CHAPTER TWO

Cystatin C: A Kidney Function Biomarker

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Abstract

Renal function is essential for homeostasis. The kidneys play important pleiotropic roles including removal of metabolic waste products and maintenance of water–electrolyte balance and blood pressure. Early diagnosis of renal dysfunction and institution of appropriate therapy are vital to survival. Unfortunately, common indicators of renal function lack necessary sensitivity and specificity. Recent evidence has, however, suggested that cystatin C (cysC) may be useful as a marker for glomerular filtration. CysC is a protein belonging to a group of cysteine proteases inhibitors produced primarily by nucleated cells. Due to low molecular weight and positive p*I*, it is easily filtered. Moreover, its serum concentration is independent of gender, age, or muscle mass, i.e., typical confounders in assessing glomerular filtration rate (GFR). This chapter discusses the structure and biologic function of cysC, its role as an indicator of GFR, and the most frequently used methods for its determination.

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1. INTRODUCTION

The role of the kidneys is pleiotropic. They excrete unnecessary and often toxic metabolic waste products (i.e., urea), regulate the volume and composition of body fluids, maintain water–electrolyte and acid–base balance, and promote normal blood pressure. Renal function is, therefore, essential for homeostasis due to its unique ability to shape and maintain the intraorganic biochemical environment. Disorders of glomerular filtration negatively impact the organism as a whole. As such, it is imperative to not underestimate any pathologic lesion within the urinary system. Early detection and resolution of the pathologic component or process is critical for survival. Once identified, immediate action is required to inhibit irreversible change in order to extend the timeframe preceding renal replacement therapy [1]. The diagnostic hallmark of renal failure is glomerular filtration rate (GFR), i.e., the amount of plasma filtered per unit time by the glomeruli.

2. CYSTATIN C—STRUCTURE AND BIOLOGIC FUNCTION

The name "cystatin" was used for the first time in 1981 by Barrett to describe a substance of protein nature that demonstrated the ability to inhibit lysosomal cysteine proteases. Since then, numerous proteins of similar activity have been described and are grouped as the cystatin superfamily [2,3].

Cystatins belong to a group of proteins possessing a characteristic tertiary structure and a capacity to tightly but reversibly bind the active site of cysteine proteases (cathepsins) and thus inhibit their activity. These competitive inhibitors are vital to controlling protease activity [3] in immune system modulation, antiviral, and antibacterial activity and participate in the body's response to brain injury. Physiologic imbalance of cystatins and protease activity may be the basis of many pathologic conditions [4].

Although cystatins are different biochemical molecules that vary in specificity, they are characterized by a common cystatin domain that consists of approximately 100 amino acid residues and the presence of stabilizing disulfide bonds. Variations within the cystatins are distributed into three families: stefins, competent cystatins, and kininogens [3,5].

Cystatin C (cysC), also called cystatin 3, is synthesized continuously by nucleated cells. It has a ubiquitous distribution in human tissue as well as

Figure 1 Dimeric form of human cystatin C [6].

body fluids. It is composed of 120 amino acids residues and has two disulfide bonds. The dimeric form of human cysC is shown in Fig. 1.

Physiologic isoelectric point (p*I*) of cysC is pH 9.3. As such, it has a net positive charge in body fluids. Interestingly, an additional cysC isoform has been observed at p*I* 7.8 in patients with various kidney disorders [1].

Human cysC performs a mainly protective function against cysteine peptidases including the cathepsin family. Cathepsins are a group of proteases involved with a variety of physiologic and pathophysiologic processes, i.e., the processing and presentation of antigens, as well as inflammatory and cancerous processes [3,4]. CysC is the strongest inhibitor of cathepsins B, H, L, and S [7,8]. Due to its role in regulation of extracellular proteolysis, increased body fluid or tissue cysC concentration may be suggestive of pathology. Although this inhibitor may be involved in injury response in brain, its exact role remains unclear. However, cysC is synthesized by glial cells in response to amyloid deposition, which suggests a role in the pathogenesis of Alzheimer's disease. In cerebrospinal fluid, decreased cysC results in insufficient deactivation of cathepsin B. Extensive nerve sheath demyelination then occurs leading a variety of pathologies including multiple sclerosis, autoimmune disorders, or mental disease.

3. METABOLISM AND CONCENTRATION IN BODY FLUIDS

Human cysC is present in all extracellular body fluids and is the most abundant of all relevant cystatins [8,9]. The kidneys play a major role in its metabolism. Due to biochemical properties, i.e., positively charged at physiologic pH and a low molecular weight (13.3 kDa), cysC is freely filtered by the glomerulus [7,9]. Following filtration, it is reabsorbed by the brush

Table 1 Cystatin C concentration in adult body fluids [2,13–15]

Body fluid	Mean concentration (μg/mL)	Reference interval (μg/mL)
Semen	51.0	41.2–61.8
Cerebrospinal fluid	5.80	3.20–12.5
Milk	3.4	2.20–3.90
Tears	2.40	1.30–7.40
Saliva	1.80	0.26–4.80
Amniotic fluid	1.00	0.80–1.40
Plasma	0.96	0.57–1.79
Urine	0.10	0.03–0.29

border cells of the proximal tubules. There, with the participation of lysosomal enzymes, it undergoes almost complete degradation (>99%) [10–12]. Consequently, cysC returns to the circulation as free amino acids. A small and insignificant remainder is lost in the urine (0.03–0.29 μg/mL). In contrast, peripheral blood cysC concentration is approximately 10-fold higher (0.57–1.79 μg/mL). Semen and cerebrospinal fluid contain the highest cysC concentration (Table 1).

It is noteworthy that the concentration of cysC in serum, urine, and other body fluids is independent of gender, age, and muscle mass. It does, however, fluctuate in response to a number of pathologic conditions [11]. Increased cysC has been noted in kidney disease, HIV infection, asthma, hyperthyroidism, and corticosteroid treatment. Decreased cysC has been found in cancer, abdominal aortic aneurysm, neurologic inflammatory disorders (multiple sclerosis and Alzheimer's disease), hypothyroidism, and cyclosporine treatment.

4. cysC AS AN INDICATOR OF GFR

Current evaluation of renal function involves measurement of serum and urinary markers and may require additional imaging and histologic studies. Unfortunately, these approaches tend to be insufficient due to their poor sensitivity or invasive nature. The traditional marker, creatinine, is dependent on a number of nonrenal factors including muscle mass, gender, age, and diet [16,17]. This issue is exacerbated when early stages of renal failure

are assessed. As such, there is a definite need for markers associated with preclinical disease to facilitate diagnosis and early treatment.

CysC is a newly discovered endogenous biomarker of kidney damage which can be used to detect early as well as acute and chronic renal failure. Determination of cysC in serum and urine can be routinely used for diagnosis and treatment [18–21]. As mentioned earlier, cysC is an ideal biomarker, i.e., it is produced at constant level, freely filtered, and ultimately catabolized. Its concentration in the serum is almost completely dependent on kidney function. Studies have confirmed the usefulness of cysC in assessing GFR. These studies have additionally confirmed that the concentration of cysC is independent of age, gender, and muscle mass [22].

5. COMPARISON OF cysC AND CREATININE

Rapid and reliable assessment of GFR is an absolute necessity in clinical practice. Studies comparing cysC to creatinine have cited the former as a parameter of greater sensitivity and diagnostic power [23,24]. As such, it is worthy to compare these two markers.

Creatinine is a traditional biochemical marker to assess renal function and increased serum concentration may signal decreased kidney function. Unfortunately, any measureable increase in creatinine occurs when >50% of active nephrons (GFR <40 mL/min/1.73 m^2) are damaged [23]. In addition, serum creatinine concentration is dependent on a number of factors including tubular resorption, total muscle mass, nutritional status, age, and gender. Assessment of GFR also requires a 24-h urine collection which may be subject to additional preanalytical errors. As such, despite its traditional use, the benefit of using creatinine alone in assessing renal function should be questioned.

To increase the accuracy of GFR based on creatinine measurement, a number of anthropometric models were introduced to reduce the impact of nonrenal factors. The most frequently used are models according to the Cockcroft and Gault formula, the Modification of Diet called in Renal Disease (MDRD) (Eq. 1) and the Schwartz formula to calculate the estimated GFR (estimated GFR) in children [25]. Although these formulas have enabled significant progress in renal diagnosis, estimating renal filtration is not free of complications

$$\mathrm{GFR}_{\mathrm{MDRD}} = 186 \cdot C_{\mathrm{crea}}{}^{-1.154} \cdot \mathrm{age}^{-0.203} \cdot ab \tag{1}$$

wherein C_{crea} = serum creatinine (mg/dL), $a = -0.742$ (females), and $b = 1.212$ (non-Caucasians).

In 2009, a new formula Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) (see Eq. 2) was developed. Although using many of the same variables as the MDRD formula (serum creatinine, age, gender, and race), it provided much smaller error in calculating GFR, especially in the 60–90 mL/min range [26]

$$\mathrm{GFR}_{\mathrm{CKD-EPI}} = 141 \cdot \min\left(C_{crea}/\kappa, 1\right)^{\alpha} \cdot \max\left(C_{crea}/\kappa, 1\right)^{-1.209} \cdot 0.993^{\mathrm{age}} \quad (2)$$

wherein $\kappa = 0.7$ (females) and 0.9 (males), $\alpha = -0.329$ (females) and -0.411 (males), min = minimum of C_{crea}/κ or 1, max = maximum of C_{crea}/κ or 1, and C_{crea} = serum creatinine (mg/dL).

Similar mathematical constructs have been developed for GFR determination using cysC. Examples include equations by Hoek, Larson, Filler, Le Bricon, and CKD-EPI (Table 2) [16,25].

In 2012, the CKD-EPI equation (see Eq. 3) was developed that combined cysC and creatinine measurement.

$$\begin{aligned}\mathrm{GFR}_{\mathrm{CKD-EPI}} = {} & 135 \cdot \min\left(C_{crea}/\kappa, 1\right)^{\alpha} \cdot \max\left(C_{crea}/\kappa, 1\right)^{-0.601} \\ & \cdot \min\left(C_{CysC}/0.8, 1\right)^{-0.375} \cdot \max\left(C_{CysC}/0.8, 1\right)^{-0.711} \cdot 0.995^{\mathrm{age}}\end{aligned} \quad (3)$$

Interestingly, the combined approach performed diagnostically better than other equations and thus may prove useful as a confirmatory test for chronic kidney disease [27].

Measurement of cysC is a fairly accurate representation of GFR. In addition, cysC appears very effective for assessing chronic renal disease in patients with a creatinine-based estimated GFR in the 60–74 mL/min/1.73 m^2

Table 2 Formulas for GFR calculation based on serum cystatin C concentration [16,25,27]

Hoek	$\mathrm{GFR} = -4.32 + 80.35/\mathrm{CysC}$ (mL/min/1.73 m^2)
Larsson	$\mathrm{GFR} = 77.239 \times \mathrm{CysC}^{-1.2623}$ (mL/min)
Filler	$\mathrm{GFR} = 1.962 + [1.123 \times \log(1/\mathrm{CysC})]$ (mL/min/1.73 m^2)
Le Bricon	$\mathrm{GFR} = 78/\mathrm{CysC} + 4$ (mL/min/1.73 m^2)
CKD-EPI	$\mathrm{GFR} = 133 \cdot \min(\mathrm{CysC}/0.8, 1)^{-0.499} \cdot \max(\mathrm{CysC}/0.8, 1)^{-1.328} \cdot 0.996^{\mathrm{age}}$ (mL/min/1.73 m^2)

Table 3 Comparison of cystatin C versus creatinine in GFR

Cystatin C	Creatinine
Concentration dependent only on GFR	Concentration dependent on age, gender, and muscle weight
High sensitivity (increase noted at very low renal function impairment)	Low sensitivity (increase noted after GFR decline of ~50%)
Single blood sample	Blood and urine specimen required
Increased cost	Inexpensive
Not widely available	Extensively performed

range and gives a more accurate estimate of the GFR of patients with muscle wasting and chronic diseases [28].

Tumor growth is typically accompanied by a significant decrease in body weight, which may be exacerbated by chemotherapy. As can be expected, loss of muscle mass would contribute to uncontrolled creatinine variation, whereas cysC is unaffected.

Serum cysC concentration is extremely sensitive to changes in GFR. Endogenous cysC production is constant, and its serum concentration is not altered by binding circulating protein [19,29–31]. Measurement of cysC is not susceptible to analytical interference, i.e., ascorbic acid or bilirubin. Both are, however, stable in urine and serum [11]. Unfortunately, cysC testing can significantly increase the cost of laboratory testing and has not been widely accepted. Therefore, it is imperative that clinical and laboratory practice guidelines be developed to better define diagnostic strategies when using this marker. Comparison of cysC versus creatinine is shown in Table 3.

6. CHRONIC KIDNEY DISEASE

Worldwide, approximately 500 million people have kidney damage. CKD leads to complications each year in more than 12 million people. In Poland, kidney disease affects approximately 4 million individuals. Despite this number, too few undergo periodic examination. CKD is a disease syndrome that results from progressive and irreversible impairment of renal function, particularly glomerular filtration. It is characterized by reduced number of nephrons following glomerulosclerosis, loss of urinary coils, and interstitial fibrosis [32]. Stages of chronic kidney disease are shown in Table 4.

Table 4 Stages of chronic kidney disease [25]

CKD	Descriptive name	GFR (mL/min)	Incidence (%)
1	Kidney damage with normal or high GFR	≥90	3.3
2	Kidney damage with mild decrease in GFR	60–89	3.0
3	Moderate decrease in GFR	30–59	4.3
4	Severe decrease in GFR	15–29	0.2
5	Kidney failure	<15 or dialysis	0.1

Disturbance of protein, lipid, and water–electrolyte homeostasis and impairment of key enzyme systems lead to multiple systemic disorders that significantly impair kidney function. As such, these patients, as a group, are at increased risk for CKD. Early detection of impaired kidney function is imperative to optimize the efficacy of disease-modifying therapy. Identifying those individuals predisposed to renal function deterioration is also of paramount importance. Effective preclinical screening with early indicators will support coverage of prevention of those at increased risk. Initial stages of renal failure are most often asymptomatic. In addition, gradual adaptation by patients to gradually increased symptoms results in detection that, for many, is too late and at advanced disease stage.

cysC is considered to be a sensitive parameter of the initial renal dysfunction. Determination of cysC can diagnose early-stage renal dysfunction and monitor renal function over time. In a study of type 1 and type 2 diabetics, cysC was a more accurate indicator of early-stage diabetic nephropathy [33]. cysC may also be helpful in assessing renal dysfunction in cancer [29,30]. In renal cancer, the balance between proteases (cathepsins) and their inhibitors (cystatins) is critical to the neoplastic processes of invasion and metastasis. Tumor cell growth and dissemination is accompanied by increased cysteine protease activity and decreased cysC [34]. As can be expected, the concentration of proteases relative to inhibitor is related to disease severity, i.e., malignancy.

7. cysC METHODS

7.1. Background

The first method for determination of cysC in human biologic fluid was radial immunodiffusion developed by Lofberg and Grubb in 1979 [35]. Subsequently, many methods have been reported using a variety of techniques

including radioimmunoassay, enzyme immunoassay (EIA), or enzyme-linked immunosorbent assay (ELISA) [36]. A sandwich-based EIA that used commercially available antibodies was later developed in 1993 [37].

An important scientific breakthrough led to the development of an automated homogeneous method for determination of cysC [38]. This approach used latex or polystyrene particles coated with cysC antibodies. Nephelometic (particle-enhanced nephelometric immunoassay, PENIA) and turbidimetric (particle-enhanced turbidimetric immunoassay, PETIA) methods were developed. These approaches, along with ELISA, remain the most commonly used methods for quantification of cysC due to their significantly higher precision versus earlier methods [1].

7.2. Particle-enhanced nephelometric immunoassay

This method is based on immune complex formation (antigen–antibody complexes) that results in light scattering. Using this approach, microspheres coated anti-cysC antibodies are incubated with biologic specimens containing cysC. Immune complex formation results in light scattering proportional to the cysC concentration. The concentration of cysC in the biologic sample can then be derived by comparison to an established calibration curve [39].

7.3. Particle-enhanced turbidimetric immunoassay

This immunologic approach is similar. It uses antibody-coated microspheres against cysC. Immune complex formation results in absorbance change (transmitted light) dependent on cysC concentration. Absorbance changes can then be compared to a calibration curve using known cysC concentration [38]. The approach is adaptable to automated multichannel analyzers capable of measuring wavelengths of 340–650 nm.

Commercial assays have been developed that use both mono- and polyclonal antibodies and recombinant cysC [40]. PETIA and PENIA are characterized by high precision, sensitivity, specificity, and throughput [41].

Because nephelometry detects smaller immunoaggregates than turbidimetry, PENIA is generally considered more sensitive and, as such, is recommended for cysC quantitation in biologic samples [41]. Unfortunately, PETIA and PENIA are both sensitive to interference by lipemia, hemolysis, and bilirubinemia [42].

7.4. Enzyme-linked immunosorbent assay

Sandwich ELISA is the most frequently used assay for the quantitative determination of cysC [43–50]. This method uses two antibodies. The first is a monoclonal antibody to cysC. The second is a biotinylated polyclonal antibody to cysC. This approach tends to have higher specificity given the "sandwich" nature of the assay. Unfortunately, ELISA is time consuming and relatively more expensive.

Although results obtained by ELISA versus PENIA and PETIA are comparable, some differences in analytical performance (variance and bias) have been noted [46].

7.5. SPRI biosensors

Recently, surface plasmon resonance imaging (SPRI) biosensors have been used to quantitate cysC [51]. This method uses plasmon resonance from a metallic surface and the generation of evanescent fields upon exposure to laser light. Binding of biologic particles to the biosensor surface causes changes in the field and intensity of light reaching the CCD camera. The concentration of cysC can simply be determined by comparison with a known calibration curve.

SPRI biosensors use the enzyme–inhibitor interaction. Papain, bromelain, ficin, and chymopapain are used as receptors. Because these proteins are specifically inhibited by cysC, they are highly selective in their binding properties. As such, SPRI-based biosensors exhibit very high sensitivity.

In contrast to ELISA, the SPRI biosensors are real-time, label-free, fast, and inexpensive. Many studies have demonstrated that SPRI biosensors have similar specificity but improved sensitivity versus ELISA [52–56].

8. CONCLUSIONS

In contrast to traditional renal function markers, cysC demonstrates high sensitivity and specificity. Because it is not dependent on a number of nonrenal parameters, its measurement provides a much better diagnostic tool and may be highly applicable as an early marker of preclinical renal disease. Identification of these patients is paramount for the initiation of effective therapies to slow or delay disease progression. These preliminary reports clearly warrant further attention and more comprehensive studies to validate the usefulness of this novel marker in renal disease.

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