Estimating renal function in children: A new GFR-model based on serum cystatin C and body cell mass

Trine Borup Andersen

This review has been accepted as a thesis together with four original papers by Aarhus University 4th of May 2011 and defended on 27th of May 2011

Tutors: Jørgen Frøkiær, Jens Brøchner-Mortensen & Anni Morsing

Official opponents: Anders Grubb and Emmanuel Durand

Correspondence: Department of Nuclear Medicine, Aalborg Hospital, Aarhus University Hospital, Hobrovej 18-22, 9000 Aalborg, Denmark

E-mail: trine74@hotmail.com

Dan Med J 2012;59:(7) B4486

THE FOUR ORIGINAL PAPERS ARE:

1. GFR Prediction From Cystatin C and Creatinine in Children: Effect of Including Body Cell Mass. Trine Borup Andersen, Lars Jødal, Martin Boegsted, Erland J. Erlandsen, Anni Morsing, Jørgen Frøkiær, Jens Brøchner-Mortensen. Am J Kidney Dis., 2012 Jan;59(1)50-7. Epub 2011 Oct 29.
2. Comparison of within- and between-subject variation of serum cystatin C and serum creatinine in children aged 2-13 years. Trine B. Andersen, Erland J. Erlandsen, Jørgen Frøkiær, Anni Eskild-Jensen, Jens Brøchner-Mortensen. Scand J Clin Lab Invest. 2010 Feb;70(1):54-9.
3. Precision and within- and between-day variation of bioimpedance parameters in children aged 2-14 years. Trine B. Andersen, Lars Jødal, Anne Arveschoug, Anni Eskild-Jensen, Jørgen Frøkiær, Jens Brøchner-Mortensen. Clin Nutr., Jun;30(3):326-31. Epub 2010 Nov 11.
4. Detection of reduced renal function in children: Comparison of serum cystatin C, age-corrected serum creatinine, and three GFR-prediction models. Trine B. Andersen, Lars Jødal, Erland J. Erlandsen, Jørgen Frøkiær, Anni Morsing, Jens Brøchner-Mortensen. Prepared

1. INTRODUCTION

In pediatric nephrology a reliable and accurate method for assessment of glomerular filtration rate (GFR) is essential, for instance in cases of cytotoxic drug treatment, urinary tract malformation, renal transplantation and when monitoring decreased renal function. A single measurement of renal function will give an estimate of the child's present renal function, whereas several measurements over time are useful in detecting changes over time, which is essential in most pediatric renal pathologies.

Several methods for measuring GFR exist but none is ideal. The methods based on renal clearance of exogenous markers such as ^{51}Cr-EDTA, ^{99}Tc-DTPA, ^{125}I-iothalamate, and Iohexol are well-established and accurate but also cumbersome and time-consuming (1). They will be mentioned briefly and their limitations discussed in section 1.1.1. In contrast, the methods based on the endogenous markers such as plasma (or serum) creatinine are convenient and easy to perform but inaccurate as will be elaborated in section 1.1.2-4 (2). Therefore a new method combining precision and accuracy with easy performance is warranted.

Serum (or plasma) cystatin C (CysC), an endogenous small molecular weight protein (3), has in two meta-analyses been proposed to be a superior renal function marker when compared to serum creatinine (4;5). The advantages and limitations of CysC as a renal function marker will be summarized in section 1.2. As a serum value of neither CysC nor creatinine provides an estimate of GFR, several pediatric GFR-prediction models based on CysC and/ or creatinine as well as other variables have been published already (6-10). However, the Schwartz method based on height, serum creatinine and an empirically derived constant (11-13) is still internationally recommended for GFR estimation in children (14;15), and none of the existing GFR-models have proven reliable alternatives to the exogenous methods. A brief overview of existing pediatric studies will be presented in section 1.3. This will cover studies comparing CysC to creatinine or to the Schwartz model and all relevant pediatric GFR-prediction models.

Aims of the thesis

The main goals of this thesis can be divided into four parts:

1) Development of a new pediatric GFR-prediction model.

We will present a novel theory on the relationship between CysC and body cell mass (BCM) (section 3.1). We hypothesize that including BCM/ CysC in a GFR-model will increase the accuracy of the GFR estimate in comparison to the GFR reference method, ^{51}Cr-EDTA plasma clearance. The accuracy of the resulting models will be estimated by comparison to reference GFR, and the models´ diagnostic performance will be investigated as the ability to detect changes in renal function (total day-to-day variation). Comparison to previously published, pediatric GFR-prediction models will be performed. Study I comprises these results.

2) Biological and analytical variation of CysC and creatinine.

Both the within- and between-subject variation and the analytical precision are important factors when considering the

clinical applications of serum markers. A large variation within the same child will limit the utility of the marker in a GFR-prediction model. If using only the serum value the biological variation indicates whether a marker is most suitable for longitudinal follow up or as a screening marker (section 1.2.3). Furthermore, both the analytical and the biological variation contribute to calculation of the reference change value (RCV), which is the smallest difference between two successive measurements that signify a statistically significant change of the measured value (section 4.2).

To our best knowledge, this thesis´ study II is the first in children to evaluate both the within- and the between-subject variation of CysC in comparison to serum creatinine in the same study.

Furthermore, the resulting GFR-models´ precision and validity will be evaluated indirectly in study II as CysC and creatinine are important variables in the new models.

3) The body cell mass – precision and variation of the measurements.

The BCM included in the new GFR-prediction model is estimated by bioelectrical impedance spectroscopy (BIS), which will be presented in more detail in sections 3.6 and 4.3. Knowledge of measurement precision and biological variation is important information when applying the method to a GFR-model where good precision and low variation is desirable.

To our knowledge no existing study has examined the precision and variation of all the BIS parameters, including both electrical parameters (R_E and R_I) and physiological parameters (ECF, ICF, TBF, BCM, FFM, and percentage body fat (%BF)). (List of abbreviation is presented on page 27).

Study III in this thesis aims at determining the precision and biological variation of the BIS device used primarily for BCM estimation in study I.

4) Practical applications and clinical consequences of the novel model.

In Study IV we will investigate the capacity of the novel GFR model to discriminate between normal and reduced function by determining cut-off levels for a three-sided diagnostic procedure with the following outcomes: normal renal function, reduced renal function, indeterminable. Furthermore, we will calculate the diagnostic probabilities of reduced renal function for the indeterminable results. The lower the number of children in-between cut-off levels, the better the diagnostic performance. These results will be compared to the diagnostic performance of previously published models as well as CysC and creatinine. Furthermore, we propose that adjusting creatinine with age-specific median values (16) will improve the ability of creatinine to correctly classify the children in the correct renal function groups.

The study hypotheses and aims are summarized in section 2. The design, study population and methods are presented in section 3, and the statistics in section 4. In section 5 the results of each study are summarized.

Finally, a general discussion of the study's clinical implications and limitations is provided (section 6), which is summed up in section 7, Conclusions.

1.1 EXISTING RENAL FUNCTION METHODS IN CHILDREN

1.1.1 Exogenous methods

The most accurate technique for measuring GFR is the direct methods, which involve injection of a tracer, characterized by being filtered freely in the glomeruli, without renal reabsorption, secretion or metabolization in the kidneys. Inulin clearance involves intravenous injection of a priming dose of inulin followed by constant infusion and meticulous urine sampling (17). The method is primarily used for research purposes. Moreover, the analysis of inulin concentration is problematic as the assays are imprecise (17).

The most commonly used tracers are ^{51}Cr-EDTA, ^{99}Tc-DTPA, ^{125}I-iothalamate and Iohexol (18-20). The total plasma clearance is given by the injected dose divided by the total area under the plasma curve obtained on the basis of a number of blood samples drawn from the time of injection and up to 5 hours or more. The technique has been simplified to include only a single or a few blood samples to increase the convenience for the child (21-25). However, the simplified techniques are not precise in case of GFR values below 30 mL/min/1.73m^2 and become inaccurate at GFR values below 10–20 mL/min/1.73m^2 (1;26). Caution should be taken in case of significant edema or ascites where the tracer will disappear into the expanded extra-cellular volume leading to GFR overestimation (27). In such cases clearance determination based on both plasma and urine samples will be more accurate (22). Although they are accurate the exogenous based methods are relatively cumbersome, invasive, and expensive and can exclusively be done at nuclear medicine or biochemistry departments. Therefore much simpler methods are applied when estimating renal function and these will be described in the following.

1.1.2 Plasma creatinine

The plasma (or serum) level of creatinine is the most commonly used method for estimating renal function in clinical practice. Due to the small size and lack of protein binding creatinine passes the glomerulus freely. However, it is also actively secreted by the proximal tubules at an unpredictable rate related to the level of renal function and/or type of renal disease (2;28). With decreasing GFR the fraction of tubular creatinine secretion increases, which leads to a GFR overestimation of 10-40% compared to inulin clearance (2). In patients with glomerulopathies the overestimation may be even higher (28).

A decrease in GFR is reflected by an increase in plasma creatinine. However, because of the large inter-individual variation but relatively small intra-individual variation, plasma creatinine will remain within the normal range of a population-based reference interval in a large proportion of patients with subnormal GFR limiting its use as screening test in such patient populations (29).

In children interpretation of a plasma creatinine value is not simple. One reason is the steady and muscle mass-related increase in the plasma creatinine levels in children above 2 years of age (13;30-33) although mean GFR measured by ^{51}Cr-EDTA remains constant at 104 mL/min/1.73m^2 (34). To obtain a meaningful estimate of renal function from the level of plasma creatinine narrow age-related reference intervals are therefore needed (16;35).

Moreover, plasma creatinine levels may change independently of glomerular function in case of dietary intake of meat, malnutrition, muscle atrophia, hepatic disease or increased tubular creatinine secretion (13).

Two methods exist for analyzing plasma creatinine: The alkaline-picrate method (the so-called Jaffe method) and the enzymatic method. The Jaffe method lacks specificity as it is interfered by other proteins. Attempts have been made to correct for these bias when compared to the reference method, Isotope Dilution Mass Spectrometry (IDMS). However, the enzymatic assay is recommended as it is specific and IDMS-traceable, with no mathematical correction needed (36).

In study IV we will investigate if normalizing creatinine by dividing the plasma value with the age-specific, IDMS-traceable

enzymatic reference values determined by Pottel et al. in 2008 (16) will increase the capacity of creatinine as a diagnostic test to discriminate between normal and reduced renal function.

1.1.3 Creatinine-based formulas

To compensate for the increasing muscle mass during childhood creatinine based formulas including height and body composition have been developed. The most commonly used formula in children is the empirically derived model by Schwartz (or Counahan-Barratt) (11;12)

$$\text{Creatinine clearance}\,(\text{mL/min/1.73m}^2) = \frac{k \times \text{height}}{\text{Crea}}$$

including a proportionality constant (k), which is highly dependent on analytical methods of creatinine, body composition, age, and gender (after onset of adolescence) (13). Especially low muscle mass as found in anorectic or malnourished children may influence the value of k and thereby the accuracy of the GFR estimate (13;37). Applying an unrevised k into such a population with a low production of creatinine will result in a significant overestimation of GFR (38). The original formula published by Schwartz in 1976 (12) was based on the alcaline-picrate method. However, the availability of the IDMS-traceable international standard for creatinine calibration has prompted Schwartz et al. to revise the formula in 2009 with enzymatic creatinine results, which yielded a lower k-value (36.5 vs. 48.6 for creatinine in µmol/L and height in cm) (9). This emphasizes the importance of utilizing the identical assay as used in the formula-modeling. The updated formula was made in a population of children with chronic kidney disease (41.3 mL/min/1.73m^2), but has been validated in a population of children with GFR >90 mL/min/1.73m^2, which indicated an underestimation of approximately 18% by the revised formula (39). A meta-analysis showed higher accuracy in the children with GFR <90 mL/min/1.73m^2 than >90 mL/min/1.73m^2 (61.3 vs. 47.2% of estimates within ±30% of ^{51}Cr-EDTA clearance) (40).

The Schwartz formula allows for rapid function assessment without urine collection and provides a good approximation of measured GFR in children with normal body composition and normal to mildly reduced renal function (GFR >50 mL/ min/ 1.73m^2) (41). However, it is well-established that the Schwarz formula leads to overestimation of moderately- to severely reduced GFR (<50 mL/min/1.73m^2) (41).

Other creatinine-based models have been developed for GFR-estimation in children (42;43). The Lund-Malmö equation performed as well as the Counahan-Barrat equation, but not as well as their own CysC equation (43). The equation by Ledger proved better than the Schwartz formula in terms of accuracy and bias (42).

1.1.4 Direct creatinine-clearance

The direct creatinine-clearance method involves precise urine collection for 24 hours, which is hard to obtain in children, time-consuming and impractical for routine use. This imprecision is exemplified by a high day-to-day variation in children of 13.8%, compared to only 4.8% and 6.9%, respectively for venous and capillary blood samples for determination of ^{51}Cr-EDTA plasma clearance (44;45). Moreover, urine collection will not eliminate bias introduced by tubular secretion of creatinine. As the endogenous 24-hour-creatinine clearance is less precise then the Schwartz estimate (46), the creatinine clearance is better estimated from the plasma level of creatinine using the Schwartz formula having in mind the mentioned limitations.

1.2 CYSTATIN C

In the search for a more easily performed but still accurate and precise method for GFR estimation, considerable research has been conducted on low molecular weight proteins, of which the most promising candidate is CysC. CysC is also known as γ-CSF, γ-globulin, post-gamma protein, γ-trace, post-γ-globulin, and δ aT (47). The protein was identified for the first time in normal cerebrospinal fluid (48) and in urine from patients with tubular proteinuri in 1961 (49). Later CysC was identified in ascitic and pleural fluids as well as in plasma (50). In 1982 Grubb et al. established that the protein entitled human γ-trace consisted of a single polypeptide chain of 120 residues with a molecular weight of 13.260 kDa (3). Until 1984 the function of CysC was still unknown, but then a new protein-cysteinase inhibitor was found (51) and this new protein was named Human Cystatin. Shortly afterwards, the name CysC was proposed for Human Cystatin (52). In 1989 and 1990 the entire nucleotide sequence of the gene encoding CysC was determined and localized to chromosome 20 (53-55).

CysC is present in human body fluids and in all human, nucleated cells investigated to date (56). Its production is determined by a single gene of the housekeeping type, which is compatible with a stable production rate in all nucleated cells (55). Functionally, CysC is a potent inhibitor of cysteine proteinases, which are involved in antigen presentation, protein catabolism, tissue remodeling, and in the pathogenesis of atherosclerosis as reviewed by Bökenkamp et al. (57). CysC interacts with cysteine proteinases during tumor invasion, bone resorption, implantation of the embryo and in regulation of inflammatory processes (57).

CysC is analyzed by particle-enhanced immunonephelometry (PENIA) or particle-enhanced immunoturbimetry (PETIA), the latter resulting in up to 30% higher levels of CysC. However, discrepancies in results can also be seen between studies using identical methods most likely related to differences in calibrator material and study populations. However, very recently the first certified calibrator material for CysC in humans has become available (58). This will eliminate the problems with discrepancies in CysC models and results due to use of different CysC assays and calibrator materials.

In addition, a pilot study in children indicates that it is necessary to draw venous blood and not perform finger puncture as capillary blood samples result in significantly higher CysC values (1.16±0.80 vs. 1.21±0.81 mg/L, p=0.006) (59).

1.2.1 Renal handling

CysC as a measure of renal function was investigated for the first time in 1985 by Simonsen et al. who found that serum CysC correlated with GFR assessed by ^{51}Cr-EDTA clearance in adults (60). Because of its low molecular weight and positive charge at physiological pH CysC is practically freely filtered in the glomeruli (61). A study of radiolabelled human CysC performed in rats showed a 94% renal plasma clearance of ^{51}Cr-EDTA and a subsequent tubular reabsorption and complete catabolization without any tubular secretion (61). As opposed to the absence of CysC in urine from normally functioning kidneys, urine from patients with renal tubular dysfunction contains elevated concentrations of CysC due to defective reabsorption (49;62;63). As CysC is eliminated in the urine without metabolization it may prove a useful marker of renal tubular dysfunction (64;65). Possibly the increased urinary concentration in tubular dysfunction is caused by the inability to reabsorb and degrade the filtered CysC by competitive inhibition in the presence of massive proteinuria (66).

Because of the unique renal handling of CysC combined with a stable production rate, the level of CysC in plasma is mainly determined by GFR. However, data from nephrectomized rats indicate an extrarenal route of elimination of approximately 15% (61). The data from the same study did not report the magnitude of extrarenal clearance in the rats with intact kidneys as the total plasma clearance could not be calculated due to very short observation time. Also in humans the existence of extrarenal elimination is suggested although the magnitude is unknown (67).

1.2.2 Physiological variation

In children a maturational increase in renal function as measured by ^{51}Cr-EDTA has been demonstrated up until approximately 2 years of age after which adult values, when expressed as clearance per 1.73m^2, are reached (34). In parallel the highest concentration of CysC is found on the 1st day of life followed by a rapid decrease during the next months (30;31;33;68). After the first year of life, several studies agree that the CysC levels remain stable in contrast to creatinine, which increases steadily throughout childhood (30-33). Other studies have found, though, that CysC is not stable until after the age of 18 months (69) or 3 years (68). Furthermore, CysC has in several studies in both children and adults been proven independent of gender, height and weight (7;30-33;70), supporting the view that CysC is a robust biomarker, that may be especially useful not only in children, but also other elderly patients and other populations with low muscle mass, where serum creatinine measurements do not perform well.

1.2.3 Within- and between-subject variation

The potential of CysC as a renal function marker is related to the purpose of the testing, whether it be screening for mild or moderate reductions in GFR in a healthy population or longitudinal GFR control in renal patients. The usefulness of a marker for either purpose may be characterized by the relationship between its within-subject variation (SD_I) and between-subject variation (SD_G), which can be expressed as an index of individuality (SD_I/SD_G=IOI) (71). If IOI is <0.6, it is considered that population-based reference values are of limited value in detecting onset of renal function impairment for an individual as the limits are insensitive to the real changes reflected in the level of the biomarker. (71). In contrast, if IOI is >1.4 observed values should be suitable for comparison to the population-based limits (71). When monitoring children with possible or established renal disease the variation within the child should be small as changes thereby are easier to detect, although even highly unusual values for an individual may still be within the reference interval (72). A low variation within the child is also desirable when including a serum marker in a GFR-prediction model as this will increase the precision of the GFR estimate.

Studies investigating within- and between-subject variation of CysC and creatinine in children are few. Existing studies in children demonstrate that the within-subject variation of CysC (range 10-13%) and creatinine (range 8-15%) are basically equal (73-75) indicating that the two analytes may be equally effective in longitudinal follow up. Studies on adults are conflicting, but the majority agrees, that the within-subject variation of CysC lies in the range 4-7% (76-79), which seems lower than in children. Only one study has found a high within-subject variation in adults of 13% and a high IOI of 1.63 (80).

1.3 CYSTATIN C STUDIES IN CHILDREN

When assessing the documented performance of CysC as a marker of renal function it is important to consider the statistical methods used. Most studies compare the reciprocal of CysC and creatinine to a reference standard with determination of correlation coefficients. However, correlation coefficients measure linear association rather than agreement between two methods. Therefore studies using solely correlation coefficients will not be commented on in this thesis, though, they are mentioned for the models using other statistical methods for comparison as well.

Bland-Altman plots provide more precise evaluation of agreement (81) and receiver operating characteristics (ROC) curves provide an index of utility of a diagnostic test (82). The area under the ROC curve is a measure of accuracy. The higher accuracy the better the diagnostic test to discriminate between "disease" and "non-disease", for instance between decreased and normal GFR (82). Furthermore, it is important to consider the reference method of GFR (GFRref) as reliable when evaluating a potential new marker of renal function. Consequently, studies having creatinine clearance, estimates of GFR by the Schwartz model, or gamma camera technique as reference methods will not be commented on in this thesis.

In the following a brief summary of studies comparing CysC to creatinine (1.3.1) or to the Schwartz model (1.3.2) will be presented. Section 1.3.3 is a more critical overview of previously published CysC-based GFR-models.

1.3.1 Serum cystatin C versus plasma creatinine

CysC has been proposed a more sensitive marker of renal function compared to plasma creatinine - especially in situations in which there is only a moderate decrease in GFR, suggesting that CysC might be advantageous in "the creatinine blind area" of initial renal impairment. Furthermore, CysC has been suggested more sensitive than enzymatic creatinine measurements in detecting early GFR impairment after renal transplantation (83-85), though this has not been confirmed in children.

Numerous studies have been conducted in children comparing CysC to plasma creatinine. Mostly the population examined is not well-defined, but a heterogeneous group of children with kidney diseases, transplantations and cancer with only a referral for measurement of GFR in common. Studies comparing CysC and creatinine using ROC analysis are presented in Table 1, while studies with merely correlation coefficients are disregarded. In five out of twelve studies (ten publications) CysC had significantly higher AUC than plasma creatinine (32;86-89), in five studies there was no significant difference (7;88;90;91) and in two studies no statistical comparison was made (92;93), though at least one study is clearly in favor of CysC when judging the AUC. No study found creatinine to be significantly better than CysC (see Table 1 for summary of 12 studies). Regarding correlation coefficients there was no statistically significant difference between CysC and creatinine in eight of twelve studies. In children with spina bifida the non-significant correlation between creatinine and GFRref in contrast to that of CysC indicates the latter as the marker of choice in this population (88).

TABLE 1
Comparison of CysC and serum creatinine (Crea) to reference method.
Correlation coefficients (CC) for CysC and Crea to reference GFR (GFRref) and Receiver Operating Characteristics (ROC) for CysC and creatinine are shown.

Reference	Number (Boy/girl)	Age (Years)	Population	Reference method, GFR cut-off (mL/min/1.73m^2)	Superior Marker according to ROC	CC with GFRref CysC vs. creat *p*-value	ROC (AUC) CysC vs. Creat *p*-value
Bökenkamp 1998 (7)	101 (Application group)	0.2-18	Nephr. disorders	Inulin (84)	?	0.88 vs. 0.85 NS*	0.97 vs 0.89 NS†
Helin 1998 (32)	69	1-16	Referred to 5[1]Cr-EDTA	^{51}Cr-EDTA (Age-depending (94))	Cys>Crea	0.83 vs 0.67 p<0.05*	AUC_{CysC}>AUC_{Crea} p<0.05
Stickle 1998 (90)	26	4-12	Nephr. disorders	Inulin (90)	Cys=Crea	0.77 vs. 0.84 p>0.3	0.88 vs 0.79 p=0.29
Stickle 1998 (90)	34	12-19	Nephr. disorders	Inulin (90)	Cys=Crea	0.87 vs. 0.89 p>0.3	0.94 vs 0.96 p=0.99
Ylinen 1999 (89)	52 (56/44%)	2-16	Nephr. disorders	^{51}Cr-EDTA (89)	Cys>Crea	0.89 vs. 0.8 p=0.073	0.99 vs 0.92 p=0.037
Filler 1999 (92)	381	1.7-18	Nephr. disorders	^{51}Cr-EDTA (90)	?	0.64 vs 0.55 NS*	0.90 vs 0.88 No p-value
Filler 2002 (86)	225 (60/40%)	0.2-18	Nephr. disorders	^{99}mTc-DTPA/ ^{51}Cr-EDTA (90)	Cys>Crea	0.77 vs. 0.5 p<0.05	0.94 vs 0.84 p<0.001
Pham-Huy 2003 (88)	201	1-20	Nephr. disorders	^{99}mTc-DTPA (90)	Cys>Crea	0.84 vs. 0.60 p<0.001	0.97 vs 0.86 S†
Pham-Huy 2003 (88)	27	1.4-20	Spina bifida	^{99}mTc-DTPA (90)	Cys=Crea	0.45 vs. 0.17 NS*	0.95 vs 0.88 NS†
Willems 2003 (91)	66 (62/38%)	1.3-21.9	Nephr. disorders	Inulin (80)	Cys=Crea	0.94 vs. 0.90 NS*	0.97 vs 0.92 NS†
Martini 2003 (87)	99 (52/48%)	1-17.9	Nephr. disorders, oncologic, miscellaneous	Inulin (100)	Cys>Crea	0.64 vs. 0.54 NS*	0.73 vs 0.6 S†
Samyn 2005 (93)	62 (48/52%)	0.6-18.7	Liver transplants, before and after	^{51}Cr-EDTA (60, 70, 80, 90)	?	0.78 vs. 0.4 p<0.001	0.93 vs 0.76‡ No p-value

*p-values calculated by author
†Stated significant (S) (p<0.05) or non-significant (NS) (p>0.05) in paper, though p-value not stated
‡Only AUC (area under curve) values calculated in 34 children with a GFR cut-off <80 mL/min/1.72m^2 are shown

1.3.2 Serum cystatin C versus the Schwartz formula

A single measurement of a serum value of CysC is clearly advantageous in easy performance over the Schwartz formula, which requires a height and a proportionality constant, which will vary in the pediatric population according to adolescence and in case of abnormal body composition. Comparing CysC and the Schwartz formula using ROC analysis CysC had statistically significantly higher AUCs than did the Schwartz formula in two of six studies in five publications (88), in two studies there was no difference (86;92) and in only one study was the Schwartz formula superior to CysC (87). In one study no statistical comparison was made (93) (see Table 2 for a summary of the 6 studies). The studies by Samyn et al. (93) and Pham-Puy et al. (88) show remarkably low correlation of 0.12 and -0.09, respectively, between the Schwartz estimate and GFRref and likewise between creatinine and GFRref (see Table 1 and 2). This is most likely attributable to the unreliability of creatinine in the studied populations consisting of malnourished children prior to liver transplantation and spina bifida patients, both groups with low muscle mass.

Comparing CysC to the Schwartz formula CysC is at least equal to Schwartz in predicting normal or reduced renal function, though an estimate in mL/min/1.73m^2 will not be obtained.

In this thesis´ study IV we will calculate AUC with ROC analysis as a supplement to another approach, and we will demonstrate that the AUC do not provide sufficient information on a method´s ability to discriminate between normal and reduced renal function.

1.3.3 Previous GFR-prediction models

All previously published GFR-prediction models are based on regression analyses in which both the dependent and independent variables are either logarithmic transformed or in absolute numbers. The models are summarized in Table 3.

The first pediatric model published by Bökenkamp et al. in 1998 was developed in a baseline group of 84 children and tested in a comparable group of 101 children (7) (Table 3). Bland-Altman plots showed a high level of accuracy expressed as the mean difference between inulin clearance and predicted GFR but rather wide limits of agreement (Table 4). These results were similar to the results using the Schwartz model indicating no advantage over creatinine based models.

The log transformed model developed in 2003 by Filler et al. was comparable to the Bökenkamp equation in the resulting accuracy and limits of agreement (8) (Table 4). However, as the Schwartz model estimates had wider limits of agreement and a lower accuracy resulting in an overestimation of GFR in the lower range of GFR, the CysC-based model proved superior to the Schwartz model. This overestimation of GFR by the Schwartz and Counahan-Barrett models was also demonstrated by Grubb et al. in 2005 (95). This was probably partly due to insufficient calibration of the Schwartz formula to the enzymatic creatinine assay used in the study. Moreover, the CysC-based equation was tested in the same population in which it was developed. The results of the study were therefore biased in favor of CysC, which was concluded to be clearly superior to the Schwartz formula (Table 4).

TABLE 2

Comparison of CysC and the Schwartz formula to reference method. Correlation coefficients (CC) for CysC and Schwartz (Schw) to reference GFR (GFRref) and Receiver Operating Characteristics (ROC) (area under curve (AUC)) for CysC and Schwartz are shown.

Reference	Number (Boy/girl)	Age (Years)	Population	Reference method GFR cut-off (mL/min/1.72m^2)	Superior marker according to ROC	CC. with GFRref CysC vs. Schwartz *p*-value	ROC (AUC) CysC vs. Schwartz *p*-value
Filler 1999 (92)	381	1.7-18	Nephr. disorders	^{51}Cr-EDTA (90)	Cys=Schw	0.64 vs. 0.78 *p*<0.001	0.90 vs. 0.97 *p*=0.12
Filler 2002 (86)	225 (60/40%)	0.2-18	Nephr. disorders	^{99m}Tc-DTPA/ ^{51}Cr-EDTA	Cys=Schw	0.77 vs 0.71 NS*	0.94 vs. 0.92 NS†
Pham-Huy 2003 (88)	201	1-20	Nephr. disorders	^{99m}Tc-DTPA (90)	Cys>Schw	0.84 vs. 0.81 NS*	0.97 vs. 0.94 *p*<0.024
Pham-Huy 2003 (88)	27	1.4-20	Spina bifida	^{99m}Tc-DTPA (90)	CysC>Schw	0.45 vs. -0.09 *p*<0.05*	0.95 vs. 0.76 S†
Martini 2003 (87)	99 (52/48%)	1-17.9	Renal, oncologic, miscellaneous	Cl-inulin (100)	Cys<Schw	0.64 vs.0.69 NS*	0.73 vs. 0.81 S†
Samyn 2005 (93)	62 (48/52%)	0.6-18.7	Liver transplants, before and after	^{51}Cr-EDTA (60, 70, 80, 90)	?	0.78 vs. 0.12 *p*<0.001	0.93 vs. 0.52‡ No *p*-value

**p*-values calculated by authors

†Stated significant (S) or non-significant (NS) in paper, though *p*-value not stated

‡Only AUC (area under curve) values calculated in 34 children with a GFR cut-off <80 mL/min/1.72m^2 are shown

Bouvet et al. constructed a model including creatinine, age and weight as these covariates increased the reliability of the resulting estimates significantly (6). However, the limits of agreement were still wide (Table 4).

Zappitelli et al. developed two models. One was based on CysC only and the other on creatinine, height and CysC (CysC/Crea model) (10) (Table 3), which they compared to the Schwartz model and the models developed by Bökenkamp, Filler and Grubb using local constants and coefficients. The best agreement and proportion of estimated GFR (eGFR) within 30% of GFRref, was obtained with the CysC/Crea model.
However, accuracy was similar and close to zero in all models, apart from the Filler and Schwartz models demonstrating respectively 10 and 6.9% overestimation of predicted GFR. Furthermore, the CysC/Crea-model using the constant for spina bifida performed extremely well in the population of children with spina bifida (95% LOA -18 to 16 mL/min/1.73m^2). However, as the model is logarithmic the 95% LOA ought to have been calculated as percentages and not in absolute numbers (mL/min/1.73m^2). Consequently, the limits are not applicable to all levels of GFR.

In 2009 Schwartz et al. developed a new model based on CysC and creatinine as well as other variables (Table 3), which they compared to previously published pediatric formulas (9). They found their formula to have the highest percentage of eGFR within 30 and 10% of measured GFR, though probably not statistically significant when comparing to the revised Zappitelli model, which had the second highest percentages. The reported limits of agreement were quite narrow for all studies, though the low median GFR (41 mL/min/1.73m^2) should be borne in mind. Moreover, they also calculated the limits in absolute numbers (mL/min/1.73m^2) for all seven models, even though five of them, including their own, were made on logarithmic transformed variables. The differences should have been percentages as was the case with the Zappitelli study. A rough estimate of the 95% LOA in percentages can be estimated as the LOA in absolute numbers (-17; 12 mL/min/1.73 m^2) as a percentage of the median GFR (41 mL/min/1.73m^2), which is -41; 29%.

Table 3

Cystatin C-based models to predict GFR in children

Reference	No. (Boy/girl)	Age (Years)	GFR method and level in mL/min/1.73m^2	CysC GFR prediction model (mL/min/1.73m^2)
Bökenkamp 1998 (7)	184 (53/47%)	0.2-18	Median inulin-GFR 77 (7-209)	GFR = (162/CysC) – 30
Filler 2003 (8)	536 (59/41%)	1-18	Mean 99mTc-DTPA-GFR 103±41	GFR = 91.62 × (1/CysC)$^{1.123}$
Grubb 2005 (95)	85 (56/44%)	0.3-17	Mean iohexol-GFR 113 (37-240)	GFR = 84.69 × CysC$^{-1.68}$ [× 1.384 if child < 14 ys]
Bouvet 2006 (6)	100 (58/42%)	1.4-22.8	Mean ^{51}Cr-EDTA-GFR 95 (18-200)	GFR (mL/min) = 63.2 × [(Crea/96)$^{-0.35}$] × [(CysC/1.2)$^{-0.56}$] × [(BW/45)$^{0.30}$] × [(age/14)$^{0.40}$]
				*GFR (mL/min) = 38.4 × Crea$^{-0.35}$ × CysC-0.56 × weight$^{0.30}$ × age$^{0.40}$
Zappitelli 2006 (10)	103 (60/40%)	1-18	Mean iohexol-GFR 74±36	GFR = (75.94/CysC$^{1.17}$) [× 1.2 if renal transplant]
				GFR = (507.76 × e$^{0.003\times height}$)/CysC$^{0.635}$ × Crea$^{0.547}$ [× 1.165 if renal transplant], [× 1.57 × Crea$^{0.925}$ if spina bifida]
Schwartz 2009 (9)	349 (61/39%)	1-16	Median iohexol-GFR 41.3	GFR = 39.1 × (height/Crea)$^{0.516}$ × (1.8/CysC)$^{0.294}$ × (30/BUN)$^{0.169}$ × (1.099)^{male}× (height/1.4)$^{0.188}$ Crea in mg/dL; BUN in
				†GFR = 25.7 × (height/Crea)$^{0.516}$ × CysC$^{-0.294}$ × BUN$^{-0.169}$ × (1.099)^{male} × height$^{0.188}$

CysC (mg/L), Crea (µmol/L), BW (kg), height (cm). For Schwartz 2009 in original formulation see "Results – study I"

*Constants combined.

†Constants combined and units changed to those used in the other formulas.

Table 4
Assessment of predicted GFR by models with CysC (GFRcys) and Schwartz (GFRsch) compared to reference GFR (GFRref) expressed as accuracy, 95% limits of agreement (LOA) and percentages of estimated GFRcys and GFRsch within 30% of GFRref

Reference	GFRcys versus GFRsch accuracy	GFRcys vs. GFRsch 95% LOA	GFRcys vs. GFRsch within 30% of GFRref
Bökenkamp 1998 (7)	2 vs 5 mL/min/1.73 m^2	-46 to 42 vs. -39 to 48 mL/min/1.73 m^2	-
Filler 2003 (8)	0.3% vs.-11%	-44 to 43% vs. -58 to 37%	-
Grubb 2005 (95)	-2% vs 51%	-	78 vs. 25%
Bouvet 2006 (6)	5% vs 3%	-31 to 41% vs. -37 to 43%	82 vs. 79%
Zappitelli 2006 (10)	-1 vs 7 mL/min/1.73 m^2	-31 to 28 vs. -42 to 56 mL/min/1.73 m^2	87 vs. 65%
Schwartz 2009 (9)	-2 vs -0.1 mL/min/1.73 m^2	-17 to 12 vs. -18 to 18 mL/min/1.73 m^2	83 vs. 71%

The CysC-based prediction models in children all have a low agreement compared to GFRref in the range of 30-40% at best. This means that a child having an eGFR of 90 mL/min/1.73m^2 may have a measured GFR somewhere between approximately 50 and 130 mL/min/1.73m^2. This variation is clearly unacceptable when considering a CysC-based model as an alternative to an exogenous GFR method. However, there is little doubt that CysC based prediction equations are at least as good as the Schwartz formula, though, it is difficult to clearly favor one equation over another.

The first study of this thesis aimes to develop an original model based on a novel theory of the relationship between CysC and body cell mass (see 3.1) to improve the current status of GFR-estimation in children.

1.5 THE BODY CELL MASS

The body cell mass plays a central role in the novel theory behind the new GFR prediction model and this will be explained in section 3.1.

The total body mass can be divided into two main compartments, the fat-free mass and the fat mass. The fat-free mass comprises the cellular mass, bone mineral content and extracellular fluid. BCM is defined as the fat-free mass without bone mineral content and extra-cellular fluid. Anatomically BCM consists of the skeletal, cardiac and smooth muscles, the parenchymal viscera, the intestinal tract, blood, the glands of the body, reproductive organs, all connective tissues and the cellular components of the brain, fat and bone, though the cellular components of the latter only represent a very small part of its total mass (96). Functionally it constitutes all the oxygen-exchanging, potassium-rich, glucose-oxidizing, work-performing tissues of the body as defined by Moore et al. in 1963 (96). This latter definition excludes the bone collagen tissues along with ECF, as energy-exchange is almost absent in these cells (96). By the functional definition of BCM the intra-cellular fluid approximates the BCM most closely. As potassium is present almost exclusively in the intra-cellular water (>95%), the reference method for quantifying BCM is whole-body counting of the naturally occurring radioactive isotope potassium 40 (40K). 40K constitutes 0.012% of natural potassium, and can therefore provide a value of total body potassium, which serves as an index of BCM (97). However, as this method was not available in Aalborg or Aarhus, we searched for a more practical and easily accessible method and discovered bioelectrical impedance spectroscopy (BIS), in our case the Xitron Hydra 4200. The BIS technique has the potential to estimate BCM by measuring electrical resistance (impedance) in the body (98). The BCM estimate is based on the assumption that the average water content of BCM is 70% in healthy adults:

$$BCM = \frac{ICF}{0.7}$$

Furthermore, total body fluid (TBF), extra-cellular fluid (ECF) and intra-cellular (ICF) as well as fat-free mass (FFM) can be determined in the individual child. The technical details will be described in section 3.6.

It is a relatively inexpensive field method that requires a minimum of operator training and maintenance, and the rapid measurements can be repeated frequently with immediately available results after each measurement. BIS has been validated against dilution methods in many clinical studies as reviewed by Earthman et al. (99). The level of accuracy for estimating BCM is not consistent, but BIS has been shown to accurately measure changes in ICW (and thus BCM), though validation is recommended in the population studied (99). However, as monitoring of bioimpedance parameters is important in many clinical settings in both children and adults, for instance loss of BCM in HIV patients (100), protein wasting in dialysis patients (101) or during weight gain treatment of adolescents with anorexia nervosa (102), it is also important to know the precision of a measurement and the minimal value necessary for a statistically significant change between measurements, defined as the reference change value (RCV) (72). This is also true in relation to the current thesis as good precision and low variation will increase the precision of the GFR model. To our knowledge only two studies in adults (103;104) and a small study in children (105) have investigated the variation within day and between days, whereas several studies have addressed the precision question using a Xitron 4000 (103;106-109) or 4200 (104;110;111). However, none of these studies examined the precision and variation of all the BIS parameters, including both electrical parameters (R_E and R_I) and physiological parameters (ECF, ICF, TBF, BCM, FFM, and percentage body fat (%BF)). It should be noted, that the electrical parameters are not linearly correlated with the physiological parameters, therefore precision and variation in the former do not simply transfer to the latter.

In study III in this thesis the variation within- and between days and the precision of all BIS measurements will be presented.

2. AIMS AND HYPOTHESES

Aims

1. To develop and investigate the accuracy and diagnostic performance of a new GFR-prediction model from serum CysC and body cell mass
2. To determine the analytical and within- and between-subject variation of serum CysC and creatinine in children aged 2-14 years
3. To compare parallel data for serum CysC and creatinine
4. To examine the precision and within- and between-subject variation of all BIS parameters.

Hypotheses

1. GFR can be estimated in children aged 2-14 years by a prediction model based on regression analysis of primarily serum CysC and BCM on GFR
2. The analytical and within- and between-subject variation of serum CysC and creatinine are low
3. GFR determined by the new prediction model is a more accurate estimate of renal function than GFR determined by previously published GFR-models based on CysC and/ or creatinine.
4. The new model is superior to other methods to discriminate between normal and reduced renal function.
5. The precision of BIS measurements is good and the within- and between-subject variation of BIS parameters is low.

3. MATERIALS AND METHODS

3.1 NOVEL THEORY ON CYSTATIN C AND BODY CELL MASS

Our novel model for predicting GFR from CysC is based on general kinetics principles of clearance and known physiological features of CysC, including rate and site of production, as will be described in the following.

As the production of CysC in all nucleated cell is determined by a single gene of the housekeeping type compatible with a constant production rate (55), this has lead us to theorize that the production rate of CysC is proportional to body cell mass (BCM):

$$\text{Production rate for CysC} = k_1 \times \text{BCM}. \qquad [1]$$

For an endogenous marker in steady state (in this case CysC), the production rate equals the excretion rate (u). Thus, for CysC the excretion rate is coupled to BCM.

$$u = \text{excretion rate} = \text{production rate} = k_1 \times \text{BCM} \qquad [2]$$

At any given time, the total plasma clearance (Cl) of a substance is determined as the ratio between the excretion rate and the plasma concentration (P(t)):

$$Cl = u(t)/P(t). \qquad [3]$$

Denoting plasma concentration of CysC as CysC, and combining with our theoretical dependence on BCM [Eq.2] we get

$$Cl = k_1 \times \frac{BCM}{CysC}. \qquad [4]$$

CysC is excreted by glomerular filtration (61), and provided no extrarenal clearance the total plasma clearance of CysC is equal (or proportional) to GFR:

$$GFR = k_2 \times \frac{BCM}{CysC}. \qquad [5]$$

Note that the model describes GFR in mL/min, not in mL/min/1.73m^2, i.e., before any normalization to body surface area (BSA).

In case of extrarenal clearance, the total clearance (Cl) will be higher than GFR. This will result in a negative intercept in the relation between GFR and BCM/CysC:

$$GFR = k_2 \times \frac{BCM}{CysC} - a \qquad [6]$$

the value of the constant a being the average of extrarenal clearance in the children studied.

However, as Figure 5 in the results section will show there is a proportional relationship between GFR and BCM/CysC, without a statistically significant intercept, i.e., without the need to include extrarenal clearance in the model.

From previously published pediatric GFR-prediction models we know that inclusion of other variables related to renal function will increase the accuracy of the GFR estimate (6;9;10). Consequently, we set out to investigate if the known relation between height and creatinine would add further to our model in addition to gender, age, BMI, BSA, BCM, 1/BUN, 1/creatinine, and albumin, which all were considered possible explanatory variables by forward, stepwise regression in the model selection procedure as described in section 4.1.

Serum creatinine: The excretion rate of creatinine per 1.73 m^2 BSA is proportional to the child's height in cm (12):

$$GFR\ (mL/min/1.73m^2) \times \text{creatinine} = k_3 \times \text{height}, \qquad [7]$$

which can be rearranged to:

$$GFR\ (mL/min/1.73m^2) = k_3 \times \text{height/creatinine} \qquad [8]$$

To allow for combination with the theoretical CysC relation above, we convert to absolute GFR (mL/min) by multiplying with BSA/1.73m² on both sides of the equation. Incorporating 1.73m² into the constant we get:

$$GFR\ (mL/min) = k_4 \times (\text{height} \times \text{BSA/creatinine}) \qquad [9]$$

where $k_4 = k_3/1.73m^2$.

Thus, the variable height×BSA/creatinine may supplement the variable BCM/CysC in estimation of GFR (mL/min).

Table 5
Patient characteristics of the three populations from study III in mean values and (range).

	Precision population	Within-day sub-population	Between-day sub-population
Number	133	44	32
Gender boy/ girl	81/ 52	22/ 22	22/10
Age (years)	8.8 (2.3-14.9)	10.2 (2.4-14.9)	8.0 (2.4-13.7)
No. aged ≥6/<6 years	99/ 34	38/ 6	22/ 10
Weight (kg)	32.4 (12-84.8)	38.5 (12.3-84.8)	28.5 (12.0-61.3)
Height (cm)	132.5 (84-181)	142.5 (91-181)	125.5 (84-173)
BCM (kg)	13.2 (3.9-38.6)	16.1 (5.0-37.6)	11.8 (3.1-31.5)
TBF (L)	17.2 (5.5-46.0)	20.6 (6.6-45.1)	15.3 (5.3-39.3)
ECF (L)	7.9 (2.9-19.0)	9.3 (3.1-18.8)	7.1 (2.7-17.3)
ICF (L)	9.2 (2.7-27.0)	11.3 (3.5-26.3)	8.2 (2.6-22.02)
FFM (kg)	7.2-62.1 (22.8)	27.5 (8.8-60.9)	20.4 (6.9-52.6)
RE (Ohm)	796 (538-1065)	771 (554-947)	800 (551-966)
RI (Ohm)	1917 (1082-3088)	1784 (1084-2406)	1958 (1157-2857)
%BF (%)	28.4 (5.8-46.9)	27.6 (11.2-46.1)	28.0 (13.4-44.3)
Measurements*	One series day 1	Two series day 1	One series day 1 and day 2

*Each measurement series consisted of 3 repeated measurements. All 133 children had one series measured on day one (precision population). Forty-four children had a second series on day one (within-day sub-population). Thirty-two children had a series measured on the next day (between-day sub-population).

Table 6.
Patient characteristics in mean values and (range) from study I. GFR subcategories in numbers.

	Boys	Girls	All
Number	79	52	131
Age (years)	8.7 (2.4-14.9)	9.0 (2.3-14.9)	8.8 (2.3-14.9)
Height (cm)	132.6 (84-181)	133.1 (90-168)	132.8 (84-181)
Weight (kg)	32.3 (12-85)	32.7 (12-66)	32.5 (12-85)
BMI*	17.1 (12.8-30.0)	17.3 (12.8-27.5)	17.2 (12.8-30.0)
BSA†	1.07 (0.51-2.04)	1.08 (0.54-1.69)	1.08 (0.51-2.04)
Crea (Creatinine) (µmol/L)	56.7 (22-128)	52.6 (25-313)	55.1 (22-313)
Cys C (Cystatin C) (mg/L)	0.92 (0.53-1.93)	0.84 (0.55-3.63)	0.89 (0.53-3.63)
BUN (blood urea nitrogen) (mmol/L)	6.1 (2.5-11.9)	5.6 (2.5-22.2)	5.9 (2.5-22.2)
Body cell mass (BCM) (kg)	13.8 (3.9-38.6)	12.4 (4.7-22.4)	13.3 (3.9-38.6)
BCM/CysC (kg/(mg/L))	15.7 (4.9-39.4)	15.9 (3.4-32.7)	15.7 (3.4-39.4)
Height×BSA/Crea (cm×m^2/ µmol/L)	2.8 (1.0-5.9)	3.1 (0.4-5.4)	2.9 (0.4-5.9)
GFR (mL/min/1.73m²)	93.8 (38.1-147.4)	100.9 (13.7-135.2)	96.6 (13.7-147.4)
GFR >90 (mL/min/1.73m²)	48	38	86
GFR 60-90 (mL/min/1.73m²)	20	13	33
GFR <60 (mL/min/1.73m²)	11	1	12

*Body mass index = weight (kg)/ height (m)
†Body surface area = $0.007184 \times [\text{Weight}]^{0.425} \times [\text{Height}]^{0.725}$ (The Dubois & Dubois formula (120))

Table 7.
Patient characteristics mean values (range) from study IV.

	GFR reduced*	GFR normal
Number	37	94
Age (years)	8.5 (2.4-14.9)	8.9 (2.3-14.9)
Height (cm)	128.0 (86-168)	134.5 (84-181)
Weight (kg)	27.4 (12.0-52.6)	34.5 (12-85)
BMI†	16.0 (12.8-21.5)	17.6 (12.8-30.0)
Creatinine (µmol/L)	77.9 (29-313)	46.1 (22-89)
Cystatin C (mg/L)	1.22 (0.78-3.63)	0.76 (0.53-1.21)
Age-corrected creatinine-ratio	1.85 (0.994-7.08)	1.08 (0.66-1.65)
GFR (mL/min/1.73m²)	63.4 (13.7-81.9)	109.8 (86.3-147.4)

*GFR ≤ 82 mL/min/1.73m^2
†Body mass index = weight (kg)/ height^2(m)

Table 8.
Patient characteristics in mean values and (range) from study II.

	Females	Males	All
Number	11	19	30
Age (years)	9.0 (3.0-12.8)	7.9 (2.4-13.3)	8.3 (2.4-13.3)
Height (m)	1.28 (0.94-1.57)	1.25 (0.84-1.68)	1.26 (0.84-1.68)
Weight (kg)	29.4 (13.3-52.6)	27.8 (12-61.3)	28.4 (12-61.3)
BMI (kg/m^2)	17.1 (14.6-23.9)	16.7 (12.8-30.0)	16.9 (12.8-30.0)
GFR (mL/min/ 1.73m^2)	93 (68-135)	102 (61-140)	99 (61-140)

3.2 STUDY POPULATION

The studies included children referred for routine measurement of GFR on an outpatient basis from March 2006 to December 2009 at the Department of Nuclear Medicine, Aalborg Hospital and Department of Clinical Physiology and Nuclear Medicine, Skejby Hospital. The main indications for referral were known or suspected nephro-urological disorders: congenital renal malformations (29.3%), hydronephrosis or reflux nephropathy (26.3%), recurring urinary tract infections (14.3%), parenchymal renal disorders (6.8%), and miscellaneous (13.5%).

The inclusion criteria were: age 2-14 years, parental, informed, written consent and referral to GFR measurement by ^{51}Cr-EDTA. The exclusion criteria for the studies involving CysC were: steroid treatment, rheumatoid arthritis, thyroid dysfunction, renal transplantation due to the fact that these conditions have been proven to affect levels of CysC independently of renal function (112-119). Ascites was an exclusion criterion due to possible inaccuracies when measuring GFR in patients with an expanded extra-cellular space. However, a homogenous distribution of body fluids is also assumed for the BIS measurements. To exclude the possibility of influencing the pacemaker with the electrical current, pacemaker was an exclusion criterion for BIS measurements. A total of 133 patients were enrolled into the study. All 133 were included in determination of BIS precision in study III (Table 5); 131 children were included in study I and IV (Table 6 and 7); 32 children had BIS measurements performed on the second day for determination of between-day variation in study III (Table 5); of these 32 children, 30 children were included in study II (Table 8); 28 children participated in calculation of GFR-estimates on two separate days for determination of the between-day variation of the BCM-model; and finally 44 children had BIS repeated after renography for determination of within-day variation of BIS in study III (Table 5).

3.3 STUDY DESIGN

The study was a cross-sectional study. On day one the children had height and weight measured. Height (cm) was measured to the nearest 0.5 cm with a fixed stadiometer. Body weight (kg) was measured to within 0.1 kg with electronic scales, the child dressed in light clothing. An intravenous line was inserted into the cubital vein for administration of ^{51}Cr-EDTA and for blood sampling. In between blood sampling for ^{51}Cr-EDTA-clearance all children (n=133) had additional samples taken for serum analysis of creatinine, CysC, blood urea nitrogen (BUN) and albumin, and all had a series of BIS measurements performed. Additionally, a subpopulation of Aalborg children (n=44) had a BIS series repeated after renography to assess within-day variation in study III.

On day two a second venous sample was obtained in a subpopulation (n=30) for analysis of serum CysC and for determination of day-to-day variation in study II. Furthermore, additional BIS measurements were performed in almost the same subpopulation (n=32), also to determine day-to-day variation, though in study III. Blood samples were obtained on two consecutive days between 9 AM and 3 PM with 20 - 29 hours (mean 23) between measurements (In one patient 47 hours elapsed between measurements and was not included in the calculation of mean hours between measurements). Duplicate analysis was performed to ascertain the analytical variation of CysC and creatinine.

Regarding the BIS measurements each series consisted of three repeated measurements within a few minutes. All 133

children had one series measured on day one (precision population). For determination of within-day variation a subpopulation of 44 children had a second series on day one, after renography with 1.5 - 4.8 (mean 2.8) hours between the two series.

Thirty-two children had a series measured on the next day between 9 AM and 3 PM with 20 - 28 (mean 23.5) hours between measurements (between-day sub-population).

As we wanted this study to reflect daily routine practice, no BIS-measurement was excluded if a child was crying or moving a little during the investigation. No restrictions regarding fluid and food-intake or toileting were given. The children, who were also referred to renography, were encouraged to drink water. Renography in itself will not affect BIS measurements, and neither is the nephro-urological disorders excepted to do so, while any fluid change will be reflected in the BIS results.

3.4 CYSTATIN C AND CREATININE ASSAYS

The venous blood samples of 1.2 mL were collected without stasis. They were centrifuged at 3000*g* for 10 minutes, and serum was separated from the blood cells and stored at -20 ˚C on the day of collection. Every 3 months the blood samples were packed on freeze-dried ice and delivered to Department of Clinical Biochemistry, Viborg Regional Hospital for analysis. CysC was measured using the N Latex cystatin C assay on the Behring Nephelometer II (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) (121). Creatinine was assayed with an IDMS-traceable, enzymatic method (Crea Plus), albumin was assayed with a bromcresol green method (ALB plus), and BUN was analyzed by a kinetic UV assay (BUN) - all on the Modular Analytics P (Roche Diagnostics, Mannheim, Germany). Before analysis, the serum samples were thawed and centrifuged at 3000*g* to ensure clarity. The serum samples were analyzed in duplicates for CysC and creatinine. One technician performed all assays.

3.5 ^{51}CR-EDTA CLEARANCE

GFR was determined as ^{51}Cr-EDTA plasma clearance by a standard five-sample technique with sampling 5, 15, 60, 90, 120 min following a bolus injection (0.11 MBq / kg, maximum 3.7 MBq) (21;44). In children aged 13-14 years the method differed slightly between the two hospitals since the 5–sample technique was used at Aalborg Hospital while a 4-sample technique with blood samples 180, 200, 220 and 240 minutes after bolus injection was used at Skejby Hospital (122). An intravenous line with a multi adapter was used to administer a minimum of 1 mL standard dose followed by 20 mL 0.9% NaCl. The venous blood samples were drawn through the same intravenous line but with a new multiadapter (123). A small amount of blood was discarded of before each sample and the intravenous line was rinsed with 0.5 mL heparin after each sample. Exact injected activity was determined by weighing the syringes before and after injection (maximum activity was 0.01 mSv). The total ^{51}Cr-EDTA plasma clearance was calculated from the injected dose divided by the total area under the plasma curve according to either a single-exponential model with corrections (122) or a double-exponential model, and the clearance was converted to GFR by (^{51}Cr-EDTA clearance – 3.6 mL/min/1.73m^2)×1.1. (44) The children were encouraged to stay in the bed during the investigation.

3.6 BIOELECTRIC IMPEDANCE SPECTROSCOPY

Whole-body BIS measurements were obtained by a multifrequency spectrum analyzer with Cole-Cole modelling software (Hydra ECF/ICF, Xitron Hydra 4200, Xitron Technologies, San Diego, CA). Prior to measurements the children had been resting

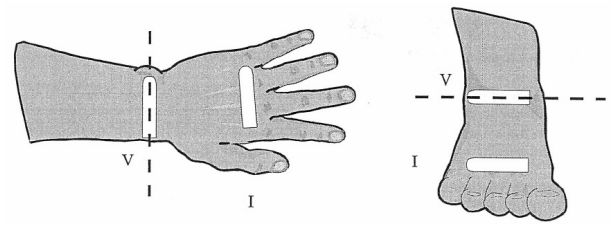


Figure 1
Correct locations of electrodes from the Xitron Manual p. 45

in the supine position for at least 10 min. Electrodes type IS 4000 (Xitron Technologies) were attached to the dorsal surfaces of right hand and foot located as follows: The electrodes for voltage measurements were applied at midline between the prominent bone ends on wrist and ankle, respectively, while the current injection electrodes were placed with the midline 5 centimeters distal to these positions, in accordance with the manufacturer's instruction manual (see Figure 1). When this was not possible because of small hands or feet, the current electrode was placed as distally as possible on the hand (not on the fingers), and the other electrode was placed with midline 5 centimeter proximal to this position.

Three repeated measurements of BIS were performed at each series. Electrodes were removed and replaced between series both within and between days.

Bioimpedance calculations: The built-in modeling software of the Xitron Hydra 4200 calculates the physiological parameters as described in the following text.

The extra- and intra-cellular resistance (R_E and R_I) are calculated from the Cole-Cole model limits for zero and infinite frequency.

The equation for calculation of extra-cellular fluid volume V_{ECF} in liters is:

$$V_{ECF} = k_{ECF} \cdot \left(\frac{H^2 \cdot \sqrt{W}}{R_E} \right)^{2/3} \qquad [10]$$

H = height of the person in cm, W = weight of the person in kg and k_{ECF} is a constant (female = 0.299, male = 0.307). The value of this constant can be calculated by the equation:

$$k_{ECF} = \frac{1}{100} \cdot \left(\frac{K_B{}^2 \cdot \rho_{ECF}{}^2}{D_b} \right)^{1/3} \qquad [11]$$

where K_B is a body geometry factor (4.3), ρECF is the resistivity of the extra-cellular fluid (female = 39.0 Ω·cm, male = 40.5 Ω·cm) and D_b is the overall body density (1.05 kg/liter). The constants used by the Xitron Hydra 4200 for calculating ECF was based on the distribution volume of bromide (124).

The volume of TBF is calculated by the formula:

$$V_{TBF} = V_{ECF} \cdot \left(\frac{\rho_{TBF}}{\rho_{ECF}} \cdot \frac{R_E + R_I}{R_I} \right)^{2/3} \qquad [12]$$

where ρTBF is the resistivity of the mixture of intra- and extra-cellular fluid. The value of ρTBF will vary from person to person. For a given individual, the value is calculated by the formula:

$$\rho_{TBF} = \rho_{ICF} - (\rho_{ICF} - \rho_{ECF}) \cdot \left(\frac{R_I}{R_E + R_I} \right)^{2/3} \qquad [13]$$

ρICF is the resistivity of the intra-cellular fluid (female = 264.9 Ω·cm, male = 273.9 Ω·cm).

The volume of intra-cellular fluid (ICF) is calculated from the volume of total body fluid and volume of extra-cellular fluid:

$$V_{ICF} = V_{TBF} - V_{ECF}. \quad 14]$$

Furthermore, the Xitron Hydra reports BCM by use of Moore's formula (96):

$$BCM = ICF/0.70 \quad [15]$$

which is based on the assumption that the body cells on average contain 70% water calculated by weight.

FFM is estimated by the formula:

$$FFM = (d_{ECF} \cdot V_{ECF}) + (d_{ICF} \cdot V_{ICF}) \quad [16]$$

where, according to the Xitron manual, d_{ECF} = 1.106 kg/liter and d_{ICF} = 1.521 kg/liter are the densities of extra/intra-cellular fluid and its associated materials.

%BF is percentage of non-FFM:

$$\%BF = 100\% \cdot (W - FFM)/W \quad [17]$$

Technical details on the methods and calculations used by the Xitron Hydra 4200 device can be found in Refs. (124;125). Ref. (126) are lecture notes on BIS with the Xitron Hydra 4200 as main example. Ref. (98) evaluates various bioimpedance methods with clinical emphasis.

4. STATISTICS

4.1 STUDY I: NEW GFR PREDICTION MODEL

All statistics were performed using the statistical software package STATA 11.0 (Statacorp, Texas, USA).

Test for correlation: Due to repeated referrals, the 131 investigated children consisted of 119 individual children, nine of which were investigated twice and one child being investigated four times. The median time between visits was 413 days, and only in a single case (98 days) less than 11 months, giving reason to assume that the repeated inclusions could be treated as independent data. To validate this assumption, a linear mixed effects model was used to assess possible within-subject correlation. No such significant correlation was found (p=0.16). Consequently, no adjustment for correlated observations was made in the further analysis.

Model selection: To select the variables to be included in the new, final GFR prediction model we used forward, stepwise regression. By this procedure, variables were added to the model in a forward selection procedure at significance level p<0.01 and removed again if their significance level dropped to below p<0.05 by addition of other variables. Following our model (section 3.1), GFR in mL/min was the dependent variable. BCM/CysC, height×BSA/creatinine, weight/CysC, gender, age, BMI, BSA, BCM, 1/BUN, 1/creatinine, and albumin were considered as possible explanatory variables. Weight, height, and 1/CysC could not be included in the selection procedure because of colinearity. As residuals lacked homoskedasticity, we repeated the stepwise regression with log-transformed variables resulting in identical selection of explanatory variables, while reducing heteroskedasticity.

The advantage of forward stepwise regression is that it automatically searches a large space of possible models among a large selection of variables. However, there is a possibility of overfitting the data, which means that the regression fits better in-sample than on a new sample. However, the cross-validation procedure described in the following will reveal if this is the case. Furthermore, the problem can be minimized by setting the criteria low for adding a variable, which is why we chose p<0.01. As we have only 11 possible variables, overfitting is not a serious problem. The ratio of possible variables to observations is recommended not to exceed 0.25, and the ratio in the present study is (11/131) = 0.08 (127).

To further validate the selection procedure a backward selection was also made, yielding identical variable selection at p<0.01 level.

Determination and validation of the new prediction model: The prediction model was determined by linear regression on the selected logarithmic variables. The prediction model was validated by a random sub-sampling cross-validation procedure (128) dividing the 131 children into two randomly chosen groups of approximately equal size. First, one group was used to estimate model parameters, while the other group was used as a test set to determine residuals. Then the roles of the two groups were reversed. In total, this gave us a full set of 131 non-biased residuals (i.e., all residuals were based on points not used for determining the model).

From the residuals, the following parameters were calculated for validation of the model:

- Root-mean-square error (RMSE)
- R^2 expressed as 1 – (residual variance) / (total variance of the reference GFR)
- Percentage of predictions being within ±10% and within ±30% of the reference GFR

As the residuals and thus the validation parameters will to some extent depend on how the points were divided into groups, the whole 2-fold procedure was repeated 1000 times. Based on the 1000 runs, median values and 2.5th and 97.5th quantiles of the validation parameters were calculated.

This model was chosen to avoid testing the procedure on the data that gave it birth to as this is almost certain to overestimate performance.

Previously published models: For each of the previously published models, GFR-residuals were estimated using the same random sub-sampling cross-validation procedure as described above. This includes the calculation of, "local" constants and coefficients by regression on the present dataset, thereby attempting to minimize any difference in GFR-method or analysis of serum CysC and creatinine. According to the original formulas estimates were calculated on the absolute values (7;12) or log-transformed values (6;8-10).

Finally, Bland-Altman plots of all models, including the new formulas based on BCM/CysC and weight/CysC were computed to illustrate the agreement between the estimated and measured GFR (mL/min/1.73m²) tested on all 131 included children. The fit was made on logarithmic variables and the differences between estimated and measured GFR were de-logarithmized and converted into percentages by the following formula:

$$DIF_{\%} = (\exp(DIF_{log}) - 1) \times 100\% \quad [18]$$

This was also the case for the bias and 95% limits of agreement. These plots do not reflect the unbiased results of the random sub-sampling cross-validation as calculation of 95% limits of agreement by this procedure is not possible. The plots illustrates

the "best-case scenario" when the models are tested on the same 131 children from which the models are derived.

Between-day variation: A linear mixed effects model was used to estimate the between-day variation of the BCM-model in 28 children. This statistical model estimated the standard deviations of variance for the GFR-estimates from two separate days. Data were log-transformed to obtain a constant variance across all levels of the response variable. The assumption of variance normality was tested graphically.

The two estimates were based on the median BCM-values (as recommended in the results from study III) and the first analysis of the serum sample for CysC and creatinine measured on to different days. Body weight and height was only registered the first day.

4.2 STUDY II: ANALYTICAL AND BIOLOGICAL VARIATION OF CYSTATIN C AND CREATININE

All statistics were performed using the statistical software package STATA SE 9.0 (Statacorp, Texas, USA), Microsoft Office Excel 2003 and GraphPad PRISM ver. 4.01 for Windows NT (GraphPad Software Inc., CA, USA).

The biological variation of creatinine and CysC including analytical variance (SD_A^2), within-subject variance (SD_I^2) and between-subject variance (SD_G^2) was calculated based on the principles given by Fraser and Harris (129).

SD_A^2 was calculated from the differences between duplicate analyses obtained both days:

$$SD_A^2 = \frac{\sum_{i=1}^{n}(x(i)_1 - x(i)_2)^2 + \sum_{i=1}^{n}(y(i)_1 - y(i)_2)^2}{4n} \quad [19]$$

where $x(i)_1$ and $x(i)_2$ denote the first and second analysis on the sample taken day one, likewise for y(i) at day two, and n is the number of subjects.

The total day-to-day variance on mean values, SD_T^2, was determined by:

$$SD_T^2 = \frac{\sum_{i=n}^{n}(\overline{x}(i) - \overline{y}(i))^2}{2n} \quad [20]$$

Subtracting $SD_A^2/2$ from SD_T^2 gives the within-subject variance:

$$SD_I^2 = SD_T^2 - \frac{SD_A^2}{2} \quad [21]$$

Subtracting SD_I^2 and SD_A^2 from the overall variance day one (SD_p^2) determined the between-subject variance (SD_G^2).

$$SD_G^2 = SD_P^2 - \left(SD_I^2 + \frac{SD_A^2}{2}\right) \quad [22]$$

It should be noted that SD_P^2 was determined only on data from 21 children with normal standard GFR (>82 mL/min/1.73m^2) (130) as the variance of CysC and creatinine when also including children with decreased renal function would be higher and would not provide a true picture of the normal variance. In addition, SD_G^2 adjusted for the age-related increase in creatinine was calculated using linear regression of mean creatinine on age. The index of individuality (IOI=SD_I/ SD_G) was calculated for both the adjusted and unadjusted SD_G^2 for creatinine and for CysC. Finally, the reference change value (RCV) for a single determination was determined as 2.77×SD_T, corresponding to a 5% confidence level when assuming normal distribution (72). RCV is the smallest difference between two successive measurements that signify a statistically significant change of the measured value.

Since the individual variance and differences for both analytes in absolute terms (µmol/L and mg/L) did not correlate significantly with the level of serum concentrations, all calculations were done in absolute terms. Comparison of first and second analysis, and mean value of duplicate analysis day one and two, was made by Students´ paired *t*-test. Normality of differences was tested graphically. To compare our results with the literature, the analytical, within-subject and between-subject variations were also expressed as coefficients of variation (CV_A, CV_I, CV_G) calculated as the ratio between SD and the corresponding mean value.

4.3 STUDY III: PRECISION AND VARIATION OF BIOIMPEDANCE MEASUREMENTS

All statistics were performed using the statistical software package STATA SE 9.0 (Statacorp, Texas, USA). A linear mixed effects model was used to estimate the analytical variation (SD_A), within-day variation ($SD_{I(1)}$) and between-day variation ($SD_{I(2)}$)) of both electrical and physiological BIS parameters. This model estimated the standard deviations for each of the variance components mentioned above (the random effects). In this analysis no fixed effects were assumed. Data were log-transformed (except %BF) to obtain a constant variance across all levels of the response variable. The assumption of variance normality was tested graphically. It is possible to calculate the three SDs in a single analysis with the mixed effects model if there is no variation in residual variation, which is the analytical variation. However, analysis of SD_A, $SD_{I(1)}$ and $SD_{I(2)}$ was done in three separate datasets as SD_A varied both within- and between days.

Standard deviations calculated in the log scale were transformed back to the original scale as percents with the formula

$$SD_{\%} = (\exp(SD_{log}) - 1) \times 100\% \quad [23]$$

A percent standard deviation has the same interpretation as the coefficients of variation (standard deviation divided by the mean). The variation of %BF was computed on the original scale and should be interpreted as percent-points.

For all BIS-parameters the individual variation between the three repeated measurements showed a tendency to be highest in small children, especially in those younger than six years of age. As a consequence we chose to age-divide the analysis of precision into two groups: ≥6 years and <6 years.

If changes over time (either within-day or between-days) are to be measured, individual and analytical variance will be seen together as the total variance: $SD_T^2 = SD_I^2 + SD_A^2$. Total variance on a single measurement can be calculated from SD_I and SD_A. We found, however, that from time to time one of our three repeated measurements was an outlier, thereby increasing variance unnecessarily. To deal with this problem we chose the median values of the three repeated measurements in each series to calculate total variation within- and between days ($SD_{T(1)}^2$ and $SD_{T(2)}^2$). Thus, the calculation was made directly from the data (instead of from SD_I and SD_A), and we report the total variation on median values instead of total variation on single values.

Levene´s test was used to test for significant difference between age-groups in analytical variation, between-day variation and total between-day variation on median values. This was not done for within-day variation and total within-day variation as only six children were <6 years. P-values were considered significant if $p<0.05$.

Reference change values (RCV) were determined as described in 4.2.

4.4 STUDY IV: DIAGNOSTIC PERFORMANCE OF SERUM MARKERS AND GFR MODELS

Definitions: Reduced renal function was defined as GFR ≤75% of normal mean value in children (109 mL/min/1.73m^2) (131) and normal GFR was defined as GFR >75% of normal value (130). Thus, the limit between normal and reduced renal function was 82 mL/min/1.73m^2.

We investigated the following variables as predictors of GFRref:

- CysC = serum cystatin C (mg/L)
- Creatinine = serum creatinine (µmol/L)
- Crea-norm = Crea / (age-dependent normal creatinine level), using age-dependent creatinine levels by Pottel et al. (16)
- $eGFR_{Sch\_old}$ = estimated GFR from Schwartz old formula (mL/min/1.73m^2)
- $eGFR_{Bök}$ = estimated GFR from Bökenkamp formula (mL/min/1.73m^2)
- $eGFR_{Fil}$ = estimated GFR from Filler formula (mL/min/1.73m^2)
- eGFRBou = estimated GFR from Bouvet formula (mL/min/1.73m^2)
- $eGFR_{Zap}$ = estimated GFR from Zappitelli formula (mL/min/1.73m^2)
- $eGFR_{Sch\_new}$ = estimated GFR from Schwartz new formula (mL/min/1.73m^2)
- $eGFR_{BCM}$ = estimated GFR from the BCM-model, normalized to 1.73m^2 (mL/min/1.73m^2)
- $eGFR_{weight}$ = estimated GFR from the Weight-model, normalized to 1.73m^2 (mL/min/1.73m^2)

The previously published models were revised with local constants and coefficients in study I to fit the present data (see Table 9), though the currently recommended Schwartz formula was assessed with both the published constant (k=36.5) (9) and the constant revised to this study (k=35.4).

The following probability analysis was only carried out for CysC, creatinine, $eGFR_{Sch\_old}$, $eGFR_{BCM}$, and $eGFR_{weight}$ as the principle is the same for all models. However, the cut-off levels were calculated for all variables.

For all variables except creatinine, we found it possible to fit a linear relation

$$\ln(GFR_{ref}) = a \times \ln(\text{variable}) + b \qquad [24]$$

with normal and (reasonably) homogeneous distribution of the residuals. The standard deviation SD was computed as the root mean square error (RMSE) of the residuals. Thus, if the given variable has value x, then $\ln(GFR_{ref})$ will be normally distributed with mean value $a \times \ln(x) + b$ and standard deviation SD, where a, b, and SD are known from the fit.

Since the normal distribution is mathematically well-known, we can calculate the probability that $\ln(GFR_{ref}) < \ln(82)$, i.e. the probability that renal function is reduced by our definition above. The value ln(82) will be Z standard deviations above the regression line, where

$$\ln(82) = a \times \ln(x) + b + Z \times SD \qquad [25]$$

$$\Updownarrow$$

$$Z = \frac{\ln(82) - a \times \ln(x) - b}{SD} \qquad [26]$$

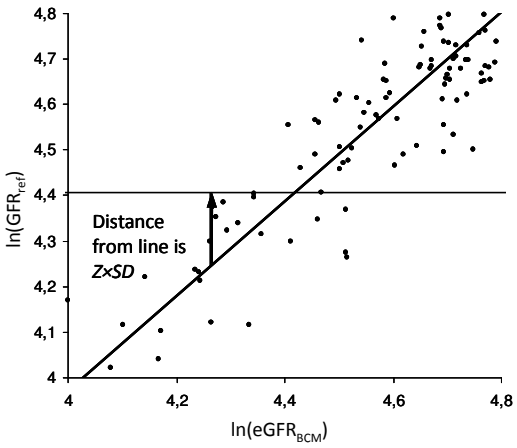


Figure 2

Regression line and data for data point near the value GFR_{ref} = 82 mL/min/1.73m^2, using x = $eGFR_{BCM}$ as an example. The line $\ln(GFR_{ref})$ = ln(82) delimits reduced renal function (below the line) from normal renal function (above the line). The arrow shows an example where x = $eGFR_{BCM}$ = 72 mL/min/1.73m^2 $\Longrightarrow$ ln(x) = 4.28. Using Table 19, it is found that Z = +1.60.

See Figure 2 for an example. A high positive value of Z means that the limit value ln(82) is considerably higher than the value of $\ln(GFR_{ref})$ determined by the regression line, thus giving a high probability that GFR_{ref} < 82 mL/min/1.73m^2. Likewise, large negative Z values correspond to low probability of reduced renal function. Using Table 19, it is found that Z = +1.60.

For a given value of Z, the probability of reduced renal function equals the area under the standard normal distribution up to the value of Z (see Figure 3). This can be calculated as Φ(Z) where Φ is the cumulative distribution function for the standard normal distribution.

It should be noted that the probability is one-sided: Only positive Z gives high probability. Thus, Z ≥ +2.33 gives 99% probability of reduced renal function, while Z ≤ –2.33 gives 1% probability of reduced renal function. Accordingly, 98% of the logarithmic data will lie within a (two-sided) interval ±2.33×SD around the regres-

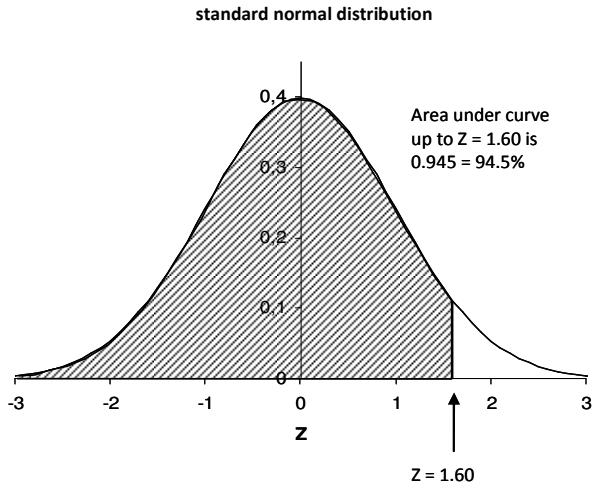


Figure 3

Probability calculation. For Z = +1.60 the probability of reduced renal function is 94.5%. For negative Z values, probability of reduced renal function will be <50%.

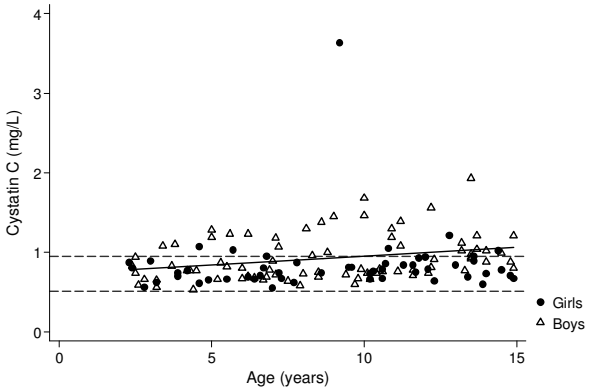


Figure 4
Correlation between cystatin C and age.
Regression line for boys (solid line): CysC = age × 0.02 + 0.72; (95% confidence interval on slope = 0.007; 0.040). Regression line for girls (not shown): CysC = age × 0.008 + 0.78; (95% confidence interval on slope = -0.023; 0.039). Reference interval for CysC is illustrated with dotted lines (0.51-0.95 mg/L) (33).

sion line, and 98% limits of agreement (LOA) were computed as

LOA = exp(regression line ± 2.33×SD) [27]

Cut-off levels were determined from Z = ±2.33, corresponding to the intersections between LOAs and the value 82 mL/min/1.73m² (see Figure 10).

The risk of misclassification at a cut-off level is only 1% (one-sided probability), and farther from the cut-off values, the risk is even lower. So, outside the range delimited by the cut-off values correct classification can be given with at least 99% certainty. In contrast to this "black or white" situation, there is a "grey zone" between cut-off levels where, however, the probability of reduced renal function can be calculated. Furthermore, the percentage of children in the grey zone was calculated for all models.

The probability calculation assumes homogeneity and normality of the residuals found with $\ln(GFR_{ref})$ as dependent variable. For creatinine, the residuals were not normally distributed. It was, however, possible to fit ln(Crea) as a linear function of $\ln(GFR_{ref})$ (instead of the other way around), with normally distributed residuals. This allows calculation of 98% agreement intervals for creatinine, given GFR_{ref}, and tentative cut-off values for creatinine were computed by the intersection between these agreement intervals and the value GFR_{ref} = 82 mL/min/1.73m^2, but without probability analysis.

The area under curve (AUC), which is an indicator of accuracy, was calculated for each method by receiver-operating characteristic analysis (ROC) (82) and compared by *t*-test. *p*-values were considered significant if $p<0.05$.

All statistics were performed using the statistical software package STATA 11.0 (Statacorp, Texas, USA).

5. RESULTS

5.1 STUDY I

A slight but significant correlation was found between CysC and age for boys (R^2=0.086, p=0.009), but not for girls (R^2=0.005, p=0.62), suggesting an increase in serum CysC with age (Figure 4). Gender specific regression of age on GFR (mL/min/1.73m^2) does not reveal an incidental dependency of GFR with age (95% confidence interval on slope: -1.5; 1.9), which could explain the age-dependency of CysC for boys.

As expected a highly significant positive correlation between age and creatinine was also found in our population (R^2=0.13 and $p<0.0001$).

Determination of GFR-models: Figure 5 shows that 85% of the variation of GFR (mL/min) can be explained by its relation with BCM/CysC (R^2=0.85), supporting our hypothesis of a linear correlation between GFR in mL/min and BCM/CysC with no evidence of extrarenal clearance. In comparison 72% of the variation of GFR (mL/min/1.73m²) can be explained by its relation with height/Crea (R^2=072) (Figure 6).

Accordingly, it is not surprising that the described selection procedure selected the two variables: BCM/CysC and height×BSA/Crea for prediction of GFR (mL/min).

GFR = 29.8 × height/Crea + 16.3 mL/min/1.73m^2 (R^2=0.72).

As described prediction was made on logarithmic values, yielding

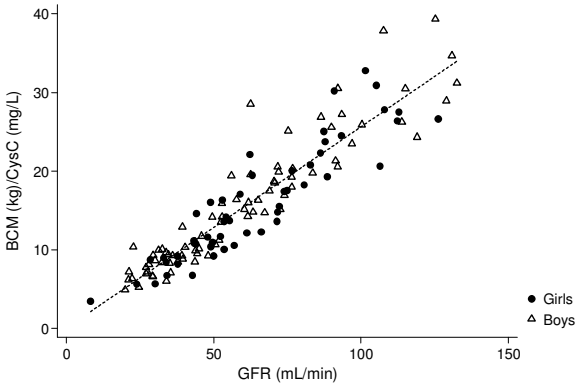


Figure 5
Relationship between GFR (ml/min) and BCM/CysC. Regression line is superimposed.
BCM/CysC = 0.26 × GFR + 0.025
⇔ GFR = 3.9 × BCM/CysC – 0.1 (R^2= 0.85).
Intercept is not significantly different from 0.0 (p = 0.967).

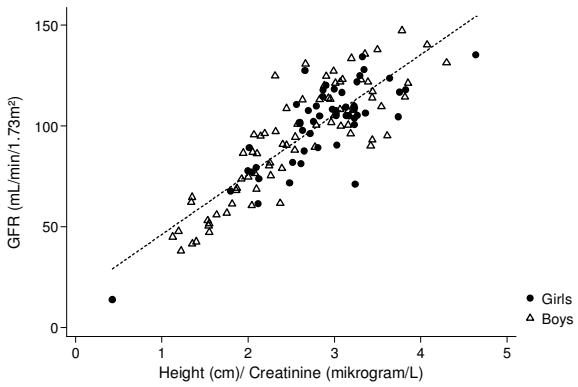


Figure 6
Analysis of GFR (mL/min/1.73m^2) and height/Crea Regression line is superimposed.
GFR = 29.8 × height/Crea + 16.3 mL/min/1.73m^2 (R^2=0.72).

Table 9.
Revised models

Reference	Estimated GFR (mL/min/1.73m2)
Schwartz_old	GFR = 35 × height/Crea
Bök_eq	GFR = (65.4/CysC) + 16.8 ((112.8/CysC) – 41.0)
Fil_eq	GFR = 78.4 × $(1/CysC)^{0.997}$
Bou_eq	GFR (mL/min) = 19.9 × $Crea^{-0.587}$ × $CysC^{-0.48}$ × $weight^{0.85}$ × $age^{0.185}$
Zap_eq	GFR = (322.1 × $e^{0.0073 \times height}$) × $CysC^{-0.481}$ × $Crea^{-0.586}$
Schwartz_new	GFR = 11.5 × $(height/Crea)^{0.57}$ × $CysC^{-0.46}$ × $BUN^{-0.068}$ × $(1.044)^{male}$ × $height^{0.319}$

$$\log(GFR) = 2.32 + 0.40 \times \log\left(\frac{BCM}{CysC}\right) + 0.65 \times \log\left(\frac{height \times BSA}{Crea}\right) \quad [28]$$

where log denotes the natural logarithm. R^2 was 0.96. De-logarithmizing, the resulting model is

$$GFR(mL/min) = 10.2 \times \left(\frac{BCM}{CysC}\right)^{0.40} \times \left(\frac{height \times BSA}{Crea}\right)^{0.65} \quad [29]$$

GFR can subsequently be converted to standardized GFR by GFR × 1.73m^2/ BSA.

Allowing for the possibility that BCM is not available the selection procedure was also run without the variables based on BCM. The selected variables were then weight/CysC and height×BSA/Crea, resulting in the following model:

$$GFR\,(mL/min) = 7.5 \times \left(\frac{weight}{CysC}\right)^{0.39} \times \left(\frac{height \times BSA}{Crea}\right)^{0.63} \quad [30]$$

The weight-model yields an R^2 = 0.95, and an RMSE = 0.117 (corresponding to 12.4% after de-log transforming the values).

Table 9 summarizes all resultant models with local constants and coefficients yielded by our population.

For the original Schwartz model, revised in 2009 (12) we derived a local constant (k) of 35 by regression without an intercept of GFR (mL/min/1.73m^2) on height/Crea. The constant k was independent of age and gender. The local model is referred to as Schwartz_old.

The CysC-based models by Filler et al (8) and Grubb et al (95) are of similar form with the only exception that Grubb et al included a constant for age <14 years due to an observed systematic difference between predicted and measured GFR values. Our data could not confirm such age effect and the resultant model is herein referred to as Fil_eq.

Table 10
Conversion factors from units used by Schwartz (left) and our units (right).

	Schwartz	Present
height	1 m	= 100 cm
Crea	1 mg/dL	= 88.4 µmol/L
CysC	1 mg/L	= 1 mg/L
BUN	1 mg/dL	= 0.357 mmol/L

The new equation by Schwartz et al. from 2009 (9) used different units than ours. These were converted to our units (Table 10) enabling us to compare their model to the new model with local coefficients (Schwartz_new).

Schwartz's 2009 formula:

$$GFR(mL/min/1.73m^2) = 39.1 \times \left[\frac{height(m)}{Crea(mg/dL)}\right]^{0.516} \times \left[\frac{1.8\,mg/dL}{CysC}\right]^{0.294} \times \left[\frac{30\,mg/dL}{BUN}\right]^{0.169} \times [1.099]^{male} \times \left[\frac{height}{1.4\,m}\right]^{0.188} \quad [31]$$

Combining all constants and changing units (Table 4) we find the following equivalent formula:

$$GFR(mL/min/1.73m^2) = 25.7 \times \left[\frac{height(cm)}{Crea(\mu mol/L)}\right]^{0.516} \times \left[\frac{1\,mg/dL}{CysC}\right]^{0.294} \times \left[\frac{1\ mmol/L}{BUN}\right]^{0.169} \times [1.099]^{male} \times [height(cm)]^{0.188} \quad [32]$$

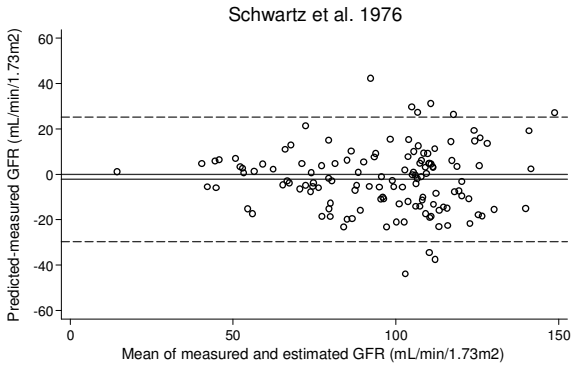
The models by Bökenkamp et al., Bouvet et al., and Zappitelli et al. were also assessed and the local models are referred to as Bök_eq, Bou_eq and Zap_eq (Table 9).

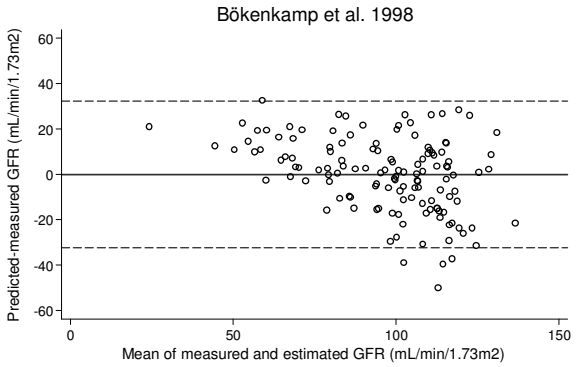
Performance evaluation of all models: The results of the random sub-sampling cross-validation are given in Table 11, with RMSE of the logarithmic models converted to percents. The highest R^2 and the highest percentage within 30% and 10% of measured GFR were found for the present study with BCM, only with slightly overlapping quantile intervals when comparing to Schwartz_new. Furthermore, the present model yielded the lowest RMSE with no overlapping quantile intervals.

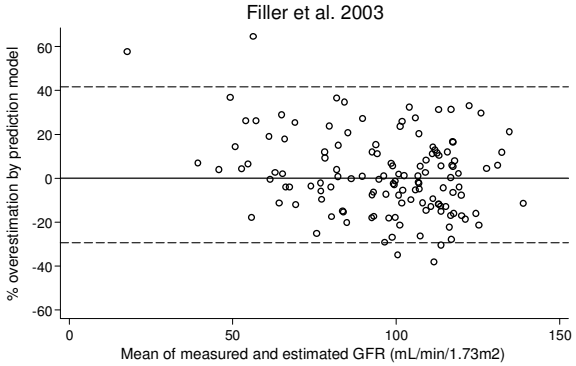
Our Weight-model, derived with log(weight/CysC) instead of log(BCM/CysC), had the second highest R^2 and estimates within 10% of measured GFR. No significant differences were seen, though, when comparing the results of the Weight-model with the results of Zapp_eq and Schwartz_new.

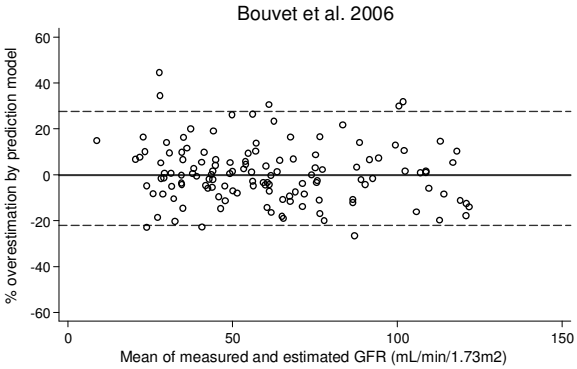
Table 11
Median values and 2.5% and 97.5% quantiles from the random subsampling validation, calculated for R^2, RMSE and percentages of GFR estimates within 30% and 10% of measured GFR.

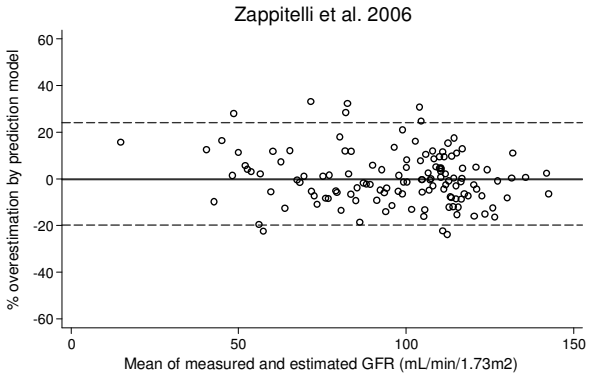
Reference	R^2	RMSE	GFR (%) within 30%	GFR (%) within 10%
Schwartz_old*	0.69 (0.65;0.69)	14.2 (14.1; 14.9) mL/min/1.73m^2	95.4 (94.7;96.2)	51.2 (47.3; 54.2)
Bök_eq*	0.57 (0.50; 0.58)	16.7 (16.4; 18.0) mL/min/1.73m^2	89.3 (84.7;90.8)	42.0 (38.9; 45.0)
Fil_eq	0.7 (0.64; 0.72)	20.0% (19.4%; 22.0%)	90.1 (86.3; 92.4)	42.0 (38.2; 45.0)
Bou_eq	0.93 (0.91;0.94)	14.2% (13.5%; 15.6%)	96.2 (94.7; 97.7)	57.3 (51.2; 61.8)
Zap_eq	0.88 (0.85; 0.89)	12.3% (11.9%; 13.7%)	97.0 (95.4; 98.5)	62.6 (58.0; 65.7)
Schwartz_new	0.87 (0.85; 0.89)	12.5% (11.9%;13.8%)	97.0 (95.4; 98.5)	62.6 (56.5; 67.2)
BCM-model	***0.96 (0.95; 0.96)***	***11.0% (10.7%; 12.1%)***	***98.5 (97.7; 99.2)***	***67.2 (62.6; 71.0)***
Weight-model	0.95 (0.94; 0.95)	12.4% (11.9%; 13.5%)	97.7 (96.2;98.5)	63.4 (58.8;67.2)

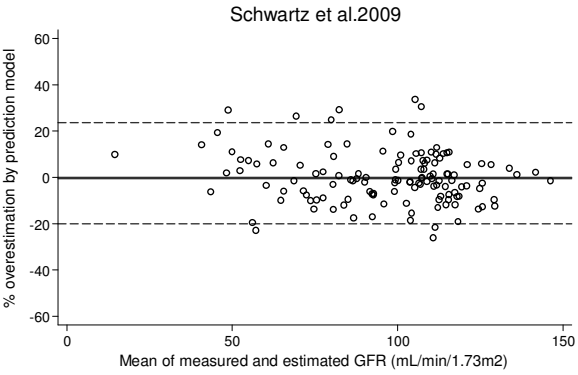

Schwartz et al. 1976
Predicted-measured GFR (mL/min/1.73m2)
Mean of measured and estimated GFR (mL/min/1.73m2)

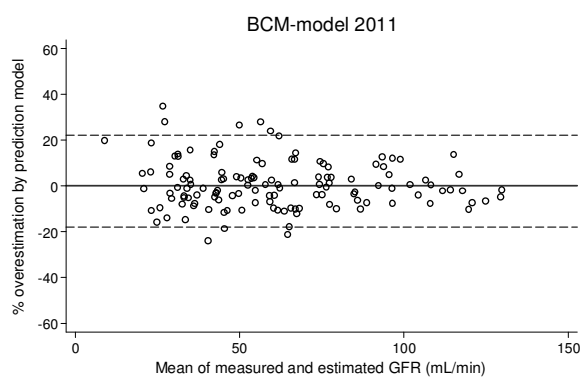

Bökenkamp et al. 1998
Predicted-measured GFR (mL/min/1.73m2)
Mean of measured and estimated GFR (mL/min/1.73m2)

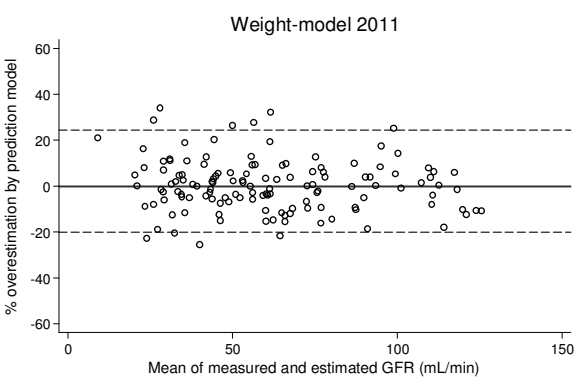

Filler et al. 2003
% overestimation by prediction model
Mean of measured and estimated GFR (mL/min/1.73m2)


Bouvet et al. 2006
% overestimation by prediction model
Mean of measured and estimated GFR (mL/min/1.73m2)


Zappitelli et al. 2006
% overestimation by prediction model
Mean of measured and estimated GFR (mL/min/1.73m2)


Schwartz et al.2009
% overestimation by prediction model
Mean of measured and estimated GFR (mL/min/1.73m2)


BCM-model 2011
% overestimation by prediction model
Mean of measured and estimated GFR (mL/min)


Weight-model 2011
% overestimation by prediction model
Mean of measured and estimated GFR (mL/min)

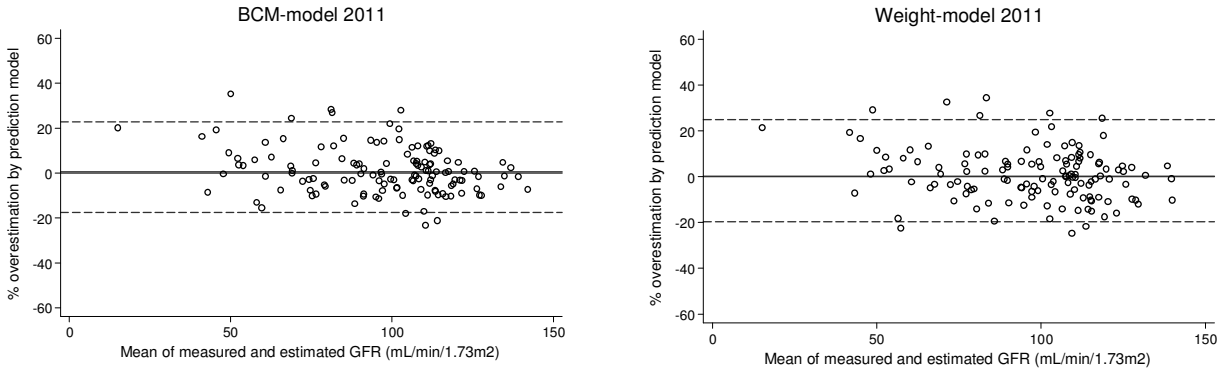


Figure 7
Bland-Altman plots of all revised models. 95% LOA: Schwartz_orig (-29;25 mL/min/1.73m2), Bök_eq (-32;32 mL/min/1.73m2), Fil_eq (-29;42%), Bou_eq (-21;28%), Zap_eq (-19;24%), Sch_new (-19;24%), BCM-model (-18;22%), Weight-model (-19;24%).
The BCM- and Weight-model are both illustrated with mL/min and mL/min/1.73m^2 yielding the same limits of agreement.

Figure 7 illustrates Bland-Altman plots for all models when the models are applied to all 131 included children outside the random sub-sampling procedure and are therefore the best possible outcomes in this population.

The 95% LOA for BCM-model were the narrowest (-18;22%), but probably not statistically lower than the models with the second narrowest 95% LOA, which was the Weight-model, followed by the Schwartz_new and Zap_eq all having the same 95% LOA (-19;24%).

Between-day variation: The total variation between days was estimated in 28 children who had BCM, CysC and creatinine determined on two separate days. The median hours between measurements was 23.2 hours (± 4.9), though 47 hours elapsed between measurements for one child. The total variation was 7.7%.

5.2 STUDY II

The distribution of data from study II is illustrated graphically in Figure 8. Table 12 lists the results of the variability analysis for CysC and creatinine. The total-day-to-day variation was 6.6% for CysC and 6.9% for creatinine. For CysC the analytical variance contributed 1.9% of the total variance, the within-subject variance contributed 29.0% and the between-subject variance contributed 69.1%. The resulting IOI was 0.65. The reference change value for two analyses separated in time was 0.16 mg/L (18% of the mean value).

For creatinine the analytical variance contributed 1.0% of the total variance, the within-subject variance contributed 5.9% and the between-subject variance contributed 93.1%. However, adjusting for the age-related increase in serum creatinine the variance in creatinine changed the distribution of the respective variances: analytical variance: 1.8%, within-subject variance: 11.3%, and between-subject variance: 86.9%. The resulting IOIs were 0.25 (data not adjusted for age) and 0.36 (data age-adjusted).

Surprisingly, when analysing the creatinine data a significant difference (0.83 µmol/L) between first and second analysis was found (p<0.0001). Likewise, a significant difference between the mean values of creatinine analyses day one and day two was found (-1.8 µmol/L, (p<0.034). Nevertheless, adjusting for these differences (by subtracting the mean differences from the systematic difference and squaring) did not change the coefficients of variation much (CV_A=2.3%, CV_I=5.9% and CV_G=28.6%). No differences were identified for CysC.

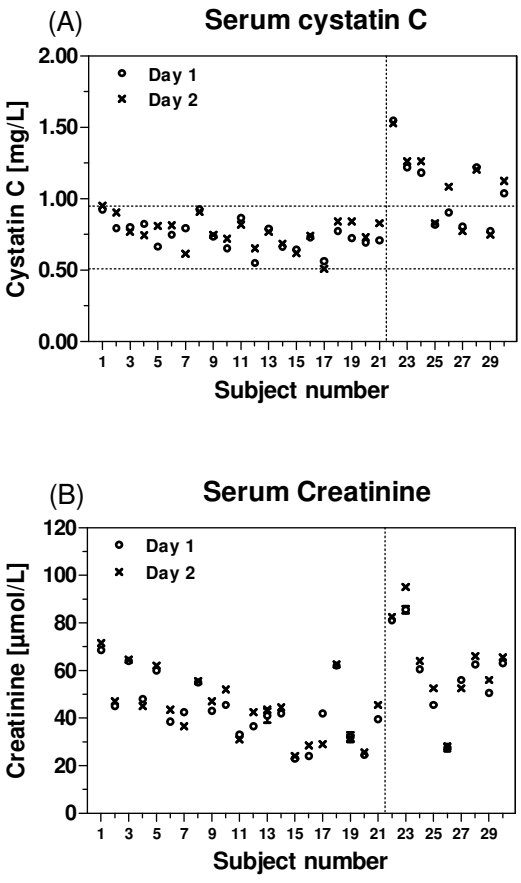


Figure 8
Distribution of data showing serum cystatin C (A) and serum creatinine (B) as mean values at day 1 and day 2 for each subject. Subject number 1-21 with normal GFR > 82 mL/min/1.73m2, and subject number 22-30 with decreased GFR ≤ 82 mL/min/1.73m2. The vertical dotted line separates the two groups. The horizontal dotted lines in figure A mark the upper limit (0.95 mg/L) and the lower limit (0.51 mg/L) of the population based reference interval of cystatin C (33).

A summary of previous results on the subject are presented in Table 13.

Table 12
Variability analysis for cystatin C and creatinine

Variable	Cystatin C (mg/L)	Creatinine (µmol/L)	Creatinine µmol/L, adjusted for age
Mean (range)	0.850 (0.51-1.56)	49.4 (22-96)	
Mean* (range)	0.752* (0.51-0.96)	43.9* (22-72)	43.9*
SD_A	0.014	1.3	
SD_I	0.054	3.2	
SD_G	0.084	12.5	8.7
CV_A	1.7%	2.5%	
CV_I	6.4%	6.4%	
CV_G	11.1%	28.4%	20.1%
RCV	18.3%	19.0%	
IOI	0.65	0.25	0.36

*Mean of 21 children with normal standard GFR.
Abbreviations: SD_A = analytical standard deviation, SD_I = within-subject standard deviation, SD_G = between-subject standard deviation, CV_A = analytical coefficient of variation, CV_I = within-subject coefficient of variation, CV_G = between-subject coefficient of variation,
RCV = reference change value (= 2.77 x $(SD_A^2 + SD_I^2)^{½}$), IOI = index of individuality (=SD_I/SD_G).

Table 13
Comparison of results from the present study to the previous studies on biological variation in children: analytical variance (CV_A), within-subject (CV_I), and between-subject (CV_G) given in percent.

Reference	Present study	Bökenkamp (75)	Sambasivan (73)	Sambasivan (73)	Podracka (74)
Population (n)	GFR 61-140* (30/ 21†)	Renal transplants (24)	GFR 90-135* (38)	GFR < 60* (54)	Solid organ transplants (20)
CystatinC					
Mean (mg/L)	0.850 (0.752)‡	-	0.8	2.81	-
CV_A	1.7	-	3.1 (6.7)**	-	-
CV_I	6.4	13.2	-	12.0	10.3
CV_G	11.1†	-	20.0	-	-
Creatinine					
Mean (µmol/L)	49.4 (43.9)‡	-	52.4	188.9	-
CV_A	2.5	-	1.8 (1.8)**	-	-
CV_I	6.4	14.6	-	13.0	7.7 (mean)
CV_G	28.4*/ 20.1§	-	30.0	-	-

*GFR is stated in mL/min/1.73m^2. †Only the 21 patients with normal GFR > 82 mL/min/1.73m^2 are included in the calculations of CV_G. ‡Mean values of 21 patients with normal GFR. §CVG adjusted for age. **At high concentrations.

Table 14
Parameters studied in this study

Abbreviation	Explanation
ECF*	Extra-cellular fluid (L)
ICF*	Intra-cellular fluid (L)
TBF*	Total body fluid. TBF = ECF + ICF (L)
BCM	Body cell mass. Total mass of cells in the body (kg)
FFM	Fat-free mass. Total body mass excluding fat (kg)
%BF	Weight-percentage of body fat (%)
R_E	Extra-cellular resistance (see text) (Ohm)
R_I	Intra-cellular resistance (see text) (Ohm)

*The classical work by Moore et al. (18) called these parameters ECW, ICW, and TBW, as abbreviations for extra-cellular water, intra-cellular water, and total-body water, respectively. However, as stressed by Matthie (21), the body fluids are not simply water. On the contrary, ions (Na+, K+, Cl-, etc.) are very important for both body physiology and electrical properties of the fluid. For this reason, we have chosen to follow Matthie's notation, calling the mix of water and ions fluid (F) instead of water (W).

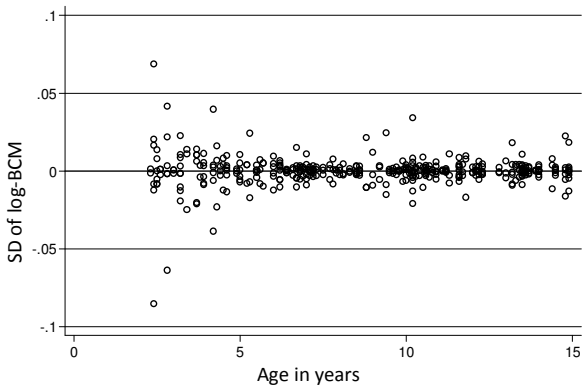


Figure 9
Standard deviation of log-BCM within measurement series shown as a function of child age. The plot shows age-dependency with larger SD for the youngest children.

5.3 STUDY 3

In Table 14 all parameters investigated are presented. Table 5 summarizes patients' characteristics and mean values and ranges of all parameters in the three sub-populations.

Precision: For all BIS-parameters a clear age-effect was found on precision, which was poorest among the youngest children. Consequently, the precision analysis was done separately for children ≥6 years and <6 years (see Table 15).

Figure 9 is an example of this age-dependency and the distribution of individual values of SD_A for BCM as a function of age illustrates why separation in two age-groups is meaningful.

Between-day variation: For the between-day variation no significant age effect was found. Calculations were made using all the data (i.e., 2 sets of 3 measurements for each child). Computing the total between-day variation we used two median values for each child because of incidental outliers, and no significant age effect was disclosed. Results are reported in Table 16. The two calculations yielded very similar results, but assumptions of normal distribution and constant variance were better fulfilled by the median data than when using all data.

Within-day variation: In the within-day dataset one of the six children <6 years (age 2.4 years) had an unusually high variation, though the child had been still during the measurement. However, the other five children had variation similar to or lower than the within-day variation found for the 38 children of age ≥6 years. Total within-day variation based on median values yielded similar results as within-day variation based on all data, but again the assumption of normality was better fulfilled by the median data.

It should be noted that the within-day and between-days variation in this study reflect daily routine practice as no restrictions regarding fluid intake and toileting were given. Consequently, the variations would most likely be lower in another setting with strict control and registrations of fluid intake and output.

Reference change value: The RCV showed that depending on BIS parameter a change of 6.6-16.5% is considered a significant change between different days and a change of 3.2-8.2% is considered significant within the same day.

5.4 STUDY IV

Of the 131 children, 94 had normal renal function (GFR_{ref} > 82 mL/min/1.73m_2), while 37 had reduced renal function (GFR_{ref} ≤ 82 mL/min/1.73m_2). Thus, the prevalence of reduced renal function in this population was 28.2%. Creatinine was corrected for age by dividing the measured creatinine with the age-specific median value (Table 17), which results in an increasing ratio (Crea-norm) with decreasing GFR.

The data points, LOAs, and cut-off levels for all models are illustrated in Figure 10, and cut-off levels are summarized in Table 18. Data points in the "grey zone" between the two cut-off levels represents cases for which further tests must be performed to either confirm suspicion of reduced GFR or confirm the assumption of normal GFR. However, the referring clinician can be guided by the probability analysis illustrated in Figure 11, in which the probability of reduced renal function may be estimated from any given level of CysC, Crea-norm, $eGFR_{Sch\_old}$, $eGFR_{BCM}$, or $eGFR_{weight}$. The probability can also be calculated by first using Eq. [26] and Table 19 to calculate Z, and then find the function Φ(Z) in a

Table 15
Analytical variation (SD_A), within-day variation ($SD_{I(1)}$) and between-day variation ($SD_{I(2)}$) for all BIS-parameters.

	Precision* (SD_A) ≥6 years	Precision* (SD_A) <6 years	Within-day ($SD_{I(1)}$) All ages	Between-day ($SD_{I(2)}$) All ages
Subjects/ obs.	99/ 296	34/ 100	43/ 257 (44/ 263)†	32/ 191
BCM	0.7%	2.1%	2.5% (3.1%)†	4.6%
TBF	0.4%	1.2%	1.4% (1.9%)†	3.2%
ECF	0.3%	0.5%	1.3% (1.4%)†	2.9%
ICF	0.7%	2.2%	2.5% (3.1%)†	4.6%
FFM	0.4%	1.3%	1.6% (2.1%)†	3.4%
R_E	0.4%	0.7%	2.0% (2.1%)†	4.4%
R_I	0.8%	2.4%	2.8% (3.7%)†	5.7%
%BF‡	0.3%	1.0%	1.1% (1.5%)†	2.4%

*Significant difference between age-groups (p<0.0001 for each parameter)
†For one child, within-day change was very large. Numbers in parenthesis include these outlier data.
‡For %BF, the numbers are given in percent-points.

Table 16
Total within-day ($SD_{T(1)}$), total between-day variation ($SD_{T(2)}$) and reference change values (RCV) on median values for all BIS parameters.

	Total within-day variation*		Total between-day variation†	
	$SD_{T(1)}$	RCV	$SDT_{(2)}$	RCV
Subjects/ obs	43/ 86		32/ 64	
BCM	2.5%	7.1%	4.6%	13.1%
TBF	1.5%	4.1%	3.1%	9.0%
ECF	1.3%	3.6%	3.0%	8.4%
ICF	2.5%	7.0%	4.6%	13.2%
FFM	1.6%	4.5%	3.3%	9.5%
R_E	2.0%	5.6%	4.4%	12.8%
R_I	2.9%	8.2%	5.7%	16.5%
%BF‡	1.1%	3.2%	2.4%	6.6%

*Significant difference between age-groups (p<0.0001 for each parameter)
†For one child, within-day change was very large. Numbers in parenthesis include these outlier data.
‡For %BF, the numbers are given in percent-points.

Table 17
Median values (by the non-parametric method) by Pottel et al. (16).

Age interval (years)	Median (mg/dL)	Median µmol/L*
2 to <3	0.30	26.5
3 to <4	0.33	29.2
4 to <5	0.36	31.8
5 to <6	0.38	33.6
6 to <7	0.43	3.0
7 to <8	0.45	39.8
8 to <9	0.47	41.5
9 to <10	0.50	44.2
10 to <11	0.52	46.0
11 to <12	0.54	47.7
12 to <13	0.57	50.4
13 to <14	0.61	53.9
14.5	0.62†/ 0.68‡	54.8†/ 60.1‡

*Crea in µmol/L = Crea in mg/dL × 88.4
†Girls. ‡Boys

standard probability table or by use of a spreadsheet function.

With exception of creatinine, the AUCs (Table 18) were almost identical and consequently only statistical comparison to creatinine was performed. In comparison to 1/Crea the AUC for 1/Crea-norm was significantly higher (p<0.001) as was the AUC for 1/CysC (p<0.005) and for the BCM-model (p<0.001). However, the percentage of children in between cut-off levels was far from similar and the BCM-model provided the narrowest grey zone with only 39% (n=51) of children between the cut-off levels (Table 18).

The BCM-model (and Weight-model) was derived from the same data as used in this work, whereas the Schwartz model is independent of our data. To control if our data were biased against the Schwartz model, a local constant for the Schwartz model was derived by fitting the model to the data in the study. The resulting change was small: The local constant was 35.4 instead of 36.5, and using this constant the cut-off values decreased by 3% from 118.5 to 115 mL/min/1.73m² and from 56.6 to 55 mL/min/1.73m², while the AUC was exactly the same. The values reported in the tables are for the unmodified Schwartz model.

Table 19
Regression data for probability analysis.

Variable	slope a	intercept b	SD
CysC (mg/L)	-0.997	4.362	0.178
Crea-norm	-0.957	4.715	0.150
Schwartz model GFR (mL/min/1.73m²)	0.924	0.327	0.143
BCM-model GFR (mL/min/1.73m²)	1.040	-0.186	0.101
Weight-model GFR (mL/min/1.73m²)	1.022	-0.102	0.113

Table 18
Cut-off levels for normal and reduced renal function and percentage of children in the grey zone in between cut-off levels for each method

Method	Cut-off level for normal renal function (>82 mL/min/1.73m^2)	Cut-off level for reduced renal function (≤82 mL/min/1.73m^2)	% of children between cut-off levels	AUC (area under curve)
Cystatin C (µmol/L)	0.63	1.45	88%	0.949 (0.914-0.983)
Creatinine (mg/L)	30.6	102.4	90%	0.841 (0.759-0.922)
Crea-norm	0.96	1.99	75%	0.954 (0.914-0.993)
Schwartz_old (mL/min/1.73m^2)	118.5	57.6	71%	0.949 (0.905-0.993)
Bök_eq (mL/min/1.73m^2)	121.5	60.7	89%	0.949 (0.914-0.984)
Fil_eq (mL/min/1.73m^2)	124.2	54.1	88%	0.949 (0.914-0.984)
Bou_eq (mL/min/1.73m^2)	110.0	61.1	56%	0.984 (0.966-1.000)
Zap_eq (mL/min/1.73m^2)	106.2	63.0	44%	0.982 (0.965-0.998)
Schwartz_new (mL/min/1.73m^2)	105.8	63.1	43%	0.982 (0.965-0.999)
BCM-model (mL/min/1.73m^2)	104.0	66.1	39%	0.989 (0.976-1.000)
Weight-model (mL/min/1.73m^2)	106.6	63.7	44%	0.985 (0.970-1.000)

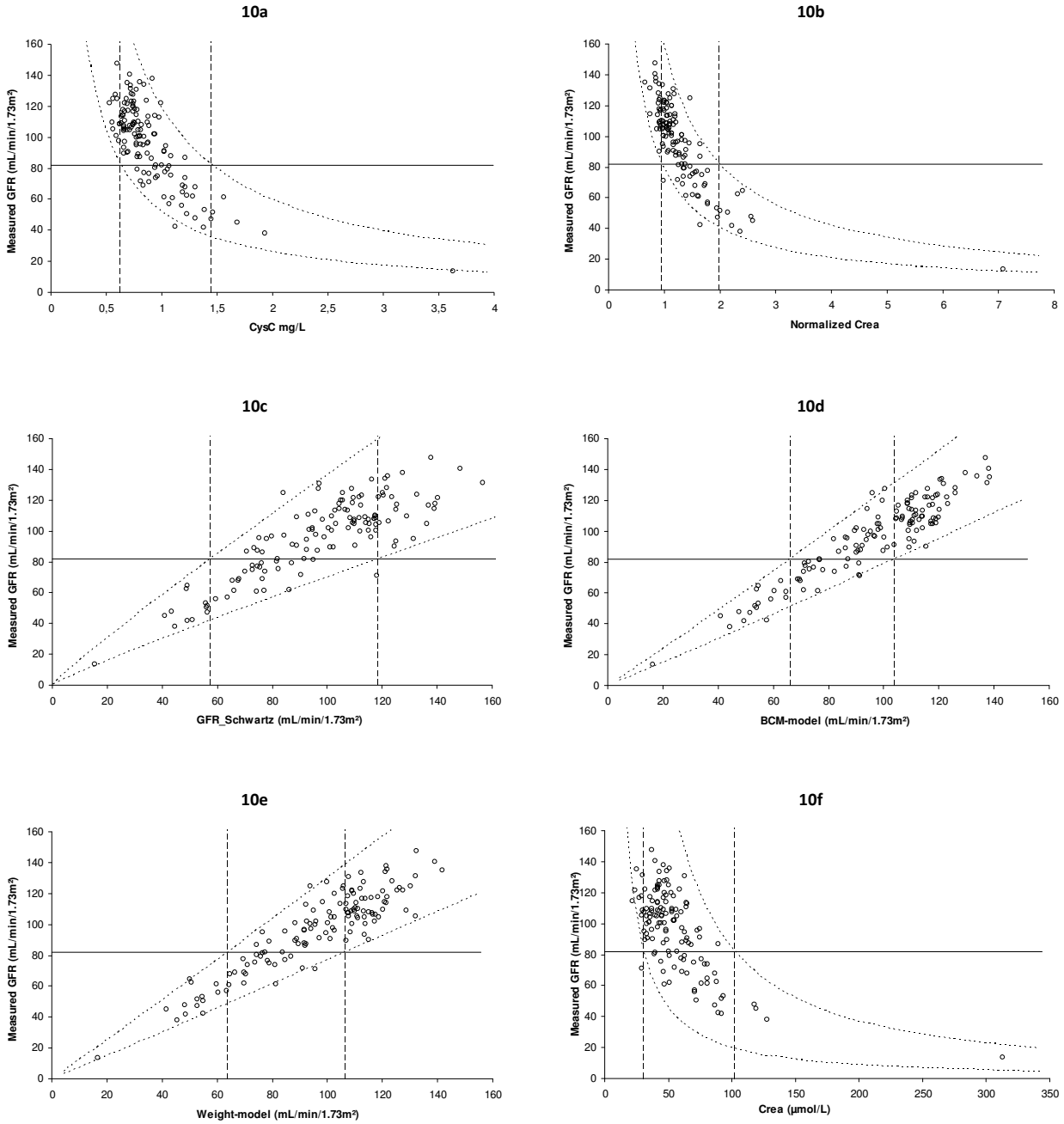


Figure 10

Cut-off levels (long dashed lines) were determined as interception of limit for reduced renal function (82 mL/min/1.73m^2) (solid line) with limits of agreement (short dashed lines). The data-points in the area between the two cut-off levels need further renal functions test to determine the level of renal function. In the case of creatinine, the limits of agreement were determined differently, resulting in only tentative cut-off levels (see text).

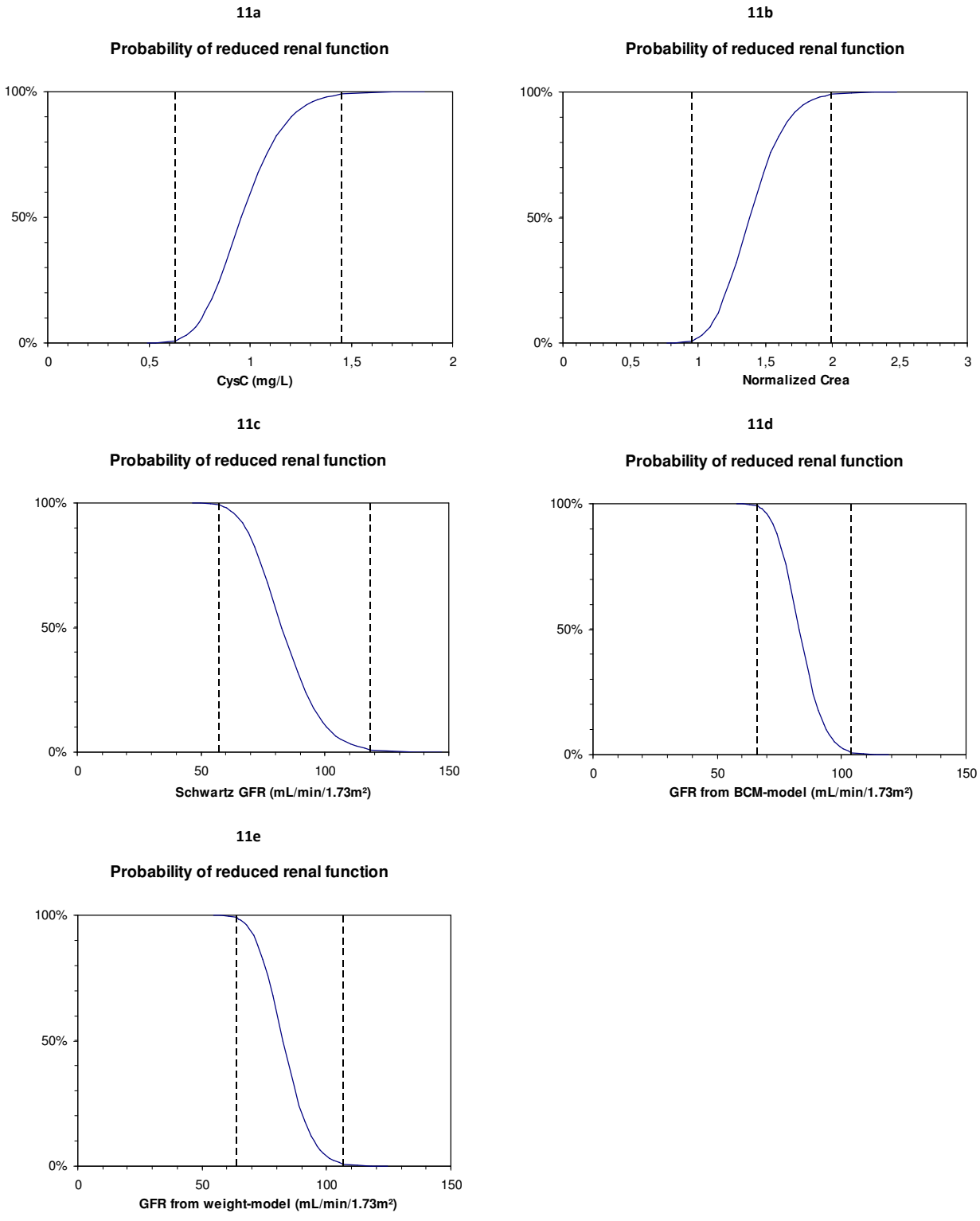


Figure 11

Probability of reduced renal function can be estimated from the measured value (CysC, Crea-norm, or eGFRSchwartz, eGFRBCM, or eGFRweight) and the corresponding probability curve. Cut-off levels determined in Figure 10 are illustrated with long dashed lines.

6. DISCUSSION

6.1 NEW PREDICTION MODEL

We aimed at developing a new prediction model based on a novel theory of the correlation between CysC and BCM, which we compared to previously published pediatric models. Inclusion of BCM in a GFR-prediction model with CysC has not previously been investigated. Our results show that it is possible to achieve a more accurate estimation of GFR by consideration of BCM when comparing to previously published models. Substituting BCM with weight in the resulting GFR-model provided results comparable to the best published models. We find that both the BCM-model and the Weight-model are reliable methods for estimating GFR in children with higher accuracy than the currently recommended Schwartz model. Furthermore, the BCM-model provided the lowest number of indeterminable results when using the model as a screening method to identify children with reduced and normal renal function or for recommendation of referral to further tests. However, the accuracy of the model is still not sufficiently high to replace exogenous methods in a clinical setting.

The main purpose of the study was to develop a new prediction model based on a novel theory correlating CysC with BCM. We then subsequently compared our model to previously published pediatric GFR models. As different assays of serum markers, calibration methods and GFR reference methods could favor our own two models, all models were compared using local constants and coefficients. Although not completely eliminating methodological differences, it is the best solution available and common practice when comparing GFR-models (6;9;10). The lack of standardized calibration of CysC assays is also a substantial problem in the development of models aimed at being generally applicable. Recently, however, the first certified calibrator material for CysC in humans has become available (58). However, until implementation of uniform calibration of assays becomes standard, CysC-based GFR-models developed in one study are not necessarily transferable to another lab. Furthermore, one should be aware that discrepancies in study populations may make direct model-comparison difficult.

All models compared in the present study show a quite high percentage of estimated GFR within 30% and 10% of measured GFR (Table 11) when compared to the work by Schwartz et al. who also tested the same models (9). Their study was conducted in a population of children with median GFR = 41.3 mL/min/1.73 m^2, and they found that their new model performed the best with 88% and 46% of estimated GFR within 30% and 10%, respectively, of measured GFR. In comparison, we found the Schwartz_new model to predict 97% and 63% of GFR within 30% and 10%, respectively of GFR_{ref}. The improvement of their model (and all other models), is not explained by evaluation-bias in the present study as the random sub-sampling cross-validation allows for performance assessment without biasing the model by including the tested data points in the prediction. However, our study has a higher mean GFR and potentially a more homogenous study population than the study of Schwartz et al. and possibly therefore yields consistently better results.

Using the forward stepwise regression procedure in variable selection is not unproblematic, though the problems are minor for the present study as noted in the Statistics section 4.1. Other regression procedures exist, but none are perfect – it will always depend on the study at hand. A simple approach is the univariate procedure, in which one variable is tested at the time and only variables significantly associated with Y (in this case GFR_{ref}) are entered into the model. However, it may be the case that variables work together, in which case an important variable is only found to be significant if certain other variable(s) are also included. For instance, BCM may not be significant alone, but may be significant in the presence of 1/CysC. One way to reduce this risk is to make theoretical considerations to find certain promising combinations of variables to be tested (e.g., BCM/CysC as one variable). Another way to tackle the problem is to use a backward procedure that eliminates the least significant variables. Since all variables are initially present, all synergetic effects are given a chance. The risk here is that the significance of a variable may be drowned by the others, leading to disadvantageous elimination.

In the current study, a combination of approaches is used to increase the reliability of the selection: Theoretical considerations lead to promising variables. These are still tested with the forward selection procedure that first has to include them, secondly keep them also in the presence of other variables. And finally, the selection was validated with a backward selection. Most importantly the cross-validation procedure confirms the validity of the model as the high level of performance achieved would not have been accomplished by an overfitted model.

Another point of criticism on the statistical methods could be the fact that we did not adjust for repeated measurements in the cross-validation procedure as the test for possible within-subject correlation was non-significant (p=0.16). It is possible that the repeated measurements will repeatedly appear in the same subgroup and bias the estimate, but even more often they will not. We therefore estimate that the small number of potentially correlated measurements will influence the validation very little with 1000 repetitions of the cross-validation procedure.

Our study confirms our theory on proportionality (linear with no constant) between GFR (mL/min) and BCM/CysC, giving no evidence of extrarenal clearance. If estimating GFR solely by BCM/CysC in the random sub-sampling procedure the outcome is inferior to the Schwartz formula (12) as only 89% and 44% will be estimated within 30% and 10% of measured GFR compared to 95% and 51% for the Schwartz formula. This is a clear indication of the validity of the Schwartz formula, especially when using a local constant, k.

Combining BCM/CysC with height×BSA/Crea, we were able to derive an equation (the BCM-model) with only two parameters which yielded better results than all other tested equations (see Table 11). From a clinical perspective the performance of the BCM-model was only marginally better than those of Zappitelli and Schwartz, and cannot replace exogenous methods. With the BCM-model, 95% of the eGFR will be within -18 to 22% of measured GFR (Figure 7), which is insufficient when accurate measurements of renal function are needed.

When deriving a GFR-prediction model based on measured variables, we may consider what level of agreement between reference and prediction it is actually possible to achieve? Since the tracer needs to be cleared from the blood before a new examination it is not possible to examine the precision of repeated measurements or variation within a day for the ^{51}Cr-EDTA method. However, the total day-to-day variation (which includes the unknown analytical precision) has been reported to be 4.8% (SD) for GFR with ^{51}Cr-EDTA plasma clearance using a standard five-sample in children with normal GFR (44). If an independent prediction method achieves the same precision, the standard deviation found in the comparison will be SDdiff = √2 × 4.8% =

6.8%, corresponding to 86% of predictions being within ±10% of reference GFR. As the present study reaches "only" 67% within ±10% of reference GFR (median value from Table 11), there is still room for improvement. It seems unlikely, though, that a GFR-model, which measures several variables (BCM, CysC, creatinine) with each its own precision, will be able to reach the same level of precision as the reference method (an unknown value below 4.8%). However, the results on precision of CysC (1.7%), creatinine (2.5%), and BCM (0.7 or 2.1% depending on age) from study II and III indicate that there is only little negative influence on the precision of the GFR-estimate, though this is difficult to quantify. The day-to-day variation of CysC and creatinine (within-subject variation between two days) also proved very low (6.4% for both analytes, Table 12), and likewise for BCM (4.6%, Table 15). This means that CysC, creatinine and BCM are very stable variables, which is an extremely important quality when estimating renal function. If there were large fluctuations around the unknown homeostatic set-point, then the variables only had little value as renal function markers.

All the positive results from study II and III on precision and biological variation of the BCM-model's measured variables are also reflected in the relatively low total day-to-day variation (7.7%) of the GFR-estimate. It is almost as low as the total day-to-day variation for the ^{51}Cr-EDTA plasma clearance (4.8%) and a lot lower than the corresponding values for 24h endogenous creatinine clearance (13.8% and 20.8%, respectively at normal and reduced renal function) (44). This level indicates the ability of the BCM-model to detect changes in renal function and clearly validates the reliability of the GFR-estimate.

Our data showed a slight but significant correlation between CysC and age in boys (Figure 4). The reason for this phenomenon is not obvious. However, it is in line with a study on US adolescents (aged 12-19 years), which reported CysC in boys, but not girls, to rise from 12 to 14 years and peak at age 14 after which CysC correlated negatively with age (132). The explanation for our findings is not found in a correlation of GFR (mL/min/1.73m^2) with age. We notice, though, that the effect was too small for age and gender to be included by the variable selection procedure.

Finally, a few notes on measuring BCM with bioimpedance spectroscopy. Applying our model to clinical practice requires access to estimation of BCM by bioimpedance spectroscopy. It may seem excessive to acquire this device for the sole purpose of GFR estimation. However, as described in section 3.6 the technique has many possible clinical applications besides estimating BCM. Alternatively, the variable BCM can be substituted by weight, which will result in less accurate GFR estimates, though still as accurate as the Schwartz$_{_new}$, but without the need for measuring BUN. It should be noted that a homogenous distribution of body fluids is assumed for the BIS measurements. If edemas are present, as can be the case in some children with severely reduced renal function, then the estimate of BCM is not valid. Furthermore, as the model is not generated in this population it can not be assumed valid in this population.

We may speculate that a more accurate estimate of BCM will increase the accuracy of the GFR estimate. All constants in the Xitron Hydra 4200 are developed with dilutions methods as reference methods in an adult population, which do not necessarily apply to children. However, as we do not have access to an ICF reference method, direct recalibration of ICF in the device is not an option.

Why does body weight provide almost as good an estimate of GFR as BCM? The simple answer is that there is a highly significant correlation between logaritmized BCM and logaritmized weight (R^2=0.94). This means that weight can be used almost interchangeably with BCM in the GFR-model with other constants and coefficients, but with less accurate results. This finding does not diminish the validity of the BCM/CysC theory as such; it just tells that for the present context BCM can be estimated reasonably well from weight.

The original purpose of developing the new GFR-model was to achieve a sufficiently accurate estimate of GFR as an alternative to exogenous markers. However, another approach to utilizing the GFR estimate is to apply it in a screening process prior to an eventual referral to exogenous GFR measurement to determine if the renal function is normal or reduced – and if indeterminate, with what probability the function is decreased. In study IV we examined this ability of all GFR-models and of CysC, creatinine and age-normalized creatinine (Table 18). Instead of using a two-sided diagnostic procedure with one cut-off level (population based reference intervals), which inevitably will result in false classifications, we derived a three-sided diagnostic procedure with the following outcomes: 1) Normal renal function; 2) Reduced renal function; 3) Indeterminable. The wider the grey zone between normal and reduced renal function, the larger the number of indeterminable estimates (Figure 10). In between cut-off levels the clinician should consider referral to GFR-measurement by plasma clearance of an exogenous marker. The closer the result in the grey zone is to the limit for normal renal function the less likely reduced renal function is and vice versa. This is very clearly illustrated in Figure 11, in which the probability for reduced renal function at any given level of a method (not creatinine) can be read at the y-axis.

If the test variable falls outside the grey zone then normal or reduced renal function can be assumed with at least 99% probability. The 98% significance level was chosen to minimize the risk of false negative and false positive results in a screening procedure. It can be assumed that the probability analysis is widely applicable as the residuals from the regression analysis were log-normally distributed and we have no reason to believe that the present sample should not be representative of the population from which it was drawn. Of course this can be validated in a larger population.

The main finding was a superior ability of the BCM-model to discriminate between normal and reduced renal function. Moreover, serum creatinine improved significantly after correction with the age-specific median values, yielding the same AUC as CysC. However, as can be concluded from the wide variation of numbers of children in the grey zone between cut-off levels, the AUCs alone do not provide sufficient information of the clinical impact of the results as will be discussed in the following.

For the BCM-model normal function (>82 mL/min/1.73m^2) can be assumed with an eGFR$_{BCM}$ >104 mL/min/1.73m² and reduced function <66 mL/min/1.73m² resulting in approximately 39% of the 131 included children in the zone in between the cut-off levels in the present population. This may seem like a large number, but not in comparison to all other methods, which all have higher percentages. The difference, though, is probably non-significant comparing to the Schwartz_new; Zap_eq, and the Weight-model (Table 18).

The ROC analysis with determination of AUCs is one way of evaluating and comparing the diagnostic accuracy of different methods. However, as can be deducted from Table 18 the percentage of children in the grey zone between cut-off levels can differ quite a lot, though the AUCs are very similar. One of the most prominent examples is the comparison of CysC to Schwartz_old. The AUC only differs 0.0006, though the

Schwartz_old classifies correctly 22 children more than CysC. This clearly illustrates that AUCs alone do not provide sufficient information of the clinical impact of the results.

We could have made several other statistical comparisons of AUC with *t*-tests, but chose not to do so as the risk of finding an erroneous significant difference (type 1 error) increases with increasing number of comparisons.

Furthermore, the ROC analysis is commonly used to identifying the cut-off levels reflecting 100% specificity and 100% sensitivity, which we also attempted at initial analysis. However, in this population it turned out that the distribution of data was greatly influenced by the number of children in the grey zone. Especially, the results for the BCM-model turned out very favourably with only 16 children between such determined cut-off levels. However, examining the data-distribution we disclosed a random lack of data-points around 82 mL/min/1.73m^2, which lead to a misleadingly high method accuracy. This clearly demonstrates that great caution is warranted if setting cut-off limits based solely on ROC analysis based on a relatively small number of subjects. These limits will be much more dependent on data-distribution than the method described in the present study.

The serum markers do not give an estimate of GFR, but are traditionally considered to aid the clinician in evaluating renal function. Creatinine is the most widely used biomarker to assess renal function in pediatric clinical practice in spite of its many limitations. Some of these limitations can be circumvented by estimating GFR by the Schwartz model, but this necessitates knowledge of height, which is usually only available to the referring clinician. As the enzymatic method is IDMS-traceable, the recently reported pediatric reference intervals by this method should be applicable to all clinical labs using enzymatic creatinine analysis and will increase the utility value of creatinine. Dividing the measured serum value with the age-specific median value results in an increasing ratio with decreasing GFR. The present study demonstrates that this ratio increases the diagnostic performance of creatinine in children considerably and yields the same level of performance as CysC and the Schwartz_old when judging the AUC. However, though the AUCs are almost identical Crea-norm classifies correctly 17 children more than CysC, but 13 less than the Schwartz model, which once more underlines the care that should be taken in interpretation of ROC analysis.

Figure 10f shows that for a pediatric population, creatinine has a very large dispersion for a given renal function. For a population similar to ours, creatinine alone tells only little about renal function. In combination with other knowledge, however, creatinine can be a valuable parameter, as demonstrated by both normalized creatinine and the GFR-estimation formulas including creatinine.

Comparing our results on CysC vs. creatinine and CysC vs. Schwartz_old to the literature we find agreement with previous ROC analyses summarized in section 1.3.1-2, which overall indicates superiority of CysC over creatinine and equality of CysC and the Schwartz_old. Furthermore, Brøchner-Mortensen et al. (130) have previously shown that age-normalized creatinine could predict the level of renal function with >95% probability in 63% of the children, which is seemingly better than the present results. However, their cut-off levels were set by a method that was dependent on the ratio between the number of children with normal and reduced renal function. Our cut-off levels were set in a population-independent way and for >99% certainty. A high certainty comes at the price of more indeterminate results (i.e., larger grey zone). Accordingly, our lower cut-off level for age-normalized creatinine was 0.96 compared to 1.18 in the work of Brøchner-Mortensen et al. If cut-off values based on their results are set by the same method as used in the present study, the results will not differentiate much.

If using the population based upper reference limit for CysC (0.95 mg/L) to discriminate between normal and reduced renal function (33), only one 11-years-old and six 13-14-years-old boys with normal renal function were falsely classified by CysC. These boys contribute to the gender-specific age-dependency for CysC reported in Study I.

One limitation to this study was the relatively low number of children with GFR ≤55 mL/min/1.73m^2 (n=10).

For this reason it was not possible to determine the cut-off levels for considerably and severely reduced renal function, which would have been desirable. Furthermore, a greater number of children in total would increase the certainty of the cut-off levels, though the significance is expected to be very small.

6.2 BIOLOGICAL VARIATION OF CYSTATIN C AND CREATININE

The main purpose of this study was to investigate the analytical and biological variation of CysC and creatinine and furthermore to indirectly investigate the validity of the new model. As both CysC and creatinine are widely used outside GFR-models for either longitudinal follow up or for screening for reduced renal function, the knowledge of within- and between-subject variation is crucial as will be discussed in the following. To our best knowledge, this is the first pediatric study to investigate both the within- and between-subject variation of CysC. Furthermore, existing studies on biological variation of CysC in children are limited (73-75) and two of the studies are on organ transplant patients, which is a different population compared to children with nephro-urologic disorders (Table 13).

The main finding was a low and identical within-subject CV for CysC (6.4%) and creatinine (6.4%) indicating that both are equally suitable biomarkers for longitudinal follow-up of renal function. In addition, our data show that neither CysC nor creatinine is suitable for screening a population of healthy children using population based reference intervals.

A number of studies have previously addressed the various coefficients of variations (CV) of CysC. The within-subject variation on both CysC and creatinine in the present study were lower than values reported in previous studies of comparable age groups (73-75). However, to compare CVs between studies, an identical mean value, from which the CV is derived, is necessary. Characteristically, the studies in Table 13 do not have a mean value similar to ours (73) or do not provide mean values (74;75), making comparison difficult. Still, the observed differences in within-subject CV may partly be explained by the population studied, since our population consisted of children with moderately reduced or normal renal function, whereas other studies, who found higher within-subject CV, included children with organ transplantations or chronic renal disease. Furthermore, the differences in study design may also contribute to these discrepancies. The present study included only two points of measurement with approximately 24 hours apart and therefore examined the day-to-day variation of CysC and creatinine rather than the true biological variation. This has the advantage that decreasing or increasing GFR and consequently fluctuations of CysC and creatinine are less likely as opposed to a design over several weeks or months. However, a potential limitation to our study is the fact that the two blood-samples were not taken at the exact same time on the two consecutive days and not under fully stan-

dardized conditions with regard to food intake and physical activity (133).

Our findings are supported by three studies in healthy adults showing CysC within-subject CV of 4.5%, 4.6% and 5.4% and creatinine within-subject CV of 6.1%, 5% and 5.8%, though the prior was not performed with the enzymatic method (76;77;79). Only the study by Keevil et al conflicts with our findings (80). They found a high within-subject CV for CysC of 13.3%, which has been suggested to be caused by a combination of using the first DAKO CysC PETIA method and postprandial blood samples (134).
The low analytical variance of CysC (1.7%) is in accordance with what some have found (76), though others have described a slightly higher analytical CV using the same nephelometric method (73;79). Creatinine analytical CV (2.5%) was not quite as low as what has been described in the literature: 0.97% (76) and 1.5% (79). Excluding two outliers from our study only reduces the CV_A to 2.2%. However, using the definition that the maximum allowable analytical variation should be less than or equal to half the average within-subject variation ($CV_A/CV_I \leq 0.50$) (129), the analytical imprecision of creatinine is acceptable ($CV_A/CV_I=0.40$) and of course similarly for CysC ($CV_A/CV_I=0.26$),

When assessing the utility of reference values between-subject CV has to be determined. It is well-known that creatinine rises with age in children as opposed to CysC, which is constant after approximately one year of age (30-33), though as mentioned in the previous section we found an inexplicable CysC age-dependency in boys, that is supported by an other study (132). For creatinine the age-dependency was reflected in the unadjusted creatinine between-subject CV of 28% decreasing to 20% after age-adjusting. However, creatinine between-subject CV was still higher than CysC between-subject CV (10%). As the within-subject CV of CysC and creatinine are identical, the lower IOI of creatinine compared to CysC can be fully explained by the higher between-subject CV. Only one study in children has investigated between-subject CV, which was considerably higher compared to our data (CysC: 20%; creatinine: 36%) (73). This may be explained by the longer study period over 18 months, and the lacking adjustment of the age-related increase in creatinine.

In general, a good screening test for impaired renal function in a healthy population is characterized by a relatively large within-subject variation (SD_I) and a small between-subject variation (SD_G) resulting in a high IOI. One study by Keevil et al has found a high CysC IOI of 1.64 (80). In this setting the distribution of values from a single, healthy individual will cover most of the reference interval, but unusual values will most likely lie outside the reference limits. In contrast we found that although creatinine IOI (0.25) is lower than CysC IOI (0.65), none of them are suitable as a screening tool in a population of healthy children. The reason for this is that the values found for each child will span only a small part of the reference interval and depending on the distance from the upper limit of the reference interval, relatively large changes in the serum level are necessary to detect disease. On the other hand, the equally low within-subject variation indicates that both creatinine and CysC are equally suitable for longitudinal follow-up of children suspected of or already diagnosed with renal disease. Furthermore, it sustains the applicability of the analytes in GFR-models and it is an indirect validation of the BCM- and Weight-model´s ability to detect changes in renal function.

Calculation of the RCV between two measurements sustains these claims. For creatinine a change in biological concentration of 9 µmol/L, equivalent to 19% of the mean value, is regarded as significantly different. Likewise, a change in CysC of 0.15 mg/L, equivalent to 18% of the mean value, will be considered significant. Of course the 18% change is only valid for children with serum values close to the observed mean.

6.3 PRECISION AND VARIATION OF BIOIMPEDANCE PARAMETERS

The main purpose of this study was to investigate the precision and biological variation of BIS parameters and indirectly verify the applicability of the BCM-estimate in the GFR-model as has been discussed in section 6.1. However, as the BIS technology has many other important clinical applications this section will discuss the main findings with comparison to the literature. As bio-impedance has many clinical applications, it is very important to know the precision of a measurement and the minimal value necessary for a statistically significant change between measurements (RCV). Clinicians need to know these results not only for the electrical resistances, which are what the device measures, but also for the physiological parameters, which are not easily deducted from R_E and R_I.

The precision of the Xitron Hydra 4200 proved very good – even in the youngest children who did not always lie completely still. However, we recommend three repeated measurements where only the median value should be chosen in order to avoid an incorrect measurement – especially in the youngest children.

Regarding the 2.4 years old child with the unusually high variation within the same day, this may be explained by difficulty in positioning the electrodes on so small hands and feet. As described in the Methods section, the 5 cm distance constraint was respected, meaning that the position was more proximal than the ideal position. Such a position, not at an anatomical landmark, can be expected to be less reproducible. Being a thin part of the body, the arm represents a large part of the total electrical resistance, and thus a small change in arm length measured will give a relatively large change in the results of the measurement.

Comparing our precision results with the literature, we find our precision to be in line with or better than others' findings. Ref. (111) found a precision of 0.3% on FFM in adults, which is comparable to our 0.4% in the ≥6 year old group. Ref. (109), also on adults, found precision of 0.5% on R_E and 0.3% on ECF, again in line with our results (Table 15). However, the latter study had considerably poorer precision for R_I (6.1%) and TBF (2.8%). A third study on adults (107) found precision on TBF and ECF to be 1.3% and 1.9%, respectively, again poorer than our results. This may be due to repositioning of electrodes between measurements, which was not done in our precision population.
Ref. (108) reported precisions for R_E of 1.8% and 2.8% for malnourished and dehydrated children, respectively, but as poor as 30.7% and 41.2% on R_I. Another study in infants found an R_E precision of 4.5 Ohm and R_I of 73.9 Ohm (110), which is in the same range as our findings in the age-group <6 years (R_E: 6 Ohm and R_I: 51 Ohm when converting to absolute values in Ohm using mean values and precision in percent).
An explanation for the generally poorer precision of R_I than R_E may be the fact that R_I depends very much on the high-frequency measurements, which are considerably more sensitive than low-frequency measurements to wrong body positioning (contact between body parts) and electrical interference (wires being close to metal or wires crossing each other). Likewise, ECF depends on R_E only, while TBF and ICF also depends on R_I. In the case of a single outlying measurement, this can be detected when performing three repeated measurements instead of just one or two.
Within-day and between-days variation of the Xitron Hydra 4200 has been addressed by Earthman et al. who found quite low

variations (99). However, as they used somewhat different approaches to deduct these variations direct comparison to the present study is not possible.

Chumlea et al. investigated the variation within- and between observers and within- and between days of resistance and reactance at a wide range of frequencies (103). They found a lower reliability of measurements at higher frequencies, which is consistent with our findings having the poorest precision and variation for R_I and consequently ICF and BCM.

The total within- and between-day variation is very low in part because of the very good precision. As a consequence, the device should easily be able to detect real changes in body fluids. This fact is also reflected in the reference change values, which are rather low for most parameters (Table 16).

Comparing Table 15 and 16, it is seen that total variations ($SD_{T(1)}$ and $SD_{T(2)}$) on median values hardly differ from the individual variations ($SD_{I(1)}$ and $SD_{I(2)}$), although the latter do not include analytical variations. This seems to reflect the minimal analytical variation. As may be noticed the reported total between-day variation is even slightly smaller than the individual variation in a few cases, which we take to reflect the statistical uncertainty due to a small number of children.

7. CONCLUSIONS

1. We have developed a new model to predict GFR in children with mainly normal or moderately reduced renal function. The model requires measurement of CysC, creatinine, height, BSA, and BCM by bioimpedance spectroscopy (or weight). By combining the known relation height/Crea and our theoretically deducted BCM/CysC in this model, we achieved a significant increase in the accuracy of the GFR estimate when compared to previously published models. Alternatively, replacement of BCM/CysC with weight/CysC may be a reasonable alternative if requirement of a BIS device is not feasible. This model will provide estimates of GFR with accuracy equal to the best previous published models, though the accuracy is insufficient to replace exogenous methods. It should be noted that the model can only be considered applicable if using the same assays as in the present studies, but even so the lacking uniform calibration of CysC assays may still pose a problem, which will hopefully be solved in the near future by the recently developed international calibrator (58).

2. Moreover, the BCM-model is the most reliable method to classify children in a screening process for normal or reduced function using the three-sided diagnostic procedure: normal, reduced, or indeterminable. This will result in the lowest number of referrals for GFR determination by exogenous methods and will provide more substantial aid to the referring clinician than a binary system as is the case for population based reference intervals.

3. In addition to providing an estimate of renal function we have developed a method to calculate the probability of reduced renal function to aid the clinician in the cases where the estimate is in the grey zone between the cut-off levels for reduced and normal renal function (66-104 mL/min)

4. The precision of the BCM-model was validated indirectly by determination of good precision and minor biological variation of CysC, creatinine, and BCM, which was reflected in a low total day-to-day variation of the GFR-estimate in 28 children.

5. Neither CysC nor creatinine is useful as a screening tool for detection of decreased renal function in children using population-based reference intervals. However, the identical and low within-subject variation indicates equality of the two analytes in longitudinal follow-up of children diagnosed with renal disease as both are suitable for detecting changes over time. As the serum level of CysC is stable in children after one year of age, one may speculate that this gives an advantage compared to creatinine when assessing changes in renal function over time. This is due to the fact that serum creatinine levels may change independently GFR due to changes in muscle mass, dietary intake of meat and tubular secretion. Consequently, CysC may still offer the best alternative of the two when monitoring renal function in children.

6. Normalizing enzymatic creatinine with age-specific median values will increase the diagnostic performance of creatinine considerably in the three-sided diagnostic procedure when screening for possible referral to GFR by exogenous methods.

7. The Xitron Hydra 4200 device provides rapid, precise estimates of body fluids in children – especially if median values are used. We have established that both the variation within- and between-days is very low.

8. PERSPECTIVES

The present study was conducted in a population of children with a prevalence of reduced renal function of 28% and only 8% (n=10) had severely reduced renal function. Future research in this area should include larger pediatric populations with a wider dispersion of GFR-levels to investigate the validity of the GFR-models outside the range of GFR used to develop the models. Validity includes accuracy, ability to discriminate between different degrees of renal function, and detection of changes. In a population of children with a higher prevalence of reduced renal function cut-off levels for more severely reduced renal function should be deducted, and consequently the probability for reduced renal function at these levels should be calculated. Furthermore, the age-dependency demonstrated for boys can be investigated further simultaneously.

The present study focused on a pediatric population. However, model development in an adult population may very well result in revised models with different constants and coefficients, which future research will hopefully tell.

Regarding the results on precision and variation of the BIS parameters it may be interesting to see if the results from this paper on children will differ from an adult population, which supposedly is more compliant.

Furthermore, the day-to-day variation of CysC has not been examined extensively and the results are not consistent. Consequently, studies on larger pediatric populations should be conducted to confirm if this variation is just as low as in adults, which is the conclusion of this study.

The CysC and creatinine blood samples were all venous and children having capillary blood samples performed were not included in the study. A small pilot study has revealed a statistically significant difference in serum level of CysC analysed from venous and capillary blood (59). This also needs confirmation in

larger populations, though capillary samples are mostly performed in children younger than the present population.

Very importantly implementation of the new, uniform calibrator for CysC assays may improve the interchangeability of models using different laboratories. Of course the nephelometric method used in the present study needs to be calibrated by the new standard. Then it remains to see if the models need revision due to calibration corrections.

The most important future aspect is implementation into clinical practice. With the method developed in the present study we are able to give the clinician both an accurate estimate of renal function (GFR) and a reliable probability of reduced renal function. At present all calculations can be performed by the formulas/ figures given in the present thesis. However, to extend the use of this method evolution of software to PC´s would be favourable.

9. LIST OF ABBREVIATIONS

BCM	Body cell mass
BIS	Bioelectrical impedance spectroscopy
BMC	Bone mineral content
BMI	Body mass index
BSA	Body surface area
BUN	Blood urea nitrogen
Crea	Serum creatinine
Crea-norm	Age-normalized creatinine
CRP	C-reactive protein
CV	Coefficient of variation
CysC	Serum cystatin C
ECF	Extra-cellular fluid
FFM	Fat free mass
GFR	Glomerular filtration rate
eGFR	Estimated GFR
GFRref	Reference GFR
ICF	Intra-cellular fluid
IDMS	Isotope Dilution Mass Spectrometry
LOA	Limits of agreement
RCV	Reference change value
RMSE	Root mean square error
SD	Standard deviation
SDA^2	Analytical variance
SDG^2	Between-subject variance
SDI^2	Within-subject variance
TBF	Total body fluid
^{51}Cr-EDTA	Chromium-51-ethylene diamine tetra-acetic acid
^{99m}Tc-DTPA	Technetium-99m-diethylene-triamine penta-acetate
^{125}I-iothalamate	Iodine-125-iothalamate

10. SUMMARY

This PhD thesis is based on four individual studies including 131 children aged 2-14 years with nephro-urologic disorders. The majority (72%) of children had a normal renal function (GFR> 82 ml/min/1.73m^2), and only 8% had a renal function <50% of the normal mean value.

The present thesis' main aims were: 1) to develop a more accurate GFR model based on a novel theory of body cell mass (BCM) and cystatin C (CysC); 2) to investigate the diagnostic performance in comparison to other models as well as serum CysC and creatinine; 3) to validate the new models precision and validity.

The model's diagnostic performance was investigated in study I as the ability to detect changes in renal function (total day-to-day variation), and in study IV as the ability to discriminate between normal and reduced function.

The model's precision and validity were indirectly evaluated in study II and III, and in study I accuracy was estimated by comparison to reference GFR.

Several prediction models based on CysC or a combination of CysC and serum creatinine have been developed for predicting GFR in children. Despite these efforts to improve GFR estimates, no alternative to exogenous methods has been found and the Schwartz´s formula based on height, creatinine and an empirically derived constant is still recommended for GFR estimation in children (Schwartz et al. 1976, revised in 2009). However, the inclusion of BCM as a possible variable in a CysC-based prediction model has not yet been explored. As CysC is produced at a constant rate from all nucleated cells we hypothesize that including BCM in a new prediction model will increase accuracy of the GFR estimate.

Study I aimed at deriving the new GFR-prediction model based on the novel theory of CysC and BCM and comparing the performance to previously published models. The BCM-model took the form GFR (mL/min) = $10.2 \times (\text{BCM}/\text{CysC})^{0.40} \times (\text{height}\times\text{body surface area}/\text{Crea})^{0.65}$. The model predicted 99% within ±30% of reference GFR, and 67% within ±10%. This was higher than any other model. The present model also had the highest R^2 and the narrowest 95% limits of agreement. If replacing BCM with weight (Weight-model) the results were almost as convincing. The total day-to-day variation of the GFR-estimate (7.7%) was low. The two new models are, however, still not sufficiently accurate to replace exogenous markers when GFR must be determined with high accuracy.

Study II aimed at determining biological variation and analytical precision of serum CysC and creatinine. The precision of CysC (1.7%), and creatinine (2.5%) was very good and the day-to-day variation of CysC and creatinine (within-subject variation between two days) also proved very low (6.4% for both analytes). Because of a relatively low ratio between within-subject variation and between-subject variation neither CysC nor creatinine seems qualified to discriminate between normal and reduced renal function, which was also confirmed in study IV. However, the relatively low total day-to-day variation of 6.6% (CysC) and 6.9% (creatinine) indicate that both are suitable for detecting changes in renal function over time.

Study III aimed at determining biological variation and analytical precision of BCM and all other parameters given by measurement by bioimpedance spectroscopy (BIS). Depending on parameter the precision was 0.3-0.8% in children ≥6 years and 0.5-2.4% in children <6 years with a statistically significant difference between the two age-groups (p<0.001). Within-day variation was 1.1-2.8% and between-day variation 2.4-5.7%. The median value of three repeated measurements is recommended in order to avoid incorrect measurements.

Study IV aimed at investigating the diagnostic performance of the BCM-model by: 1) Determining cut-off levels for a three-sided diagnostic procedure with the following outcomes: normal renal function, reduced renal function, indeterminable; 2) Calculating the diagnostic probabilities of reduced renal function for the indeterminable results. The lower the number of children in between cut-off levels, the better the diagnostic performance. The BCM-model resulted in the smallest percentage (39%) of indeterminate children in need for further investigation.

In conclusion, with the models developed in the present thesis we are able to provide the clinician with both a reasonably accurate estimate of renal function and a probability of reduced renal function. Furthermore, the positive results from study II and III on precision and biological variation indicate that CysC, creatinine and BCM are very stable variables, which is an indirect validation of the BCM-model's precision and validity. This is also reflected in the relatively low total day-to-day variation of the GFR-estimate.

11. REFERENCE LIST

1. Piepsz A, Colarinha P, Gordon I, Hahn K, Olivier P, Sixt R, et al. Guidelines for glomerular filtration rate determination in children. Eur J Nucl Med 2001 Mar; 28(3): BP31-BP36.
2. Perrone RD, Madias NE, Levey AS. Serum creatinine as an index of renal function: new insights into old concepts. Clin Chem 1992 Oct; 38(10): 1933-53.
3. Grubb A, Lofberg H. Human gamma-trace, a basic microprotein: amino acid sequence and presence in the adenohypophysis. Proc Natl Acad Sci U S A 1982 May; 79(9): 3024-7.
4. Dharnidharka VR, Kwon C, Stevens G. Serum cystatin C is superior to serum creatinine as a marker of kidney function: a meta-analysis. Am J Kidney Dis 2002 Aug; 40(2): 221-6.
5. Roos JF, Doust J, Tett SE, Kirkpatrick CM. Diagnostic accuracy of cystatin C compared to serum creatinine for the estimation of renal dysfunction in adults and children--a meta-analysis. Clin Biochem 2007 Mar; 40(5-6): 383-91.
6. Bouvet Y, Bouissou F, Coulais Y, Seronie-Vivien S, Tafani M, Decramer S, et al. GFR is better estimated by considering both serum cystatin C and creatinine levels. Pediatr Nephrol 2006 Sep; 21(9): 1299-306.
7. Bökenkamp A, Domanetzki M, Zinck R, Schumann G, Byrd D, Brodehl J. Cystatin C--a new marker of glomerular filtration rate in children independent of age and height. Pediatrics 1998 May; 101(5): 875-81.
8. Filler G, Lepage N. Should the Schwartz formula for estimation of GFR be replaced by cystatin C formula? Pediatr Nephrol 2003 Oct; 18(10): 981-5.
9. Schwartz GJ, Muñoz A, Schneider MF, Mak RH, Kaskel F, Warady BA, et al. New equations to estimate GFR in children with CKD. J Am Soc Nephrol 2009 Mar; 20(3): 629-37.
10. Zappitelli M, Parvex P, Joseph L, Paradis G, Grey V, Lau S, et al. Derivation and validation of cystatin C-based prediction equations for GFR in children. Am J Kidney Dis 2006 Aug; 48(2): 221-30.
11. Counahan R, Chantler C, Ghazali S, Kirkwood B, Rose F, Barratt TM. Estimation of glomerular filtration rate from plasma creatinine concentration in children. Arch Dis Child 1976 Nov; 51(11): 875-8.
12. Schwartz GJ, Haycock GB, Edelmann CM, Jr., Spitzer A. A simple estimate of glomerular filtration rate in children derived from body length and plasma creatinine. Pediatrics 1976 Aug; 58(2): 259-63.
13. Schwartz GJ, Brion LP, Spitzer A. The use of plasma creatinine concentration for estimating glomerular filtration rate in infants, children, and adolescents. Pediatr Clin North Am 1987 Jun; 34(3): 571-90.
14. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation,
classification, and stratification. Am J Kidney Dis 39[Suppl. 1], S1-S266. 2002.
15. Prigent A. Monitoring renal function and limitations of renal function tests. Semin Nucl Med 2008 Jan; 38(1): 32-46.
16. Pottel H, Vrydags N, Mahieu B, Vandewynckele E, Croes K, Martens F. Establishing age/sex related serum creatinine reference intervals from hospital laboratory data based on different statistical methods. Clin Chim Acta 2008 Oct; 396(1-2): 49-55.
17. Rahn KH, Heidenreich S, Bruckner D. How to assess glomerular function and damage in humans. J Hypertens 1999 Mar; 17(3): 309-17.
18. Brown SC, O'Reilly PH. Iohexol clearance for the determination of glomerular filtration rate in clinical practice: evidence for a new gold standard. J Urol 1991 Sep; 146(3): 675-9.
19. Gaspari F, Perico N, Ruggenenti P, Mosconi L, Amuchastegui CS, Guerini E, et al. Plasma clearance of nonradioactive iohexol as a measure of glomerular filtration rate. J Am Soc Nephrol 1995 Aug; 6(2): 257-63.
20. Blaufox MD, Aurell M, Bubeck B, Fommei E, Piepsz A, Russell C, et al. Report of the Radionuclides in Nephrourology Committee on renal clearance. J Nucl Med 1996 Nov; 37(11): 1883-90.
21. Brøchner-Mortensen J, Haahr J, Christoffersen J. A simple method for accurate assessment of the glomerular filtration rate in children. Scand J Clin Lab Invest 1974 Apr; 33(2): 140-3.
22. Chantler C, Barratt TM. Estimation of glomerular filtration rate from plasma clearance of 51-chromium edetic acid. Arch Dis Child 1972 Aug; 47(254): 613-7.
23. Ham HR, Piepsz A. Estimation of glomerular filtration rate in infants and in children using a single-plasma sample method. J Nucl Med 1991 Jun; 32(6): 1294-7.
24. Groth S, Aasted M. 51Cr-EDTA clearance determined by one plasma sample in children. Clin Physiol 1984 Feb; 4(1): 75-83.
25. Fjeldborg P, Brochner-Mortensen J. Determination of 51Cr-EDTA clearance in infants by a single capillary blood sample. Scand J Clin Lab Invest 1986 Jun; 46(4): 335-40.
26. Brøchner-Mortensen J. Current status on assessment and measurement of glomerular filtration rate. Clin Physiol 1985 Feb; 5(1): 1-17.
27. Henriksen JH, Brochner-Mortensen J, Malchow-Moller A, Schlichting P. Over-estimation of glomerular filtration rate by single injection [51Cr]EDTA plasma clearance determination in patients with ascites. Scand J Clin Lab Invest 1980 May; 40(3): 279-84.
28. Hood B, Attman PO, Ahlmen J, Jagenburg R. Renal hemodynamics and limitations of creatinine clearance in determining filtration rate in glomerular disease. Scand J Urol Nephrol 1971; 5(2): 154-61.
29. Shemesh O, Golbetz H, Kriss JP, Myers BD. Limitations of creatinine as a filtration marker in glomerulopathic patients. Kidney Int 1985 Nov; 28(5): 830-8.
30. Bökenkamp A, Domanetzki M, Zinck R, Schumann G, Brodehl J. Reference values for cystatin C serum concentrations in children. Pediatr Nephrol 1998 Feb; 12(2): 125-9.
31. Finney H, Newman DJ, Thakkar H, Fell JM, Price CP. Reference ranges for plasma cystatin C and creatinine measurements in premature infants, neonates, and older children. Arch Dis Child 2000 Jan; 82(1): 71-5.
32. Helin I, Axenram M, Grubb A. Serum cystatin C as a determinant of glomerular filtration rate in children. Clin Nephrol 1998 Apr; 49(4): 221-5.

33. Randers E, Krue S, Erlandsen EJ, Danielsen H, Hansen LG. Reference interval for serum cystatin C in children. Clin Chem 1999 Oct; 45(10): 1856-8.
34. Piepsz A, Tondeur M, Ham H. Revisiting normal (51)Cr-ethylenediaminetetraacetic acid clearance values in children. Eur J Nucl Med Mol Imaging 2006 Dec; 33(12): 1477-82.
35. Ceriotti F, Boyd JC, Klein G, Henny J, Queralto J, Kairisto V, et al. Reference intervals for serum creatinine concentrations: assessment of available data for global application. Clin Chem 2008 Mar; 54(3): 559-66.
36. Myers GL, Miller WG, Coresh J, Fleming J, Greenberg N, Greene T, et al. Recommendations for improving serum creatinine measurement: a report from the Laboratory Working Group of the National Kidney Disease Education Program. Clin Chem 2006 Jan; 52(1): 5-18.
37. Brion LP, Boeck MA, Gauthier B, Nussbaum MP, Schwartz GJ. Estimation of glomerular filtration rate in anorectic adolescents. Pediatr Nephrol 1989 Jan; 3(1): 16-21.
38. Hari P, Bagga A, Mahajan P, Lakshmy R. Effect of malnutrition on serum creatinine and cystatin C levels. Pediatr Nephrol 2007 Oct; 22(10): 1757-61.
39. Staples A, LeBlond R, Watkins S, Wong C, Brandt J. Validation of the revised Schwartz estimating equation in a predominantly non-CKD population. Pediatr Nephrol 2010 Nov; 25(11): 2321-6.
40. Pottel H, Mottaghy FM, Zaman Z, Martens F. On the relationship between glomerular filtration rate and serum creatinine in children. Pediatr Nephrol 2010 May; 25(5): 927-34.
41. Seikaly MG, Browne R, Bajaj G, Arant BS, Jr. Limitations to body length/serum creatinine ratio as an estimate of glomerular filtration in children. Pediatr Nephrol 1996 Dec; 10(6): 709-11.
42. Leger F, Bouissou F, Coulais Y, Tafani M, Chatelut E. Estimation of glomerular filtration rate in children. Pediatr Nephrol 2002 Nov; 17(11): 903-7.
43. Nyman U, Bjork J, Lindstrom V, Grubb A. The Lund-Malmo creatinine-based glomerular filtration rate prediction equation for adults also performs well in children. Scand J Clin Lab Invest 2008 Mar 24; 1-9.
44. Brøchner-Mortensen J, Rohbrandt K, Lauritzen RB. Precision of single injection [51Cr]EDTA plasma clearance and endogenous creatinine clearance determinations in children. Scand J Clin Lab Invest 1977 Nov; 37(7): 625-9.
45. Brøchner-Mortensen J, Christoffersen J. Single injection (51Cr)EDTA plasma clearance determination in children using capillary blood samples. Scand J Clin Lab Invest 1977 Nov; 37(7): 631-3.
46. Aufricht C, Balbisi A, Gerdov C, Muller T, Lothaller MA, Balzar E. [Formula creatinine clearance as a substitute for 24-hour creatine clearance in children with kidney transplantation]. Klin Padiatr 1995 Mar; 207(2): 59-62.
47. Grubb A, Simonsen O, Sturfelt G, Truedsson L, Thysell H. Serum concentration of cystatin C, factor D and beta 2-microglobulin as a measure of glomerular filtration rate. Acta Med Scand 1985; 218(5): 499-503.
48. CLAUSEN J. Proteins in normal cerebrospinal fluid not found in serum. Proc Soc Exp Biol Med 1961 May; 107: 170-2.
49. BUTLER EA, FLYNN FV. The occurrence of post-gamma protein in urine: a new protein abnormality. J Clin Pathol 1961 Mar; 14: 172-8.
50. Hochwald GM, THORBECKE GJ. Use of an antiserum against cerebrospinal fluid in demonstration of trace proteins in biological fluids. Proc Soc Exp Biol Med 1962 Jan; 109: 91-5.
51. Brzin J, Popovic T, Turk V, Borchart U, Machleidt W. Human cystatin, a new protein inhibitor of cysteine proteinases. Biochem Biophys Res Commun 1984 Jan 13; 118(1): 103-9.
52. Barrett AJ, Davies ME, Grubb A. The place of human gamma-trace (cystatin C) amongst the cysteine proteinase inhibitors. Biochem Biophys Res Commun 1984 Apr 30; 120(2): 631-6.
53. Abrahamson M, Islam MQ, Szpirer J, Szpirer C, Levan G. The human cystatin C gene (CST3), mutated in hereditary cystatin C amyloid angiopathy, is located on chromosome 20. Hum Genet 1989 Jun; 82(3): 223-6.
54. Saitoh E, Sabatini LM, Eddy RL, Shows TB, Azen EA, Isemura S, et al. The human cystatin C gene (CST3) is a member of the cystatin gene family which is localized on chromosome 20. Biochem Biophys Res Commun 1989 Aug 15; 162(3): 1324-31.
55. Abrahamson M, Olafsson I, Palsdottir A, Ulvsback M, Lundwall A, Jensson O, et al. Structure and expression of the human cystatin C gene. Biochem J 1990 Jun 1; 268(2): 287-94.
56. Jacobsson B, Lignelid H, Bergerheim US. Transthyretin and cystatin C are catabolized in proximal tubular epithelial cells and the proteins are not useful as markers for renal cell carcinomas. Histopathology 1995 Jun; 26(6): 559-64.
57. Bökenkamp A, Herget-Rosenthal S, Bökenkamp R. Cystatin C, kidney function and cardiovascular disease. Pediatr Nephrol 2006 Sep; 21(9): 1223-30.
58. Grubb A, Blirup-Jensen S, Lindstrom V, Schmidt C, Althaus H, Zegers I. First certified reference material for cystatin C in human serum ERM-DA471/IFCC. Clin Chem Lab Med 2010 Oct 29.
59. van Deutekom AW, Zur B, van Wijk JA, Bouman AA, Bokenkamp A. Measurement of cystatin C in capillary blood samples in pediatric patients. Clin Biochem 2010 Feb; 43(3): 335-7.
60. Simonsen O, Grubb A, Thysell H. The blood serum concentration of cystatin C (gamma-trace) as a measure of the glomerular filtration rate. Scand J Clin Lab Invest 1985 Apr; 45(2): 97-101.
61. Tenstad O, Roald AB, Grubb A, Aukland K. Renal handling of radiolabelled human cystatin C in the rat. Scand J Clin Lab Invest 1996 Aug; 56(5): 409-14.
62. Tkaczyk M, Nowicki M, Lukamowicz J. Increased cystatin C concentration in urine of nephrotic children. Pediatr Nephrol 2004 Nov; 19(11): 1278-80.
63. Herget-Rosenthal S, van Wijk JA, Brocker-Preuss M, Bokenkamp A. Increased urinary cystatin C reflects structural and functional renal tubular impairment independent of glomerular filtration rate. Clin Biochem 2007 Sep; 40(13-14): 946-51.
64. Herget-Rosenthal S, Feldkamp T, Volbracht L, Kribben A. Measurement of urinary cystatin C by particle-enhanced nephelometric immunoassay: precision, interferences, stability and reference range. Ann Clin Biochem 2004 Mar; 41(Pt 2): 111-8.
65. Uchida K, Gotoh A. Measurement of cystatin-C and creatinine in urine. Clin Chim Acta 2002 Sep; 323(1-2): 121-8.
66. Thielemans N, Lauwerys R, Bernard A. Competition between albumin and low-molecular-weight proteins for renal tubular uptake in experimental nephropathies. Nephron 1994; 66(4): 453-8.
67. Sjostrom P, Tidman M, Jones I. Determination of the production rate and non-renal clearance of cystatin C and estimation of the glomerular filtration rate from the serum concentration of cystatin C in humans. Scand J Clin Lab Invest 2005; 65(2): 111-24.

68. Harmoinen A, Ylinen E, la-Houhala M, Janas M, Kaila M, Kouri T. Reference intervals for cystatin C in pre- and full-term infants and children. Pediatr Nephrol 2000 Nov; 15(1-2): 105-8.
69. Fischbach M, Graff V, Terzic J, Bergere V, Oudet M, Hamel G. Impact of age on reference values for serum concentration of cystatin C in children. Pediatr Nephrol 2002 Feb; 17(2): 104-6.
70. Filler G, Witt I, Priem F, Ehrich JH, Jung K. Are cystatin C and beta 2-microglobulin better markers than serum creatinine for prediction of a normal glomerular filtration rate in pediatric subjects? Clin Chem 1997 Jun; 43(6 Pt 1): 1077-8.
71. Harris EK. Effects of intra- and interindividual variation on the appropriate use of normal ranges. Clin Chem 1974 Dec; 20(12): 1535-42.
72. Fraser CG. Changes in serial results. Biological Variation: From Principles to Practice.Washington DC: AACCPress; 2001. p. 67-90.
73. Sambasivan AS, Lepage N, Filler G. Cystatin C intrapatient variability in children with chronic kidney disease is less than serum creatinine. Clin Chem 2005 Nov; 51(11): 2215-6.
74. Podracka L, Feber J, Lepage N, Filler G. Intra-individual variation of cystatin C and creatinine in pediatric solid organ transplant recipients. Pediatr Transplant 2005 Feb; 9(1): 28-32.
75. Bökenkamp A, Ozden N, Dieterich C, Schumann G, Ehrich JH, Brodehl J. Cystatin C and creatinine after successful kidney transplantation in children. Clin Nephrol 1999 Dec; 52(6): 371-6.
76. Delanaye P, Cavalier E, Depas G, Chapelle JP, Krzesinski JM. New data on the intraindividual variation of cystatin C. Nephron Clin Pract 2008; 108(4): c246-c248.
77. Toffaletti JG, McDonnell EH. Variation of serum creatinine, cystatin C, and creatinine clearance tests in persons with normal renal function. Clin Chim Acta 2008 Sep; 395(1-2): 115-9.
78. Le Bricon T, Leblanc I, Benlakehal M, Gay-Bellile C, Erlich D, Boudaoud S. Evaluation of renal function in intensive care: plasma cystatin C vs. creatinine and derived glomerular filtration rate estimates. Clin Chem Lab Med 2005; 43(9): 953-7.
79. Bandaranayake N, nkrah-Tetteh T, Wijeratne S, Swaminathan R. Intra-individual variation in creatinine and cystatin C. Clin Chem Lab Med 2007; 45(9): 1237-9.
80. Keevil BG, Kilpatrick ES, Nichols SP, Maylor PW. Biological variation of cystatin C: implications for the assessment of glomerular filtration rate. Clin Chem 1998 Jul; 44(7): 1535-9.
81. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986 Feb 8; 1(8476): 307-10.
82. Zweig MH, Campbell G. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clin Chem 1993 Apr; 39(4): 561-77.
83. Christensson A, Ekberg J, Grubb A, Ekberg H, Lindstrom V, Lilja H. Serum cystatin C is a more sensitive and more accurate marker of glomerular filtration rate than enzymatic measurements of creatinine in renal transplantation. Nephron Physiol 2003; 94(2): 19-27.
84. Herget-Rosenthal S, Marggraf G, Husing J, Goring F, Pietruck F, Janssen O, et al. Early detection of acute renal failure by serum cystatin C. Kidney Int 2004 Sep; 66(3): 1115-22.
85. Le Bricon T, Thervet E, Benlakehal M, Bousquet B, Legendre C, Erlich D. Changes in plasma cystatin C after renal transplantation and acute rejection in adults. Clin Chem 1999 Dec; 45(12): 2243-9.
86. Filler G, Priem F, Lepage N, Sinha P, Vollmer I, Clark H, et al. Beta-trace protein, cystatin C, beta(2)-microglobulin, and creatinine compared for detecting impaired glomerular filtration rates in children. Clin Chem 2002 May; 48(5): 729-36.
87. Martini S, Prevot A, Mosig D, Werner D, van MG, Guignard JP. Glomerular filtration rate: measure creatinine and height rather than cystatin C! Acta Paediatr 2003 Sep; 92(9): 1052-7.
88. Pham-Huy A, Leonard M, Lepage N, Halton J, Filler G. Measuring glomerular filtration rate with cystatin C and beta-trace protein in children with spina bifida. J Urol 2003 Jun; 169(6): 2312-5.
89. Ylinen EA, la-Houhala M, Harmoinen AP, Knip M. Cystatin C as a marker for glomerular filtration rate in pediatric patients. Pediatr Nephrol 1999 Aug; 13(6): 506-9.
90. Stickle D, Cole B, Hock K, Hruska KA, Scott MG. Correlation of plasma concentrations of cystatin C and creatinine to inulin clearance in a pediatric population. Clin Chem 1998 Jun; 44(6 Pt 1): 1334-8.
91. Willems HL, Hilbrands LB, van de Calseyde JF, Monnens LA, Swinkels DW. Is serum cystatin C the marker of choice to predict glomerular filtration rate in paediatric patients? Ann Clin Biochem 2003 Jan; 40(Pt 1): 60-4.
92. Filler G, Priem F, Vollmer I, Gellermann J, Jung K. Diagnostic sensitivity of serum cystatin for impaired glomerular filtration rate. Pediatr Nephrol 1999 Aug; 13(6): 501-5.
93. Samyn M, Cheeseman P, Bevis L, Taylor R, Samaroo B, Buxton-Thomas M, et al. Cystatin C, an easy and reliable marker for assessment of renal dysfunction in children with liver disease and after liver transplantation. Liver Transpl 2005 Mar; 11(3): 344-9.
94. Piepsz A, Pintelon H, Ham HR. Estimation of normal chromium-51 ethylene diamine tetra-acetic acid clearance in children. Eur J Nucl Med 1994 Jan; 21(1): 12-6.
95. Grubb A, Nyman U, Bjork J, Lindstrom V, Rippe B, Sterner G, et al. Simple cystatin C-based prediction equations for glomerular filtration rate compared with the modification of diet in renal disease prediction equation for adults and the Schwartz and the Counahan-Barratt prediction equations for children. Clin Chem 2005 Aug; 51(8): 1420-31.
96. Moore, Olesen, McMurry, Parker, Ball, Boyden. The Body Cell Mass and Its Supporting Environment. Body composition in health and disease. 19-28. 1963. Philadelphia, WB Saunders Company.
97. Schoeller DA. Hydrometry. In: Roche AF, Heymsfield SB, Lohman TG, editors. Human Body Composition. Champaign, IL; 1996. p. 25-44.
98. Matthie JR. Bioimpedance measurements of human body composition: critical analysis and outlook. Expert Rev Med Devices 2008 Mar; 5(2): 239-61.
99. Earthman C, Traughber D, Dobratz J, Howell W. Bioimpedance spectroscopy for clinical assessment of fluid distribution and body cell mass. Nutr Clin Pract 2007 Aug; 22(4): 389-405.
100. Wanke C, Polsky B, Kotler D. Guidelines for using body composition measurement in patients with human immunodeficiency virus infection. AIDS Patient Care STDS 2002 Aug; 16(8): 375-88.
101. Edefonti A, Mastrangelo A, Paglialonga F. Assessment and monitoring of nutrition status in pediatric peritoneal dialysis patients. Perit Dial Int 2009 Feb; 29 Suppl 2: S176-S179.

102. Moreno MV, Djeddi DD, Jaffrin MY. Assessment of body composition in adolescent subjects with anorexia nervosa by bioimpedance. Med Eng Phys 2008 Jul; 30(6): 783-91.
103. Chumlea WMC, Guo SS, Bellisari A, Baumgartner RN, Siervogel RM. Reliability for Multiple Frequency Bioelectric Impedance. Am J Hum Biol 1994 Aug 24; 6: 195-202.
104. Earthman CP, Matthie JR, Reid PM, Harper IT, Ravussin E, Howell WH. A comparison of bioimpedance methods for detection of body cell mass change in HIV infection. J Appl Physiol 2000 Mar; 88(3): 944-56.
105. Oldroyd B, Truscott JG, Holland PC, Smith MA. Multifrequency bio-electrical impedance: precision in normal children. Appl Radiat Isot 1998 May; 49(5-6): 529-30.
106. Ellis KJ, Shypailo RJ, Wong WW. Measurement of body water by multifrequency bioelectrical impedance spectroscopy in a multiethnic pediatric population. Am J Clin Nutr 1999 Nov; 70(5): 847-53.
107. Hannan WJ, Cowen SJ, Plester C, Fearon KC. Proximal and distal measurements of extracellular and total body water by multi-frequency bio-impedance analysis in surgical patients. Appl Radiat Isot 1998 May; 49(5-6): 621-2.
108. Mazariegos M, Pithan C, Meyer A, Mendoza I, Furst P, Solomons NW. Bioelectrical impedance spectroscopy (BIS) in young children with acute and semi-acute hydration disorders: potentials and limitations. Appl Radiat Isot 1998 May; 49(5-6): 611-4.
109. Tengvall M, Ellegard L, Bosaeus N, Isaksson M, Johannsson G, Bosaeus I. Bioelectrical impedance spectroscopy in growth hormone-deficient adults. Physiol Meas 2010 Jan; 31(1): 59-75.
110. Sesmero MA, Mazariegos M, Pedron C, Jones J, Solomons NW. Bioimpedance electrical spectroscopy in the first six months of life: some methodologic considerations. Nutrition 2005 May; 21(5): 567-73.
111. Svantesson U, Zander M, Klingberg S, Slinde F. Body composition in male elite athletes, comparison of bioelectrical impedance spectroscopy with dual energy X-ray absorptiometry. J Negat Results Biomed 2008; 7: 1.
112. Bökenkamp A, van Wijk JA, Lentze MJ, Stoffel-Wagner B. Effect of corticosteroid therapy on serum cystatin C and beta2-microglobulin concentrations. Clin Chem 2002 Jul; 48(7): 1123-6.
113. Bökenkamp A, Laarman CA, Braam KI, van Wijk JA, Kors WA, Kool M, et al. Effect of corticosteroid therapy on low-molecular weight protein markers of kidney function. Clin Chem 2007 Dec; 53(12): 2219-21.
114. den Hollander JG, Wulkan RW, Mantel MJ, Berghout A. Is cystatin C a marker of glomerular filtration rate in thyroid dysfunction? Clin Chem 2003 Sep; 49(9): 1558-9.
115. Fricker M, Wiesli P, Brandle M, Schwegler B, Schmid C. Impact of thyroid dysfunction on serum cystatin C. Kidney Int 2003 May; 63(5): 1944-7.
116. Goede DL, Wiesli P, Brandle M, Bestmann L, Bernays RL, Zwimpfer C, et al. Effects of thyroxine replacement on serum creatinine and cystatin C in patients with primary and central hypothyroidism. Swiss Med Wkly 2009 Jun 13; 139(23-24): 339-44.
117. Kotajima N, Yanagawa Y, Aoki T, Tsunekawa K, Morimura T, Ogiwara T, et al. Influence of Thyroid Hormones and Transforming Growth Factor-beta1 on Cystatin C Concentrations. J Int Med Res 2010 Jul; 38(4): 1365-73.
118. Risch L, Herklotz R, Blumberg A, Huber AR. Effects of glucocorticoid immunosuppression on serum cystatin C concentrations in renal transplant patients. Clin Chem 2001 Nov; 47(11): 2055-9.
119. Wiesli P, Schwegler B, Spinas GA, Schmid C. Serum cystatin C is sensitive to small changes in thyroid function. Clin Chim Acta 2003 Dec; 338(1-2): 87-90.
120. DuBois D, DuBois EF. A formula to estimate the approximate surface area if height and weight be known. Arch Int Med 1916; 17: 863-71.
121. Erlandsen EJ, Randers E, Kristensen JH. Evaluation of the Dade Behring N Latex Cystatin C assay on the Dade Behring Nephelometer II System. Scand J Clin Lab Invest 1999 Feb; 59(1): 1-8.
122. Jødal L, Brøchner-Mortensen J. Reassessment of a classical single injection 51Cr-EDTA clearance method for determination of renal function in children and adults. Part I: Analytically correct relationship between total and one-pool clearance. Scand J Clin Lab Invest 2009; 69(3): 305-13.
123. Brøchner-Mortensen J, Haahr J, Christofferseen J, JO Lund. Simplified single injection methods for accurate assessment of the glomerular filtration rate in children and adults. In: Winkel K, Blaufox MD, Funck-Brentano JL, editors. Radionuclides in Nephrology.Stuttgart: Georg Thime Publishers; 1975. p. 289-93.
124. De Lorenzo A, Andreoli A, Matthie J, Withers P. Predicting body cell mass with bioimpedance by using theoretical methods: a technological review. J Appl Physiol 1997 May; 82(5): 1542-58.
125. Matthie JR. Second generation mixture theory equation for estimating intracellular water using bioimpedance spectroscopy. J Appl Physiol 2005 Aug; 99(2): 780-1.
126. Jødal L. Lecture notes on Electrical theory behind the measurement of body fluids with bioimpedance spectroscopy (BIS). Department of Nuclear Medicine, Aalborg Hospital, Aarhus University Hospital 2010 March 17 [cited 2010 Jun 1];Available from: URL: http://person.au.dk/fil/20320521/Lecture_notes_on_BIS.pdf
127. Freedman L.S., Pee D, Midthune N. The Problem of Underestimating the Residual Error Variance in Forward Stepwise Regression. Journal of the Royal Statistical Society Series D (The Statistician) 1992; 41(4): 405-12.
128. Picard R, Cook D. Cross-Validation of Regression Models. J Am Stat Assoc 1984; 79: 575-83.
129. Fraser CG, Harris EK. Generation and application of data on biological variation in clinical chemistry. Crit Rev Clin Lab Sci 1989; 27(5): 409-37.
130. Brøchner-Mortensen J, Hammerich B, Christoffersen J. Assessment of renal function from plasma urea and plasma creatinine in children. Scand J Urol Nephrol 1982; 16(3): 229-36.
131. Brøchner-Mortensen J, Ditzel J, Mogensen CE, Rodbro P. Microvascular permeability to albumin and glomerular filtration rate in diabetic and normal children. Diabetologia 1979 May; 16(5): 307-11.
132. Groesbeck D, Kottgen A, Parekh R, Selvin E, Schwartz GJ, Coresh J, et al. Age, gender, and race effects on cystatin C levels in US adolescents. Clin J Am Soc Nephrol 2008 Nov; 3(6): 1777-85.
133. Fraser CG. Biological Variation: From Principles to Practice. Washington, DC: AACCPress; 2001.
134. Filler G, Bokenkamp A, Hofmann W, Le BT, Martinez-Bru C, Grubb A. Cystatin C as a marker of GFR--history, indications, and future research. Clin Biochem 2005 Jan; 38(1): 1-8.