equations, the aim is to produce one equation for all cystatin C assays, regardless of the analytical platform, that is widely applicable in the general population (Grubb et al., 2010; Shlipak et al., 2011). Other interests related to the standardization include studying non-GFR determinants of cystatin C (Shlipak et al., 2011).

3 AIMS OF THE STUDY

This study's objectives were to develop an immunoassay for cystatin C and to evaluate its clinical performance in estimating reduced kidney function and in predicting the risk of adverse outcomes in patients with non-ST elevation acute coronary syndrome.

The aims of the study were:

- I** To conceive three methodologically different assay designs based on time-resolved fluorescence for cystatin C and to evaluate and compare the performance of the developed methods.
- II** To develop a detection method for cystatin C by utilizing monoclonal antibodies (double monoclonal method) and to evaluate the performance of the method in plasma.
- III** To evaluate the clinical performance of cystatin C measured by the developed double monoclonal method as a marker of reduced renal function and to evaluate the biological variation of cystatin C measured by the developed method.
- IV** To assess if cystatin C, using the established double monoclonal method, can be a risk marker that predicts adverse cardiac outcome in patients with non-ST elevation acute coronary syndrome.

4 SUMMARY OF MATERIALS AND METHODS

Materials and methods are described in more detail in the original publications (**I-IV**). A short summary with some additional information is presented here.

4.1 CLINICAL SAMPLES (I-IV)

All plasma and serum samples were collected according to standard laboratory routines and stored at -70°C or at -80°C until measured. A summary of analyzed samples is shown in Table 8.

Lithium heparinized plasma samples (n=132) were used for methodological evaluation of the developed immunoassays for cystatin C (**I, II**). These samples were collected at University Hospital of Lund (Lund, Sweden) as part of routine sampling procedures, and no clinical information was available for these samples.

To establish the clinical utility of the dry-reagent double monoclonal cystatin C assay (**III**), two panels consisting of males with known weight, height, creatinine and cystatin C levels and measured iohexol clearance had plasma collected at Skåne University Hospital (Malmö, Sweden). The first panel consisted of 220 consecutive men without known renal disease. Three samples per patient were collected: first, before iohexol measurement, second, immediately after iohexol measurement (4 hours, range: 3–7 hours), and the median interval between the first and the third sample was 12 days (range: 6–38 days). No results of renal markers were available for the second and the third sample. They were used only in the biological variability analysis after measurement with the newly developed dry-reagent double monoclonal assay. The second panel consisted of 108 men with CKD, who were enrolled at routine visits for GFR determination with iohexol clearance. After exclusion of patients with missing data for creatinine, cystatin C and iohexol clearance, 170 and 104 patients were left for analysis in the first and second panel, respectively. The included men in the second cohort had been diagnosed with diabetic nephropathy (n=21), glomerulonephritis (n=22), non-specified renal disease (n=14), nephrosclerosis (n=15), or a group of miscellaneous renal diseases (n=10), or had renal transplants with stable but reduced renal function (n=22).

The cohort used in publication **IV** was obtained from the Turku University Central Hospital and it originally consisted of 541 patients suffering from chest pain, who were presented to the emergency room for evaluation of suspected MI. We selected those 245 patients, who were hospitalized due to symptoms consistent with non-ST-elevation ACS and whose admission creatinine values were available. Serum samples were drawn at admission and, for cardiac troponin I (cTnI) measurements, at 6–12 hours and at 24 hours. Follow-up data (12 months) was available for these patients. The time from symptom onset to hospital admission was recorded during patient recruitment in the emergency room as described previously (Ilva et al., 2009).

In addition, serum and plasma samples collected from volunteers at the Department of Biotechnology, University of Turku, were used to develop the cystatin C assays (**I**, **II**). Informed consent was obtained from the volunteers.

The procedures for collection of samples with available clinical data were approved by the regional ethics committees at Lund University and Turku University and they were in accordance with the Helsinki Declaration of 1975 as revised in 1996. All volunteers gave a signed informed consent to participate in the study and to allow retrieval of information from medical records.

Table 8. Summary of samples used in this study.

| Collection time | Collection place | Study population | Sample matrix | N | Publication |
|---------------------------|--|---|-----------------------|-----|-------------|
| 2005 | LUH | Deidentified leftover samples | Heparin plasma | 132 | I, II |
| October 2001 – April 2004 | Dept. of Urology, SUH | No known renal disease | EDTA plasma | 170 | III |
| 2004–2006 | Dept. of Nephrology and Transplantation, SUH | Renal dysfunction | EDTA plasma | 104 | III |
| May 2000 – July 2001 | TUCH | Chest pain patients diagnosed with nSTE-ACS | Serum | 245 | IV |
| 2004 | DBUT | Healthy volunteers | Heparin plasma; serum | 10 | I, II |

Dept., Department; LUH, Lund University Hospital, Malmö, Sweden; SUH, Skåne University Hospital, Malmö, Sweden; TUCH, Turku University Central Hospital, Turku, Finland; DBUT, Department of Biotechnology, University of Turku, Turku, Finland; nSTE-ACS, non-ST-elevation acute coronary syndrome.

4.2 RECOMBINANT CYSTATIN C AND ANTIBODIES (I-IV)

Recombinant cystatin C was used as a calibrator in all assays (**I-IV**) and was produced in *Escherichia coli* XL-1 Blue cells according to the protocol reported by Abrahamson and coworkers (Abrahamson et al., 1988; Dalboge et al., 1989). The actual calibrators were prepared by diluting recombinant cystatin C in Tris-Saline-Azide (TSA) buffer (50 mmol/L Tris-HCl, pH 7.8, 154 mmol/L NaCl, 0.5 g/L NaN₃) containing 60 g/L bovine serum albumin (BSA). Aliquots of the dilutions were stored at -70°C and a new set of calibrator aliquots were used for each measurement. Concentrations of the original stock solutions were determined spectrophotometrically. The purified recombinant cystatin C was stored at -70°C.

Seven monoclonal and one polyclonal antibody were used in the studies **I-IV**. For the development of three immunoassays for cystatin C (**I**), one monoclonal cystatin C-specific antibody, HCC3 (Olafsson et al., 1988), produced at University Hospital of Lund, was used, together with an affinity purified cystatin C-specific polyclonal antibody (Dako, Copenhagen, Denmark). Monoclonal cystatin C-specific antibodies Cyst10,

Cyst13, Cyst19, Cyst23, Cyst24 and Cyst28 (HyTest Ltd., Turku, Finland) were used for the generation of a schematic epitope map (II), development of the dry-reagent double monoclonal assay (III) and measurement of clinical samples (III, IV). The monoclonal antibody HCC3 was also included in the study II. All of the antibodies had been generated by immunization of mice (monoclonal antibodies) or rabbit (polyclonal antibody) with either recombinant cystatin C or with cystatin C purified from human urine. The monoclonal antibodies represented the IgG1 subclass, except for Cyst10, which was of the IgG3 subclass (Olafsson et al., 1988; HyTest, 2009). Data from affinity studies were provided for three of the monoclonal antibodies: HCC3, $K_a=2 \times 10^7$ L/mol (Olafsson et al., 1988); Cyst10, $K=5.55 \times 10^{-8}$ mol/L, and Cyst24, $K=1.09 \times 10^{-8}$ mol/L (HyTest, 2009), which indicate good affinity of the antibodies.

4.3 LABELLING OF CYSTATIN C AND ANTIBODIES (I-IV)

For use in the homogeneous immunoassay (I), recombinant cystatin C was labeled with Cy5 dye (Cy5 Mono-Reactive Dye Pack, GE Healthcare Life Sciences, Buckinghamshire, United Kingdom). Briefly, a 20–50-fold molar excess of Cy5-dye was left to react with cystatin C molecules in carbonate buffer (50 mmol/L sodium carbonate-bicarbonate buffer, pH 9.6) at 4°C for 30 minutes. The labeled cystatin C molecules were purified from excess free dye using disposable PD10 gel filtration columns (GE Healthcare Life Sciences) and 50 mmol/L TSA as the elution buffer.

For the heterogeneous competitive assay (I), recombinant cystatin C was labeled with an intrinsically fluorescent 9-dentate Eu^{3+} chelate, {2,2',2'',2'''}-[2-(4-Isothiocyanatophenyl) ethylimino]bis(methylene)bis{4-[4-(R-galactopyranoxy)phenyl]ethynyl} pyridine-6,2-diyl}bis(methylenenitrilo)} tetrakis(acetato)} Eu^{3+} (von Lode et al., 2003). The labeling reaction was carried out at room temperature for 16 hours with 25–35-fold molar excess of the chelate in carbonate buffer and the labeled cystatin C was separated from free chelate by using reverse phase HPLC on $\mu\text{RPC C2/C18 ST}$ (GE Healthcare Life Sciences). Increasing hydrophobic gradient was formed with acetonitrile (5% – 80%; within 20 mL). The labeling procedure is described in more detail in the publication I.

The antibodies used as captures were labeled with biotin isothiocyanate (kindly provided by Jaana Rosenberg, University of Turku) and the antibodies used in detection were labeled with the 9-dentate Eu^{3+} chelate (I-IV). Both labelings were carried out in carbonate buffer at ambient temperature, and purified with gel filtration chromatography using 50 mmol/L TSA, pH 7.8, as the elution buffer. The labeling with biotin isothiocyanate was done using 25-fold molar excess of the labeling reagent, 3- or 4-hours incubation time at room temperature and the reaction was purified using disposable NAP gel filtration columns (GE Healthcare Life Sciences), whereas the labeling with the europium chelate was done using 25–35-fold molar excess of the Eu^{3+} chelate, 16-hours reaction time at room temperature and purified with fast performance liquid chromatography (FPLC) system using Superdex 200 HR 10/30 column (GE Healthcare Life Sciences).

After the determination of the protein concentration and the number of attached label molecules per labeled protein molecule (label degree), the products were stabilized with 1 g/L BSA, filtered and stored at 4°C. Label degrees of cystatin C were 1–3 whereas label degrees of antibodies varied from 3 to 10.

4.4 IMMUNOASSAYS

In this study, three methodologically different approaches were investigated for measurement of cystatin C. Heterogeneous non-competitive (method 1), heterogeneous competitive (method 2) and homogenous competitive (method 3) assays were developed by using recombinant cystatin C, monoclonal antibody HCC3 and polyclonal antibody. The work with the heterogeneous, non-competitive assay was further continued with monoclonal antibodies and a dry-reagent format. Figure 8 shows the schematic principles of the assays. The methods are explained in more detail below. The concentrations of assay-specific reagents, i.e., the antibodies and labelled cystatin C, had been optimized regarding the sensitivity and the working range of the assays, and only the final, selected amounts of the reagents are presented.

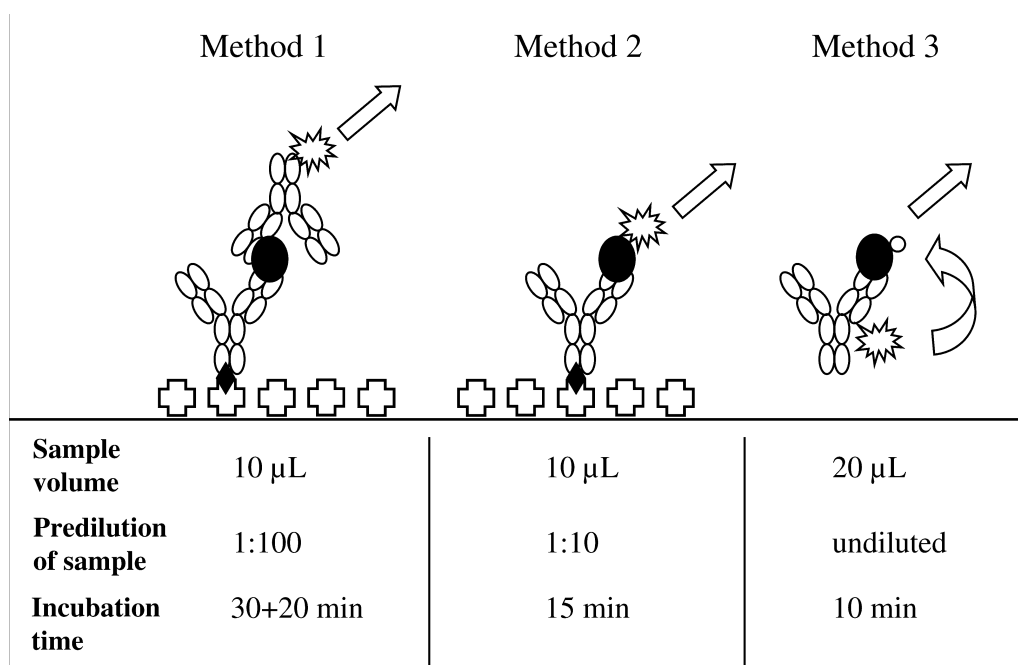


Figure 8. The signal-forming complexes and details of the three methodologically different immunoassays for cystatin C. Method 1, a heterogeneous noncompetitive assay; method 2, a heterogeneous competitive assay; method 3, a homogenous competitive assay. In methods 1 and 2, biotinylated antibodies are first bound to a solid streptavidin-coated surface, whereas the method 3 takes place in solution phase. Shapes depict compounds: black ellipsoid, cystatin C; black diamond; biotin; explosion, Eu^{3+} chelate; small open circle, Cy5 dye. The straight arrows denote fluorescence emission, and the curved arrow, a fluorescence resonance energy transfer from Eu^{3+} chelate to Cy5 dye.

4.4.1 Heterogeneous non-competitive assay (I)

In the noncompetitive excess reagent assay, a “sandwich” complex was formed by the binding of the polyclonal antibody and the monoclonal antibody HCC3 to cystatin C in two steps. The biotinylated polyclonal antibody (200 ng) was pre-bound to a solid streptavidin surface (streptavidin coated microtiter strips, Kaivogen, Turku, Finland) during 60 min incubation. In the first assay step, 10 μL of 100-fold diluted sample or standard was applied to capture wells together with 20 μL of assay buffer (50 mmol/L Tris, pH 7.8, 154 mmol/L NaCl, 0.5 g/L NaN_3 , 0.1 g/L Tween 40, 5 g/L bovine serum albumin, 0.5 g/L bovine gamma-globulin and 20 $\mu\text{mol/L}$ diethylenetriaminepentaacetate). After a 30 min incubation at 22°C, unbound reagents were washed out, and in the second assay step, 200 ng of the Eu^{3+} labeled HCC3 was added in 30 μL of assay buffer enhanced with 0.2 g/L of native mouse IgG and 0.1 g/L of denatured mouse IgG and incubated on captured cystatin C at 22°C for 20 min. After final wash step, the wells were dried under a stream of warm air, let cool to room temperature, and Eu^{3+} emission was measured with Victor™ 1420 multilabel reader (PerkinElmer, Wallac, Turku, Finland) in a time-resolved mode.

4.4.2 Heterogeneous competitive assay (I)

A heterogeneous competitive assay was based on using the biotinylated polyclonal antibody as the capture and Eu^{3+} chelate-labeled cystatin C as the competitor. In the actual assay step, 10 ng of Eu^{3+} labeled cystatin C competed for binding to immobilized capture antibody (200 ng) with calibrator cystatin C or cystatin C in sample. After 15 minutes at 37°C, the wells were washed, dried and measured as described in section 4.4.1. Total assay volume was 30 μL of which 1:10 diluted sample or standard comprised 10 μL .

4.4.3 Homogeneous assay (I)

Homogeneous competitive immunoassay for cystatin C was performed with Eu^{3+} chelate-labeled polyclonal antibody and Cy5 dye-labeled recombinant cystatin C. First, 10 ng of Cy5-labeled cystatin C and undiluted sample or standard were added to the wells. Immediately after that, the immunocomplex formation was started by addition of 200 ng of the Eu^{3+} chelate-labeled polyclonal antibody. The cystatin C in standard or sample competed for binding to the antibody at 22°C for 10 min. Without sample cystatin C, the maximum number of signal-generating Eu^{3+} -polyclonal antibody—Cy5-cystatin C complexes were able to form. The number of these complexes decreased with the amount of free cystatin C in sample. This homogeneous design was accomplished by using a resonance energy transfer (RET) principle, where excitation energy absorbed by a Eu^{3+} chelate proceeded to a Cy5 dye in close proximity through RET; when Eu^{3+} chelate and Cy5 dye lay within certain distance range and angle in space, Cy5 emitted the transferred energy as visible light (maximum emission peak at 670 nm). The presence of lanthanide label (Eu^{3+}) produced a long-lifetime emission and Cy5 emission was measured in time-resolved mode. The Eu^{3+} emission was measured at 615 nm and the interference from Eu^{3+} chelate emission was

mathematically corrected according to a method previously published (Qin et al., 2003).

4.4.4 Dry-reagent double monoclonal assay (II)

The development of a sandwich-type double monoclonal assay was started by manually assaying the functionality of all combinations with monoclonal antibodies made available during the study, resulting to a schematic epitope map of cystatin C. Prior to testing the combinations, all the monoclonal antibodies were separately biotinylated and labeled with europium chelate as described in section 4.3. All possible two-site combinations were tested for the ability to bind cystatin C (both recombinant and endogeneous cystatin C). Based on the results, the best three antibody pairs were selected and tested for assay kinetics, linearity and agreement to cystatin C concentrations obtained by a PETIA method. Finally, one pair was selected for the development of a dry-reagent immunoassay for cystatin C.

The double monoclonal assay was performed in an automated immunoanalyzer using dry-reagent wells that contained all necessary assay components and could be stored for prolonged periods (Pettersson et al., 2003). Dry-reagent wells were prepared by applying biotin-labeled antibodies to streptavidin-coated single wells (Innotrac Diagnostics, Turku, Finland), covering the resulting capture surface with a layer of strongly buffered insulating solution, drying the layer completely, and finally applying Eu^{3+} chelate-labeled detection antibody in a 1- μL volume to the top of the insulating layer. After final drying, the wells were packed in sealed aluminum foil packs with desiccants and stored at 4°C.

Samples were diluted 1:100 before analysis with automated Aio! immunoanalyzer (Innotrac Diagnostics). The analyzer applied 10 μL of diluted sample and 20 μL of combined assay and wash buffer (von Lode et al., 2004) to a dry reagent well, and each well was incubated for 10 min at 36°C with shaking. Before Eu^{3+} fluorescence measurement, the reagent well was washed and the residual humidity was dried with hot air. The Eu^{3+} fluorescence was measured directly from the solid surface in time-resolved mode, using the default measurement settings of the Aio! Immunoanalyzer.

4.5 ASSAY EVALUATIONS

4.5.1 Methodological evaluation (I, II)

Analytical sensitivity was estimated from the low-end of the dose–response curve and it was defined as the concentration deviating 3 standard deviations from background signal. Twelve replicate measurements of zero calibrator and 6 replicates of each standard concentration were assayed. Assay range was defined as the continuous range of the dose–response curve, where coefficient of variation (CV) calculated from cystatin concentrations was less than 20%. CVs were calculated as the ratio of the standard deviation to the mean concentration and expressed as percentages.

Assay kinetics, i.e., the speed of the binding of assay components to reach saturation, was studied with low and high concentrations of recombinant cystatin C in buffer and with plasma samples. The binding properties of the assays were determined by varying the incubation time and temperature.

Plasma recoveries were determined from samples enhanced with either recombinant cystatin C or endogenous cystatin C from high concentration plasma samples. The spiked volume was no more than 5 percent of the total sample volume. Recovery percentages were calculated as the increase in the cystatin C concentration of the sample divided by the expected increase.

For the dry-reagent double monoclonal assay(s), further evaluation was conducted (III).

Linearity of sample dilution series was studied by linear regression analysis from wide-ranging series of three-fold diluted plasma (3) and serum (2) samples, assayed in 6 replicates. Functional detection limit was estimated from the imprecision profiles of the sample dilution series and defined as the cystatin C concentration that could be measured with a CV of 20 percent.

Within-run and total assay imprecisions of the final dry-reagent double monoclonal assay were determined according to Clinical Laboratory Standards Institute (CLSI) Guideline EP5-A. For imprecision calculations, the cystatin C concentrations of three plasma samples were measured, twice a day, for 20 days, in duplicate.

Interference of potential interfering components (i.e., bilirubin, hemoglobin and triglycerides) were tested according to CLSI guideline EP7-A. Instead of hemolysate, purified human hemoglobin (Sigma-Aldrich, St. Louis, MO, USA) was used.

4.5.2 Comparison to conventional method (I-III)

Plasma samples described in section 4.1 were used for methodological comparison of the developed assays to PETIA method (Kyhse-Andersen et al., 1994). The PETIA reagents were either obtained from Roche and the determination was performed on COBAS 6000 analysis system (Roche) (I, II) or obtained from Dako and run on the Hitachi Modular P analysis system (Roche) (III).

4.5.3 Clinical evaluation of dry-reagent double monoclonal assay (III, IV)

Cystatin C levels measured by the dry-reagent double monoclonal assay were compared to other measures of renal function: measured GFR, serum creatinine, and estimated GFR (eGFR) based on serum creatinine measurement and individual data. The measured GFR was determined from plasma clearance of iohexol (III), according to a one-compartment model, where samples were taken after the distribution phase, in the assumed monoexponential part of the plasma decay curve. The concentration of iohexol was measured by a HPLC technique (Brochner-Mortensen, 1972; Krutzen et al., 1984). Serum creatinine was measured by a creatinase-based method using the Hitachi Modular P analysis system (application 652; Roche) (III) or by a kinetic Jaffé

procedure using Hitachi 917 automatic analyzer (Hitachi, Tokyo, Japan) (IV). Estimated GFR was calculated by using CG equation (Cockcroft and Gault, 1976) and adjusted for body surface area (eGFR-CG/BSA, mL/min/1.73 m²) (IV). Body surface area was estimated from the DuBois formula (DuBois and DuBois, 1916). To classify the stages of CKD according to the internationally recommended guidelines (National Kidney Foundation, 2002; Levey et al., 2005), eGFR values were calculated according to the four-variable version of MDRD Study equation (eGFR-MDRD) (Levey et al., 1999) (IV).

4.6 STATISTICAL ANALYSIS (I-IV)

The regression between cystatin C levels measured with the different methods was assessed by using Deming regression, and nonparametric Spearman's rank sum correlation coefficients were calculated. Agreement amongst values given by different assays was assessed by a Bland-Altman analysis (Bland and Altman, 1986) (I, II).

Comparisons between cystatin C, creatinine and measured GFR were done by using the reciprocal of the measured cystatin C and creatinine concentrations: 1/cystatin C (L/10⁻¹ g) and 1/creatinine (L/10⁻² mol). This procedure was used to facilitate a linearization of the relationship between GFR and the marker concentrations (III). To quantify the amount by which values of 1/cystatin C and 1/creatinine differed from iothexol clearance, concordance correlation coefficients and the 95% limits of agreement were calculated. To determine intra-individual variability of cystatin C, CVs were calculated from cystatin C concentrations measured at the three time points (III).

Normality was tested with Kolmogorov-Smirnov test. Baseline characteristics of the study population in the original publication IV were characterized in subgroups based on cystatin C values. Continuous variables in groups were expressed as mean (range) or as median (25th, 75th). Categorical variables were described as numbers (percentages). Comparisons between groups were evaluated using an unpaired t-test or the nonparametric Mann-Whitney *U* test for continuous variables and chi-square test for parametric variables. Bonferroni correction was applied to all multiple comparisons. The frequency of study endpoints in subgroups of patients was compared similarly. Exact *P*-values were calculated with Fisher's test when the expected cell values were less than five in over 20 percent of the cells. Nonparametric Spearman's rank sum correlation coefficients were calculated to estimate the correlation between cystatin C and other biomarkers.

The relation of cystatin C, creatinine and eGFR-CG/BSA levels to the delay (0–3 h, 3–6 h, 6–12 h, 12–24 h) from onset of symptoms to the admission blood sampling was studied in the group of patients who had reported reliable delay less than 24 h (n=135). Median concentrations (IQR) were calculated for each marker and category and Mann-Whitney *U* test was used for comparisons between the groups (IV). Kaplan-Meier survival plots and ROC analysis were performed to estimate the potential of cystatin C measured by the new dry-reagent double monoclonal assay in risk prediction (IV). To

estimate the independency of cystatin C in risk prediction, multivariate analysis by Cox proportional hazards model was conducted. All candidate variables were included in the model and backward elimination was used to exclude variables with a *P*-value greater than 0.10.

Unless otherwise stated in the original publications, the statistical analyses were conducted using Stata 10.0 (Stata Corp, College Station, TX) or SAS Enterprise Guide 4.1 and SAS software version 9.1 (SAS Institute Inc., Cary, NC, USA). In all statistical analyses, *P*-values less than 0.05 were considered significant.

5 SUMMARY OF RESULTS AND DISCUSSION

Summary of the results of the original publications is presented in this section. In addition, some unpublished data are included.

5.1 ANALYTICAL PERFORMANCE OF IMMUNOASSAYS

5.1.1 Manually performed, methodologically different assays (I)

Three cystatin C assays, based on time-resolved fluorometry, were developed using different assay formats: a noncompetitive heterogeneous (method 1), a competitive heterogeneous (method 2) and a competitive homogenous assay (method 3). The assays were constructed by using recombinant cystatin C, one monoclonal cystatin C-specific antibody and one polyclonal, affinity-purified cystatin C-specific antibody. Due to the long-lasting fluorescence of lanthanide ions, such as Eu^{3+} used in this study, the emission or – in case of the RET assay – transferred energy emitted by Cy5 could be measured in time-resolved mode, which greatly reduces the nonspecific background fluorescence (Stenroos et al., 1998). The essential analytical performance characteristics of the assays are summarized in Table 9.

Table 9. Analytical performance characteristics of manually performed immunoassays.

| | Method 1 | Method 2 | Method 3 |
|--------------------------------|---------------------------------|------------------|-----------|
| Assay range (mg/L) | 0.0005–0.2 | 0.05–1.0 | 0.25–20 |
| [undiluted samples] | [0.05–20] | [0.5–10] | [0.25–20] |
| Analytical sensitivity (mg/L) | 0.00016 | 0.027 | 0.095 |
| [undiluted samples] | [0.016] | [0.27] | [0.095] |
| Recoveries (%) | 90–106 | 87–145 | 93–122 |
| Binding (% of maximum) | 1. step 92–100 2. step 78–94 | 53–66 | 98–100 |
| Time to reach saturation (min) | 1. step 60 2. step 120 | 180 ¹ | 10 |

Method 1, heterogeneous noncompetitive assay; method 2, heterogeneous competitive assay; method 3, homogenous competitive assay.

¹ highest studied time point.

Method 3 was the easiest to perform with one incubation step, without shaking or separation. Method 1 required two incubation steps and it was the slowest of the assays. Methods 1 and 3 showed the best performance in measuring cystatin C in terms of analytical recoveries and a working range (CVs <20 %) that covered all clinically relevant cystatin C concentrations in circulation (0.4–14.1 mg/L, according to (Kyhse-Andersen et al., 1994).

The wide assay range and high sensitivity of method 1 is due to reagent excess assay design. It enabled a high dilution of sample, which – especially if combined with wash steps – is likely to remove possible interferences from sample matrix. In contrast, the homogeneous competitive design of the method 3 led to an insensitive assay that allowed samples to be measured without dilution. Homogeneous designs, where binding reactions happen in solution phases, possess negligible steric hindrance. Owing

to this, method 3 was the fastest method for quantitative detection of plasma cystatin C: the steady-state for binding was reached in 10 minutes, and in 2.5 minutes the obtained signal level was greater than or equal to 95 percent of the steady-state signal level. Method 3 could potentially be optimized to provide quantitative, rapid, high-throughput detection of plasma cystatin C.

Methods 2 and 3 had narrower assay ranges due to the reagent-limited assay design. This was particularly pronounced in method 2, where the reliable assay range did not cover all the theoretical cystatin C concentrations in plasma. Combined to the highly varied plasma recoveries that may be related to assay kinetics, method 2 did not achieve a reliable measurement of circulating cystatin C. The limited performance of method 2 may also be partly caused by interferences from sample matrix, e.g., as interference leading to unequivocal binding of free and labeled cystatin C to the immobilized antibodies. The labeling of cystatin C with a high excess of chelate and the purification with HPLC also may introduce imprecision due to potential changes in the labeled cystatin C. In contrast, the labeling of cystatin C in method 3 was done by using mild labeling and separation conditions as the lowest label degree and resulted in the best signal-to-background ratio. This observation is in agreement with previously reported results (Qin et al., 2003).

The analytical performance of these assays compared to each other and to a conventional PETIA assay was briefly demonstrated by assaying 30 lithium-heparinized plasma samples. The cystatin C concentrations measured with the PETIA method ranged from 0.69 to 5.03 mg/L. Deming regression analysis with the PETIA method produced the following data: Method 1, slope 1.454 (SD, 0.059), y-intercept 0.165 (SD, 0.114) mg/L, $Sy|x$ 0.346 mg/L; Method 2, slope 1.079 (0.033), y-intercept 0.213 (0.632) mg/L, $Sy|x$ 0.194 mg/L; Method 3, slope 1.403 (0.028), y-intercept 0.001 (0.054) mg/L, $Sy|x$ 0.166 mg/L (n=30). According to these results, method 2 had a constant difference, method 3, a proportional difference and method 1 both types of differences. Unlike the other developed methods, method 1 uses a monoclonal antibody, which may contribute to the differences observed between it and the other methods. When the results of individual samples were compared, the developed, manually performed assays showed higher cystatin C levels than the conventional method: the mean difference between the new methods and the PETIA method was 44 percent (95% limits of agreement, 17–72%) for method 1, 22 percent (3–41%) for method 2 and 34 percent (15–53%) for method 3. Potential causes for the observed differences include methodological differences, such as the substantial difference in the assay formats and detection methodologies, and the quantification of the in-house-produced calibrator that was used. Considerable calibrator-related differences in cystatin C levels have been reported for particle-enhanced assays (Stowe et al., 2001).

It was originally hypothesized that method 1 that requires a high dilution, involves washing steps, and needs two specific bindings to generate specific signal, may be less prone to interferences caused by the sample material than especially method 3 with no pre-dilution of the sample and a homogeneous design. However, this was not clearly

demonstrated with the small number of samples analyzed, although there were sample-specific differences between the three research assays observed not only as the mean difference in Bland-Altman analysis but also as a wide limits of agreement: mean difference (95% limits of agreement) was 23 percent (-10–56%) between method 2 and method 1, 11 percent (-23–44%) between method 3 and method 1 and 13 percent (-11–36%) between method 3 and method 2. One potential cause for the sample-specific differences among the three assays was that only method 3 was analysed at equilibrium, while methods 1 and 2 were far from equilibrium making them more vulnerable to variations in, e.g., assay timing. Due to high sample fraction in method 3 (20 μ L of undiluted sample in total reaction volume of 120 μ L), for further use, careful evaluation of sample interferences would need to be carried out.

5.1.2 Dry-reagent double monoclonal assay (II)

Based on the results of the three assays described above, the recent introduction of new monoclonal antibodies for cystatin C and good experience on similar assay configurations, e.g., for cTnI (Pettersson et al., 2000), the work continued with the reagent-excess assay design and aimed to improve the assay by utilizing monoclonal antibodies to form a double monoclonal “sandwich” complex capable of recognizing cystatin C. Specifically, the aim was to develop a one-step assay and reduce the total assay time to less than 20 minutes. To overcome the problems related to the manual performing of the assay, the assays utilizing the most promising antibody pairs were adapted to a dry-reagent format that allowed performing of the assays in an automated immunoanalyzer enabling precise timing and dispensing.

Generating a schematic epitope map and selection of the antibody pair

A schematic epitope map was generated by using 7 monoclonal cystatin C antibodies (Figure 9). In total, 42 combinations were tested for the ability to form 2-site complexes with monomeric cystatin C. Twenty-six of these antibody pairs produced a response for cystatin C, which differed significantly from nonspecific binding and background noise. Based on the results, the tested antibodies represented 4 different recognition sites (epitopes) on cystatin C: i) Cyst10; ii) Cyst13, Cyst19; iii) Cyst23, Cyst24; and iv) HCC3 (Figure 9). The seventh monoclonal antibody, Cyst28, detected an epitope that closely resembled the epitope recognized by Cyst13 and Cyst 19.

The antibody pair for the dry-reagent double monoclonal (2-site) assay, Cyst28 – Cyst24, was selected according to the results from epitope mapping tests and studies of assay kinetics and plasma recoveries with viable antibody pairings. A more detailed description of the selection can be found in the publication II. Briefly, those of the 26 viable antibody pairs that showed: i) consistent binding of recombinant and plasma cystatin C (difference <10%), ii) favourable kinetic properties (10- to 60-min binding ratio 76–100%), iii) uniform dose–responses over 3 decades of recombinant cystatin C, and iv) less than 10% decrease in signal per hour after the steady state of binding was reached, were selected for further evaluation. Three antibody pairs fulfilled these criteria: Cyst24 – Cyst19, HCC3 – Cyst24 and Cyst28 – Cyst24. For

practical reasons, one antibody pairing was finally selected. The selection of the pair Cyst28 – Cyst24 was based on excellent linearity, fast and consistent binding of recombinant vs. plasma cystatin C throughout the working range, and good correlation to the PETIA method.

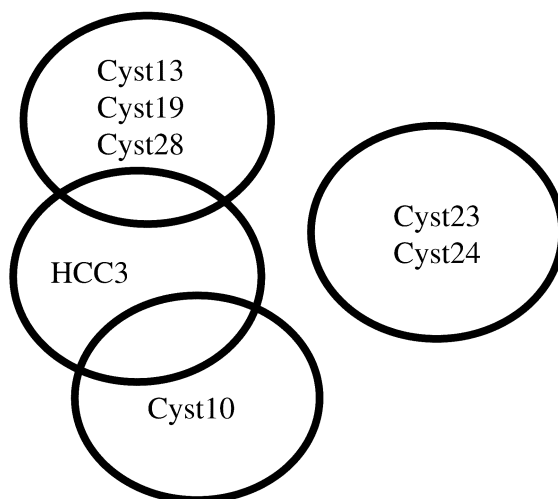


Figure 9. Schematic epitope map constructed with seven monoclonal antibodies. Overlapping ovals indicate non-functional antibody pairs whereas separate ovals indicate functional antibody pairs. The “Cyst” antibodies are all from HyTest. For the HCC3 antibody, see Olafsson et al. 1988. Adapted from (II).

Analytical performance of double monoclonal assay

The dose-response curve and CVs are shown in Figure 10. The assay, run on an automated immunanalyzer, gave a linear response from 0.001 to 0.5 mg/L. When a 100-fold dilution of sample was used, the range (0.1–50 mg/L) covered the cystatin C concentrations in human serum/plasma in health and disease (Kyhse-Andersen et al., 1994). Approximately 93 percent of the steady-state signal level was observed after the 10-minute incubation of the assay and the total assay time for one measurement was 15 minutes.

The other analytical performance characteristics of the assay were as follows: The analytical (calculated as the mean background signal + 3 SD) and functional detection limits were ≤ 0.0001 mg/L and 0.0002 mg/L (corresponding plasma concentrations ≤ 0.01 mg/L and 0.02 mg/L). Serial dilutions of three plasma and two serum samples showed a linear response (Pearson r , 0.993–0.999). Plasma recoveries varied from 94 percent to 110 percent and within-run and total assay imprecision were less than 4.7 percent and less than 5.6 percent, respectively, for control plasma samples whose cystatin C concentrations were 0.84, 2.0, and 3.2 mg/L.

In the presence of potentially interfering substances, bilirubin (257 $\mu\text{mol/L}$), hemoglobin (5 g/L) and triglycerides (5.6 mmol/L), the change in measured cystatin C values was clinically negligible (<1.5%).

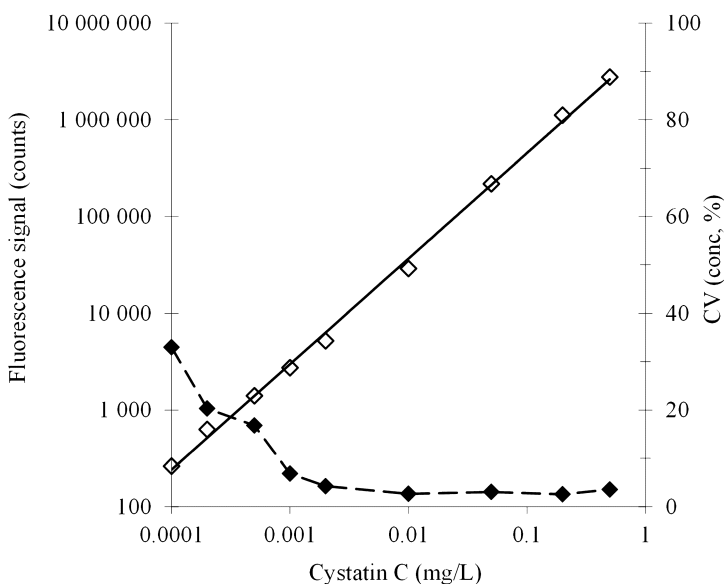


Figure 10. Dose-response curve (hollow diamonds) and the corresponding coefficients of variation (CVs) calculated from concentration values (solid diamonds) for the dry-reagent double monoclonal assay. Adapted from (II).

The analytical performance characteristics closely resembled those of the manually performed 2-site assay (method 1) described in section 5.1.1, with corresponding sensitivities, assay range, CVs, strong linearity and plasma recoveries. It was noted that within-assay imprecision did not improve by the use of an automated analyzer (3.2% vs. 4.7%), possibly due to the reagent-excess design of the assay. Similarly, as in method 1, the high sensitivity of the assay enabled high dilution of sample (1:100), which largely reduced vulnerability of the assay to interference from plasma components. A 2-site assay utilizing native (non-fragmented) antibodies from same species for capture and detection is particularly vulnerable to interference from heterophilic antibodies. The use interference blockers, specifically native and denatured mouse IgG, in the double monoclonal assay together with the high dilution of sample remarkably reduced the risk of interference from heterophilic antibodies.

The applicability of the new assay to measure plasma cystatin C was investigated by comparing the cystatin C concentrations of 132 plasma samples measured by the new assay to values obtained with the PETIA assay. The correlation between ranked cystatin C values measured by the two methods was good, 0.949 (Spearman *rho*). According to Deming regression parameters: slope, 1.391 (SD, 0.029); y-intercept, 0.152 (0.045) mg/L; $Sy|x$ 0.294 mg/L, the double monoclonal assay showed a clear proportional difference as compared to the PETIA method. This was also observed from a Bland-Altman analysis: according to the mean of the differences, the

concentrations measured with the new assay were about 20 percent higher than the PETIA values of the same samples. This differs from method 1, where a 44 percent mean difference to the PETIA method was observed. For the same 30 samples used in the evaluation of method 1, the mean difference between the new double monoclonal assay and the PETIA method was 23 percent. Potential explanations for the observed in-house difference are the antibodies used (double monoclonal vs. one polyclonal and one monoclonal), one-step vs. two-step assay format and automated vs. manual performing of the assays. Notably, the saturation of the HCC3 (monoclonal antibody) binding step in method 1 was not reached until 120 min, while equilibrium was reached for different double monoclonal combinations using HCC3 already after 10 min. A plausible explanation for this is that in method 1, polyclonal antibody partly recognized the same epitope as HCC3 and the competition for binding of cystatin C slowed down reaching the equilibrium. This could have caused differences in performance of the two 2-site assays. Differences may have also occurred due to potentially different recognition of recombinant calibrator cystatin C and endogenous sample cystatin C by polyclonal and monoclonal antibodies. Furthermore, no standardized reference material existed for cystatin C at that time and moderate measurement error may have occurred in quantification of the calibrator cystatin C.

5.2 CLINICAL PERFORMANCE OF DRY-REAGENT DOUBLE MONOCLONAL ASSAY (III, unpublished)

The clinical performance of the dry-reagent double monoclonal assay for cystatin C was evaluated in panels consisting of either men with normal renal function (median iohexol clearance 81 mL/min/1.73 m²; IQR 70–92; group A) or men with diminished renal function as a consequence of renal disease (median iohexol clearance 23 mL/min/1.73 m²; IQR 16–34; group B). The cystatin C values measured with the new assay were compared to other measures of kidney function – serum creatinine concentration and iohexol clearance that was considered equal to inulin clearance and represented an accurate GFR.

The scatter plots for 1/cystatin C and true GFR (iohexol clearance) in both groups are shown in Figure 11. The calculated concordance correlation coefficients with true GFR were 0.441 in group A and 0.680 in group B for the new assay whereas the corresponding values for the PETIA method were 0.465 and 0.593. The average difference (limits of agreement) between 1/cystatin C values and the measured GFR are shown in Table 10. At higher levels of GFR, the relationship between 1/creatinine and true GFR was not linear, which was seen as a low concordance correlation in group A: 0.161. In group B the concordance correlation was 0.499 – creatinine had closer agreement with the true GFR at greatly diminished levels of renal function (<30 mL/min/1.73 m²) – however, overall and at higher levels of GFR cystatin C measured with both assays showed closer agreement with true GFR than did creatinine.

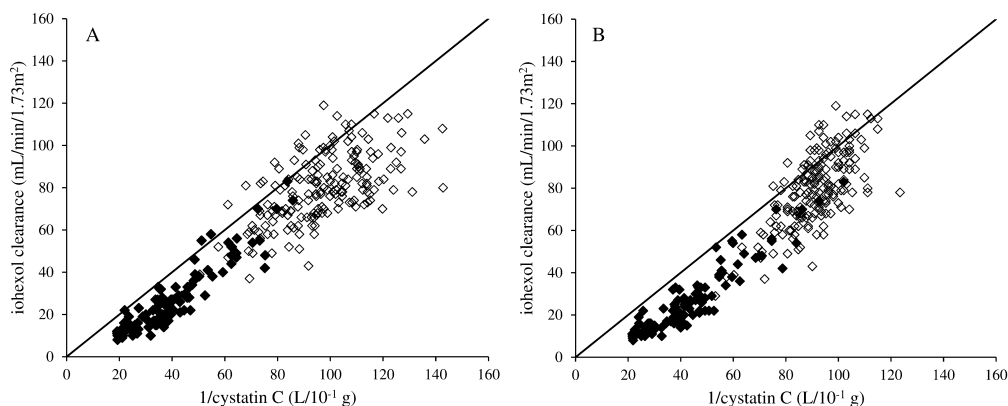


Figure 11. Scatter plots for 1/ cystatin C and iohexol clearance. A, the developed dry-reagent double monoclonal cystatin C assay; B, particle-enhanced turbidimetric assay. The black line represents perfect agreement. Hollow diamonds denote patients with normal kidney function (Group A) and solid diamonds denote patients with diagnosed chronic kidney disease (Group B). Adapted from (III).

The agreement between 1/cystatin C and iohexol clearance was higher in the patients with diagnosed renal disease (group B) than in those with normal renal function (group A). The double monoclonal cystatin C assay was equal to the conventional PETIA method in patients with normal renal function and was better in patients with renal disease (higher concordance correlation). Based on the average differences between reciprocal cystatin C values and the measured GFR, both cystatin C assays tended to overestimate GFR. However, the reciprocal of cystatin C was used to linearize the relationship between cystatin C and GFR but it is not expected to perfectly estimate GFR values without correction factors. An equation to calculate eGFR values, based on cystatin C levels, has been developed and published for use with the conventional PETIA assay (Grubb et al., 2005). It was, however, not used in this study as eGFR equations are method- and calibration-specific (Delanaye et al., 2008; Poge et al., 2008) and the accuracy of the eGFR equation would be biased in favor of the conventional assay and would not permit a fair comparison between the conventional and the new cystatin C assay.

Table 10. Agreement of 1/cystatin C values with measured GFR (iohexol clearance).

| | Group A | | Group B | |
|----------------------|----------------|---------------------------------------|----------------|---------------------------|
| | Median | Average difference¹ | Median | Average difference |
| | [IQR] | (95% limits of | [IQR] | (95% limits of |
| | | agreement) | | agreement) |
| 1/cystatin C (PETIA) | 93 [86–99] | 11.6 (-12.6, 35.8) | 41 [33–52] | 16.8 (4.4, 29.3) |
| 1/cystatin C (new) | 97 [88–110] | 17.1 (-10.8, 45.1) | 38 [29–48] | 13.1 (0.9, 25.3) |

¹Difference is the remainder of 1/cystatin C (considered as eGFR here) minus true GFR (iohexol clearance)

Group A, patients with normal kidney function; Group B, patients with diagnosed chronic kidney disease; New, dry-reagent double monoclonal assay; PETIA, particle-enhanced turbidimetric assay.

In receiver operating characteristic (ROC) analysis with pooled groups A and B, similar discrimination was observed at iohexol clearance of 60 mL/min/1.73 m² for the new cystatin C assay (area under the curve, AUC, 0.984) and the PETIA method (AUC, 0.981), $P > 0.05$.

The strength of this comparison was to include a wide range of measured GFR values as the range strongly affects the comparison; within the “normal” range of GFRs, the correlation is worse (Kyhse-Andersen et al., 1994). Only male patients were assessed; thus a similar comparison against measured GFR in females is warranted.

Intraindividual variation

Intraindividual variation of reciprocal cystatin C was measured in Group A (normal renal function) using the new dry-reagent double monoclonal assay. Three samples for each patient were available: the first sample that was drawn before measurement of iohexol clearance, the second after 4 hours (range: 3–7 hours) and third after 12 days (range: 6–38 days). The mean (SD) CV of cystatin C concentrations was 6 percent (4%) between the first and the third time point, and 5 percent (4%) between first and second or second and third time point. Thus, the new cystatin C assay exhibited small and clinically insignificant mean intraindividual variability across serial samples. Considering the imprecision of the new assay, the intraindividual variation is barely high enough to be determined reliably. However, similar intraindividual variation has been reported with a PENIA cystatin C assay: 5.4 percent over 5 months (Toffaletti and McDonnell, 2008) and 6.8 percent over 18 days (Selvin et al., 2013).

5.3 RISK PREDICTION

Among 245 patients hospitalized due to symptoms consistent with acute MI, and diagnosed with a non-ST elevation ACS, the predictive value of admission cystatin C, measured with the dry-reagent double monoclonal assay, was studied and compared to other estimates of renal function.

5.3.1 Baseline characteristics (IV, unpublished)

The mean age was 70 years (range: 36–93), and the median cystatin C measured with the new assay was 1.07 mg/L (IQR 0.89–1.34 mg/L). For statistical analyses, the study population was divided into three groups according to their admission cystatin C levels: i) <0.96 mg/L; ii) 0.96–1.21 mg/L; and iii) >1.21 mg/L. The baseline characteristics, including levels of biochemical markers and eGFRs, by tertiles of admission cystatin C are shown in Table 11.

No significant differences between the groups were found for body to mass index, diabetes, family history of CVD, hypertension, maximum cTnI, sex or smoking status. However, patients within the highest tertile of cystatin C concentrations were older, had higher admission CRP and 24-hour BNP and were more likely to have chronic HF or previous MI than patients within the lowest tertile of cystatin C levels. Of the whole

group, 33 percent had a previous MI and 32 percent had a chronic HF. Significant correlations existed between cystatin C and creatinine (0.758; $P<0.0001$), admission CRP (0.287; $P=0.0002$), eGFR-CG/BSA (-0.741; $P<0.0001$) and 24-hour BNP (0.543; $P<0.0001$). No significant correlation was observed between cystatin C and maximum cTnI (0.119; $P=0.129$).

Table 11. Baseline characteristics according to cystatin C tertiles. Adapted from (IV).

| | Cystatin C | | |
|--|------------------|------------------------------|-------------------------------|
| | <0.96 mg/L | 0.96–1.21 mg/L | >1.21 mg/L |
| n | 84 | 82 | 79 |
| Men | 51 (61%) | 43 (52%) | 37 (47%) |
| Chronic heart failure | 14 (17%) | 27 (33%), $P=0.046^1$ | 37 (47%), $P=0.0001$ |
| Current smoking | 30 (36%) | 16 (20%) | 20 (25%) |
| Diabetes mellitus | 17 (20%) | 15 (18%) | 18 (23%) |
| Family history of cardiovascular disease | 30 (36%) | 31 (38%) | 27 (34%) |
| Hypertension | 26 (31%) | 27 (33%) | 27 (34%) |
| Previous myocardial infarction | 16 (19%) | 34 (42%), $P=0.0048$ | 30 (38%), $P=0.022$ |
| Age (y) | 62 (11) | 69 (10), $P<0.0001$ | 76 (9), $P<0.0001$ |
| Body mass index (kg/m^2) ² | 27.4 (3.9) | 27.4 (4.2) | 27.4 (5.2) |
| BNP (ng/L) ³ | 7.7 [1.6–25.5] | 23.4 [10.3–59.9], $P<0.0001$ | 58.1 [20.2–109.0], $P<0.0001$ |
| CRP (mg/L) ³ | 2.15 [0.95–6.35] | 2.30 [1.00–7.20] | 4.70 [1.90–10.80], $P=0.0075$ |
| cTnI (ng/mL) | 0.03 [0.01–0.74] | 0.04 [0.01–0.63] | 0.06 [0.01–1.93] |
| Creatinine ($\mu\text{mol}/\text{L}$) | 87 [79–94] | 95 [88–103], $P<0.0001$ | 119 [103–141], $P<0.0001$ |
| eGFR-CG/BSA ($\text{mL}/\text{min}/1.73 \text{ m}^2$) ² | 76 (17.4) | 61 (15.2), $P<0.0001$ | 44 (15.5), $P<0.0001$ |

For categorical variables, data are expressed as numbers (percentages). For continuous variables, data are expressed as mean (SD), or as median [IQR].

BNP, brain natriuretic peptide (24 h); CRP, C-reactive protein on admission; cTnI, cardiac troponin I (maximum); eGFR, estimated glomerular filtration rate; CG/BSA, Cockcroft–Gault adjusted for body surface area.

¹Chi-square test for categorical variables and Mann-Whitney U -test or unpaired t -test for continuous variables. All analyses have been performed with first tertile (cystatin C <0.96 mg/L) as reference. Data without a P -value are non-significant ($P>0.05$). ² $n=242$ ³ $n=244$.

Due to known interactions between declining cardiac and kidney function, the relation of cystatin C, creatinine and eGFR-CG/BSA levels to the delay from the onset of symptoms to admission was studied. No association between kidney function and the length of the delay was found – within 24 hours, the delay to the admission blood sampling had no significant decreasing or increasing effect on levels of kidney function estimates, $P>0.05$ for all paired comparisons (Table 12). The predictive potential of decreased kidney function thus is due to other mechanisms.

Table 12. Cystatin C, creatinine and eGFR median levels [IQR] according to delay from the onset of symptoms to the admission blood sampling. (Data for creatinine and eGFR-CG/BSA are unpublished.)

| Delay (h) | N | Cystatin C (mg/L) | Creatinine (µmol/L) | eGFR-CG/BSA (mL/min/1.73 m ²) |
|-----------|----|-------------------|---------------------|---|
| 0–3 | 46 | 1.01 [0.86–1.35] | 95 [86–111] | 59 [45–76] |
| 3–6 | 40 | 0.99 [0.88–1.30] | 95 [81–106] | 63 [47–82] |
| 6–12 | 28 | 1.17 [0.97–1.58] | 94 [85–108] | 64 [39–74] |
| 12–24 | 21 | 1.02 [0.88–1.14] | 91 [82–103] | 64 [55–75] |

eGFR-CG/BSA, estimated glomerular filtration rate calculated with Cockcroft–Gault equation and adjusted for body surface area.

5.3.2 Cystatin C and outcome (IV)

Twenty-five deaths (11%) and 34 MIs (14%) occurred during the 1-year follow-up period. All-cause mortality and combined events (all-cause mortality and MI) were more prevalent with higher serum cystatin C values, 3rd vs 1st tertile $P=0.012$ and 0.0012 , respectively (Figure 12). The relation of cystatin C with MI, however, was not statistically significant ($P=0.227$). Correspondingly, in the Kaplan–Meier survival analysis, tertile groups of cystatin C showed significant differences for all-cause mortality (test of equality over strata, $P=0.012$) and combined events ($P=0.0011$).

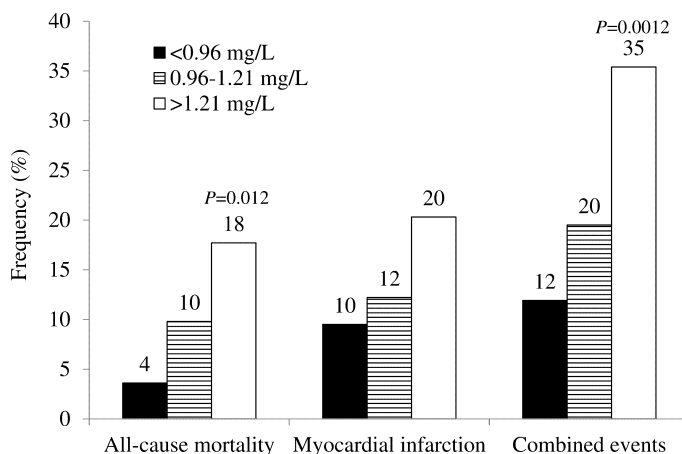


Figure 12. Frequency of study endpoints according to tertile groups of admission cystatin C. The follow-up period was 1 year. Significant differences ($P<0.05$) for comparisons *versus* the 1st tertile (<0.96 mg/L) have been marked with the actual P -values.

Although the new cystatin C assay agrees well with conventional PETIA method, some sample-specific differences occur (II, III) likely due to different assay technology. A comparison to a conventional cystatin C assay in risk prediction would have been interesting, but was not possible to study with this data set due to limited sample volume.

5.3.3 Cystatin C vs. other markers (IV, unpublished)

The predictive power of cystatin C was compared to creatinine and eGFR. Estimated GFR was calculated with CG equation adjusted for body surface area, as the use of MDRD Study equation is known to be limited for GFR values less than 60 mL/min/1.73 m². The frequency of all-cause mortality, MI and combined events in tertile groups of creatinine and eGFR-CG/BSA are collected to Table 13. Similarly as cystatin C, eGFR-CG/BSA had significantly higher rates of all-cause mortality and combined events in the third tertile compared to the first tertiles (Table 13). There seemed to be the least mortality events in the second tertile group with creatinine concentrations from 89 to 103 µmol/L. A similar association of middle quintiles of creatinine with death may exist in elderly persons, whereas cystatin C concentrations were directly associated with increased risk of mortality (Shlipak et al., 2005). Cystatin C has higher sensitivity to detect minor, though clinically significant, impairments of kidney function in the normal GFR range compared to creatinine, and steeper increase in plasma levels compared to creatinine in the elderly (Shlipak et al., 2005; Shlipak et al., 2009a; Christensson and Elmstahl, 2011).

Table 13. Frequency of study endpoints according to tertile groups of admission creatinine and estimated glomerular filtration rate calculated with Cockcroft–Gault equation and adjusted for body surface area (eGFR-CG/BSA). The follow-up period was 1 year. Significant ($P<0.05$) differences for comparisons versus the 1st tertile have been marked with the actual P -values. Note that eGFR-CG/BSA groups are from higher to lower eGFR values.

| | Creatinine (µmol/L) | | |
|-----------------------|---|--------|-------------------|
| | <89 | 89–103 | >103 |
| All-cause mortality | 8.3% | 2.5% | 19.5% |
| Myocardial infarction | 11.9% | 12.7% | 17.1% |
| Combined events | 17.9% | 13.9% | 34.2% |
| | eGFR-CG/BSA (mL/min/1.73 m ²) | | |
| | >70 | 51–70 | <51 |
| All-cause mortality | 5.1% | 4.8% | 21.3%, $P=0.012$ |
| Myocardial infarction | 9.0% | 16.7% | 15.0% |
| Combined events | 11.5% | 20.2% | 33.8%, $P=0.0027$ |

Receiver operating characteristic (ROC) analysis was performed for all-cause mortality and combined events. For all-cause mortality, the area under the ROC curve (AUC) for cystatin C, creatinine and eGFR-CG/BSA was 0.73, 0.65, and 0.72, respectively. Although cystatin C seemed to predict outcome better than creatinine and equally compared to eGFR-CG/BSA, the differences in the AUC values between cystatin C and other renal estimates were not statistically significant, $P=0.14$ and $P=0.79$ for creatinine and eGFR-CG/BSA, respectively. The AUCs for combined events were 0.67 (cystatin C), 0.61 (creatinine), and 0.65 (eGFR-CG/BSA), and the P -values for comparisons between cystatin C and creatinine or eGFR-CG/BSA were 0.19 and 0.49, respectively. Although cystatin C seemed to have the highest AUC values, the small sample size may contribute to the statistical insignificance compared to creatinine.

The Cox proportional hazards regression model was used to estimate the independency of cystatin C, creatinine and eGFR-CG/BSA in predicting the risk of all-cause mortality and combined events. In univariate analysis, an increase in tertile group of any of the

estimates of kidney function contributed to increased risk of endpoints (Table 14). The HRs calculated after adjustment to baseline characteristics and/or biomarkers, all converted to two-class categorical variables, were as shown in Table 14. Although higher cystatin C levels were associated with older age, chronic HF and previous MI, cystatin C remained an independent and significant predictor of death and combined events after adjustment to non-biomarker baseline variables. Similarly, eGFR-CG/BSA was found to be an independent predictor of endpoints. Creatinine lost its predictive value after adjustment for other factors. When non-renal biochemical markers were included in the “cystatin C” model (i.e., “All variables” in Table 14), 24-hour BNP over median (\leq / $>$ 21.9 ng/L; adjusted HR=4.48; 95% CI, 1.63–12.3; P =0.0037) and admission CRP (\leq / $>$ 2 mg/L; adjusted HR=2.98; 95% CI, 1.02–8.75; P =0.0465) were independent predictors of all-cause mortality. In case of eGFR-CG/BSA, it remained a significant predictor of all-cause mortality together with 24-hour BNP over median (adjusted HR=3.84; 95% CI 1.42–10.37; P =0.0079) and admission CRP (\leq / $>$ 2 mg/L; adjusted HR=3.03; 95% CI, 1.03–8.90; P =0.0433). However, cystatin C together with cTnI status (\leq / $>$ 0.03 ng/mL; adjusted HR=0.49, 95% CI, 0.27–0.88; P =0.0175) was an independent and significant predictor of combined events. Also eGFR-CG/BSA was a significant predictor of combined events, together with admission CRP (\leq / $>$ 2 mg/L; adjusted HR=1.95; 95% CI, 1.03–3.69; P =0.0392) and cTnI status (\leq / $>$ 0.03 ng/mL; adjusted HR=0.54, 95% CI, 0.30–0.99; P =0.0456).

Table 14. Unadjusted and adjusted hazard ratios for estimates of kidney function.

| | All-cause mortality HR (95% CI) | P-value | Combined events¹ HR (95% CI) | P-value |
|--|--|----------------|--|----------------|
| Cystatin C | | | | |
| Univariate | 2.19 (1.27–2.77) | 0.0046 | 1.86 (1.31–2.65) | 0.0005 |
| Non-biomarker baseline variables ² | 2.19 (1.28–3.78) | 0.0046 | 1.75 (1.22–2.51) | 0.0024 |
| All variables ³ | 1.58 (0.92–2.72) | 0.0967 | 1.57 (1.09–2.26) | 0.0149 |
| Creatinine | | | | |
| Univariate | 1.83 (1.09–3.08) | 0.0225 | 1.54 (1.09–2.16) | 0.0132 |
| Non-biomarker baseline variables | 1.63 (0.95–2.79) | 0.0736 | 1.39 (0.98–1.97) | 0.1538 |
| All variables | 1.32 (0.79–2.22) | 0.2934 | 1.27 (0.89–1.81) | 0.1889 |
| eGFR-CG/BSA | | | | |
| Univariate | 2.56 (1.43–4.59) | 0.0016 | 1.82 (1.27–2.60) | 0.0011 |
| Non-biomarker baseline variables | 2.56 (1.43–4.59) | 0.0016 | 1.76 (1.23–2.53) | 0.0022 |
| All variables | 1.88 (1.04–3.42) | 0.0379 | 1.54 (1.07–2.22) | 0.0022 |

Data are expressed per increase of one tertile.

CI, confidence interval; eGFR-CG/BSA, estimated GFR according to Cockcroft-Gault formula and adjusted for body surface area; HR, hazard ratio

¹Combined events include all-cause mortality and myocardial infarction.

²Candidate variables entered to the model were: age (\leq / $>$ 65 y), body mass index (median; \leq / $>$ 27.1 kg/m²), gender, chronic heart failure, hypertension, previous myocardial infarction, current smoking, diabetes mellitus, and family history of cardiovascular disease.

³Candidate variables included in the model were 24-hour B-type natriuretic peptide (\leq / $>$ 21.9 ng/L (median)), admission C-reactive protein (\leq / $>$ 2 mg/L), cardiac troponin I positivity (\leq / $>$ 0.03 ng/mL), and all variables that were entered to the first model.

Compared to creatinine, cystatin C was a better predictor of study endpoints. When eGFR was calculated according to CG formula and adjusted for body surface area, it equally predicted adjusted risk of combined endpoints compared to cystatin C, and remained a significant predictor of all-cause mortality after adjustments in this study population. The calculation of eGFR-CG/BSA requires measures such as weight and height that may not always be easily obtained. The data acquired in this study suggests that cystatin C is a superior measure compared to creatinine and is as effective as a risk marker compared to the more complicated eGFR-CG/BSA test.

Cystatin C may be linked to higher risk of death independently of kidney function (Shlipak et al., 2005; Stevens and Levey, 2005; Peralta et al., 2011a). Due to the lack of true GFR, as measured by the clearance of an exogenous substance, the association of cystatin C alone to risk of death could not be studied with this data set.

Admission CRP and 24-hour BNP, established biomarkers in ACS, were strong predictors of all-cause mortality. Cystatin C had a significant positive correlation with both markers. For BNP, the correlation is likely explained by the close connection of cardiac and renal function, as plasma levels of BNP increase with declining ventricular function. Although a significant correlation between cystatin C and CRP level was a common finding (Knight et al., 2004; Koenig et al., 2005; Keller et al., 2007), no causal relationship has been found between cystatin C and inflammation (Grubb et al., 2011). However, evidence of systemic inflammation, such as increased plasma CRP concentration, is reported to be associated with kidney disease (Shlipak et al., 2003). Inflammation may also mediate some type of progressive kidney disease (Tonelli et al., 2005). It has also been suggested that the association between CRP and cystatin C may be linked to atherogenic process (Ross, 1999). Despite the observed strong correlation, in this study, BNP and CRP were independent risk markers of all-cause mortality.

6 CONCLUSIONS

Cystatin C, a small, globular, reversible cysteine protease inhibitor is a new marker of kidney function. Cystatin C fulfills many criteria that are set to an ideal endogenous biomarker of kidney function. Still, detection of CKD in routine practice is traditionally limited to serum creatinine measurement. One of the clear advantages of cystatin C is that muscle mass, that greatly limits the potential of creatinine as a marker of kidney function, does not affect its circulating concentration. There are other factors, though, which stimulate or repress the production of cystatin C. However, non-GFR determinants affect the levels of all known endogenous markers of GFR and thus no marker perfect for all situations exists. Non-GFR factors, particularly if not yet well understood, complicate the interpretation of results. Efforts to correct for known affecting factors have been made: several formulae correcting for measures of body size, such as age and weight, are used for estimation of GFR from single creatinine measurement. Estimated GFR values are used to unify the reporting of measures of kidney function and, thus, also cystatin C is often mathematically converted to eGFR values. For cystatin C, formulae for calculation of eGFR do not incorporate other measurable variables, simplifying the data needed for the calculation. Although serum markers appear to provide adequate assessment of GFR, wide confidence limits in difference plots between eGFR and GFR measured using reference clearance methods suggest that no existing equation, whether based on creatinine, cystatin C or both, can conclusively define GFR.

The most significant potential of cystatin C as a diagnostic marker lies in diagnosing mild renal dysfunction, especially in persons with varying and extremes of muscle mass, for example newborns, small children and the elderly. These populations could benefit from cystatin C, either as a primary (“first line”) kidney function test or as a confirmative test in situations where creatinine-based estimates of GFR may be biased. Further potential of cystatin C includes its use as a marker of predicting increased cardiovascular risk, as higher levels of cystatin C appear to be associated with cardiac adverse events. While the role of cystatin C as a marker of renal function is widely acknowledged, the relevance and interpretation of cystatin C as a predictive marker of cardiac outcome is not yet as extensively studied. However, recent results highlight the superiority of cystatin C over creatinine in determining risk based on kidney function.

Plasma concentration of cystatin C is currently mainly assessed with automated turbidimetric or nephelometric immunoassay methods. Variability in reference levels, and differences between results obtained with different methods have complicated the introduction of cystatin C in clinical practice despite its advantages over creatinine as a clinical marker of kidney function. The international standardization of cystatin C measurement is an important aspect on the way to routine clinical use of cystatin C. Factors that limit the use of cystatin C include the costs of cystatin C assays, which are at least two-fold compared to creatinine.

During this study, a reliable time resolved fluorescence-based immunoassay for quantification of human cystatin C was developed. The study started with a comparison of three methodologically different assays for cystatin C. The work was continued with the non-competitive design by assaying two-site combinations of 7 monoclonal antibodies. Based on the results, one antibody pair was selected for dry-reagent double-monoclonal assay. The clinical performance of the new assay was studied in estimating renal function and in predicting increased cardiac risk.

The main conclusions based on the original publications are:

- I** Both competitive and non-competitive assay formats based on time resolved fluorescence are applicable in quantitative measurement of cystatin C. A RET-based homogeneous competitive assay format was for the first time shown to be utilizable for cystatin C and it provided rapid easy-to-perform approach without need for sample predilution. A heterogeneous, non-competitive and a homogeneous competitive assay enabled reliable detection of plasma cystatin C over a working range that covered all clinically relevant cystatin C concentrations in circulation.
- II** Four different recognition sites for monoclonal antibodies on cystatin C were mapped. By utilizing a one-step double monoclonal approach and transferring the assay to an automated immunoanalyzer, the turnaround time of the heterogeneous, non-competitive assay was significantly reduced. Possibly owing to a high amount of dilution and a separation step, no interference from bilirubin, hemoglobin and triglycerides was observed at levels reported to have caused interference for some PETIA-type assays. Advantages of the assay include utilization of monoclonal antibodies, which (compared to widely used polyclonal antibodies) greatly decreases batch to batch variation in reagent and assay performance. The identical recognition of the antigen from batch to batch is beneficial from the standardization and calibration point of view.
- III** The clinical evaluation of the new double monoclonal assay for cystatin C showed close agreement with the conventional PETIA method. Furthermore, in male patients with CKD, the new assay showed closer agreement with true GFR than the PETIA method. Biological variation measured with the double monoclonal assay was low and comparable to the determinations reported in the literature.
- IV** In patients with confirmed non-ST elevation ACS, both the risk of all-cause mortality and the risk of combined events, i.e., death and MI, was found to be increased with elevated cystatin C measured with the new assay. Cystatin C remained an independent predictor of death and combined events, including death and MI, after adjustment with non-biochemical baseline factors. This is in accordance with the earlier reports where cystatin C has been measured by using conventional methods.

The possibilities of the homogenous competitive assay were not fully exploited during the study. The applied method could enable a very rapid and simple test for quantification of cystatin C. Due to the use of undiluted sample in a homogeneous assay format further investigations of the assay should include a careful evaluation of sample interferences.

In conclusion, based on a comparison of methodologically different time resolved fluorescence assays, a dry-reagent double monoclonal assay for cystatin C was developed and evaluated and it enabled rapid and reliable quantitative measurement of cystatin C. The assay showed strong agreement with the established PETIA method, and in patients with CKD it showed a better agreement with GFR measured by a reference clearance method than PETIA. As measured with the developed assay, cystatin C is a potential predictor of outcome in patients with nSTE-ACS. This further strengthens the important clinical role of cystatin C among markers of renal function. The quantification of cystatin C with the developed assay as such is not limited to these conditions. Future studies will show whether cystatin C could be used in other clinical settings, such as those related to neurological disorders.

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