Estimation and measurement of glomerular filtration rate in children

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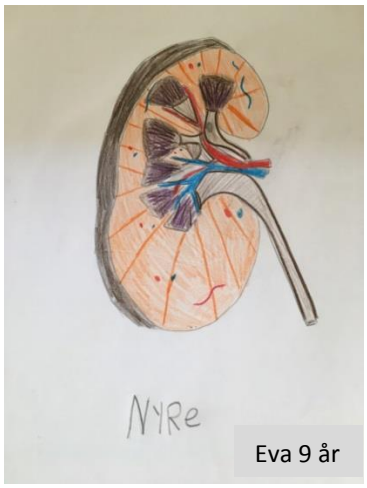


Eva 9 år

2 Abbreviations

^{51}Cr-EDTA	^{51}Cr- ethylenediaminetetraacetic acid
^{99m}Tc-DTPA	^{99m}Tc-diethylenetriaminepentaacetic acid
AGAT	arginine:glycine amidinotransferase
AKI	acute kidney injury
BSA	body surface area
CAPA	Caucasian, Asian, pediatric, and adult
CDS	creatine deficiency syndromes
CKD	chronic kidney disease
CRE	creatine
crn	creatinine
CRT	creatine transporter
CV	coefficient of variation
DBS	dried blood spot
eGFR	estimated GFR
ESRD	end stage renal disease
FAS	full age spectrum
GAA	guanidinoacetate
GAMT	guanidinoacetate methyltransferase
GFR	glomerular filtration rate
HE4	human epididymis protein 4

HPLC	high performance liquid chromatography
ID-MS	isotope dilution mass spectrometry
IQR	interquartile range
KDIGO	Kidney Disease: Improving Global Outcomes
MDRD	Modification of Diet in Renal Disease
mGFR	measured GFR
NGAL	neutrophil gelatinase-associated lipocalin
P-	plasma
S-	serum
SD	standard deviation
U-	urine

3 Publications included

Paper I: Estimating Glomerular Filtration Rate in Children: evaluation of creatinine- and cystatin C based equations

Cathrin Lytomt Salvador, Camilla Tøndel, Alexander Dominic Rowe, Anna Bjerre, Atle Brun, Damien Brackman, Lars Mørkrid. Pediatr Nephrol, 2019;34:301-311.

Paper II: Iohexol plasma clearance in children: validation of multiple formulas and single-point sampling times

Camilla Tøndel, Cathrin Lytomt Salvador, Karl Ove Hufthammer, Bjørn Bolann, Damien Brackman, Anna Bjerre, Einar Svarstad, Atle Brun. Pediatr Nephrol, 2018;33:683-696.

Paper III: Glomerular filtration rate measured by iohexol clearance: A comparison of venous samples and capillary blood spots

Cathrin Lytomt Salvador, Camilla Tøndel, Lars Mørkrid, Anna Bjerre, Atle Brun, Bjørn Bolann, Damien Brackman, Stein Bergan. Scand J Clin Lab Invest, 2015; 75:710-716

Paper IV: Renal Function Influences Diagnostic Markers in Serum and Urine: A Study of Guanidinoacetate, Creatine, Human Epididymis Protein 4, and Neutrophil Gelatinase–Associated Lipocalin in Children

Cathrin Lytomt Salvador, Camilla Tøndel, Alexander Dominic Rowe, Anna Bjerre, Atle Brun, Damien Brackman, Nils Bolstad, Lars Mørkrid. The Journal of Applied Laboratory Medicine, 2017; 2:297-308.
https://doi.org/10.1373/jalm.2016.022145

4 Abstract

Background: Glomerular filtration rate (GFR) is commonly used in daily practice for diagnosing kidney disease and for the follow-up of kidney disorders and potential nephrotoxicity, including cancer treatment and adjustment of dosage of other renal excreted drugs. There are several formulas for an approximate estimation of GFR (eGFR), based on the concentration of plasma markers or the combination of plasma and urine markers. Most common are those based on the endogenous creatinine and/or cystatin C. The plasma elimination of iohexol, a non-ionic contrast agent, is widely used for a more accurate measurement of GFR (mGFR). Venous samples are used in everyday practice, however different procedures regarding the number of samples needed and the timing after marker injection exist. Impaired kidney function could also affect the level of other non-renal diagnostic biomarkers (e.g. biomarkers used in screening for Inborn errors of metabolism).

Aims: The aims of the thesis were to 1) evaluate different equations for estimating GFR based on biomarkers in blood, 2) find the optimal time point and formula for blood sampling when using a single time point for mGFR, 3) explore the possibility of using dried capillary blood spots instead of venous sampling for mGFR, and 4) investigate the relationship between mGFR and some non-renal diagnostic markers in blood and urine and examine to which extend this could significantly affect their decision limits.

Materials and methods: We recruited 96 children (median age 9.2 years, range 0.25-17.5) with chronic kidney disease (CKD) stages 1-5. A 7-point iohexol clearance (GFR7p) was used as the reference method (median mGFR 66 mL/min/1.73m^2, total range 6-153). The performances of ten different eGFR-formulas and six different single time point mGFR formulas (GFR1p) were evaluated. In a subgroup of 32 children < 6 years old (range 0.3-6.2 years) capillary blood spot mGFR based on two-four sampling points (120-240 min post injection) was studied. Serum (S) and urinary (U) guanidinoacetate (GAA),

creatine (CRE), human epididymis protein 4 (HE4) and neutrophil gelatinase-associated lipocalin (NGAL) were analyzed for influence of age, sex and mGFR in the complete cohort.

Results: The cystatin C based $Schwartz_{cysC}$ eGFR formula had the lowest bias and both the $Schwartz_{cysC}$ and the combined $Schwartz_{CKiD}$ formula including cystatin C, creatinine, urea and body measures, had 90% of the values within ±30% of GFR7p (P30) and 44 and 48%, respectively, within ±10% of GFR7p (P10). Among the creatinine-based formulas the revised Lund-Malmø equation showed the best values with P30 of 72% and P10 of 27%.

The best scoring single-point mGFR method was found to be GFR1p with sampling 3 h after injection of iohexol and calculated with the Fleming formula that showed P10 of 92% when GFR ≥ 30 mL/min/1.73m^2, not significantly different from two-point mGFR (p=0.29). Venous GFR1p and GFR2p in the complete cohort showed P10 of 82% and 97%, respectively. Capillary blood spot GFR2p showed P10 of 59%. In the subgroup of GFR7p < 60 mL/min/1.73m^2 in children with capillary based GFR2p, the P10 was 77% and P30 was 100%.

We demonstrated decreased values for S-GAA and U-GAA/creatinine (crn) in patients with renal impairment. The level of GFR also affected S-HE4 and U-HE4/S-HE4 ratio with increased HE4 serum values in patients with lower GFR. In addition, S-NGAL increased with decreasing kidney function.

Conclusions: For evaluation of GFR based on natural biomarkers we recommend to use Schwartz' eGFR-formulas including serum cystatin C when available. Simplified mGFR based on only one blood sampling after marker injection may be a practical alternative to multipoint mGFR gold standard procedures when using Fleming formula and sampling at 3 h. If GFR < 30 mL/min/1.73m^2, a mGFR procedure with a minimum of two blood samples is recommended. mGFR based on capillary blood spots sampling is an alternative with limitations in children. Non-renal diagnostic biomarkers may be influenced by reduced renal function and

this could shift an inconspicuous value above or below the decision limits and lead to clinical misinterpretation.

5 Introduction

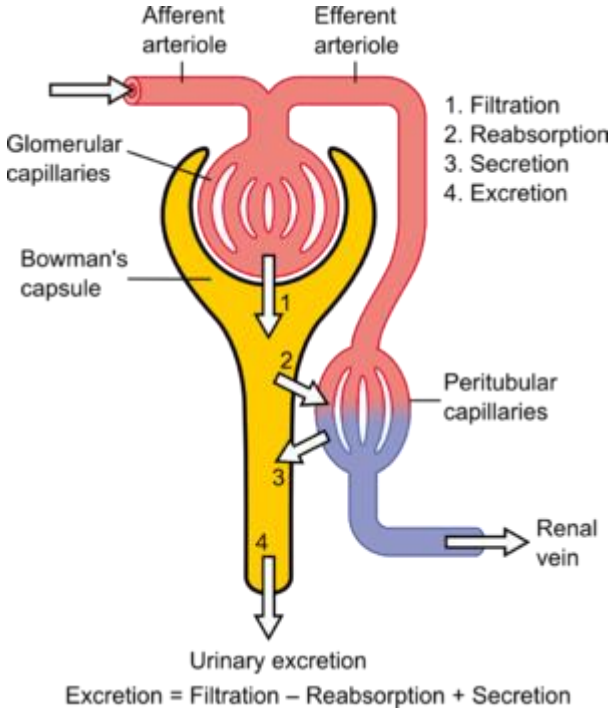
5.1 Renal embryology and anatomy

The development of nephrons begins around week 9 of gestation and ceases by week 36 of gestation, with formation of 60% of the nephrons in the third trimester (1, 2). No nephrons are formed after this time point, however, a study from 2004 showed that the kidney continuous to form postnatal in preterm children until the glomerulogenesis ceases after 40 days (3). The kidney parenchyma is divided into outer renal cortex and the inner renal medulla. Each kidney has approx. 1 million functional units called nephrons, with a range from 200.000 to >2.5 million nephrons (4, 5). These urine-producing structures span the cortex and medulla. The nephron is composed of a corpuscle with glomerulus and Bowman`s capsule, and a tubule, and the urine finally enters the ureters and bladder (6). Low birth weight (LBW), small size for gestational age (SGA) and prematurity are associated with impaired fetal nephron development, reduced glomerular surface and increased risk of hypertension and kidney disease in adult life; the Brenner hypothesis (7, 8). Vikse et al. demonstrated based on large Norwegian numbers that LBW and intrauterine growth restriction is associated with increased risk for end stage renal disease (9).

5.2 Renal function

The functions of the kidneys consist of filtration, reabsorption, secretion, excretion, regulation and production (Figure1). Fluid is filtered and the reabsorption prevents loss of important substances (e.g. proteins, smaller organic molecules and salts). Several substances, K^+, N^+, H^+, NH_3, urea, creatinine and phosphate, are secreted in the tubules, as part of the regulation process of fluid- and electrolytes and the acid/base balance. In addition, the kidneys produce hormones like renin, erythropoietin, prostaglandins and active vitamin D (6).

Figure 1



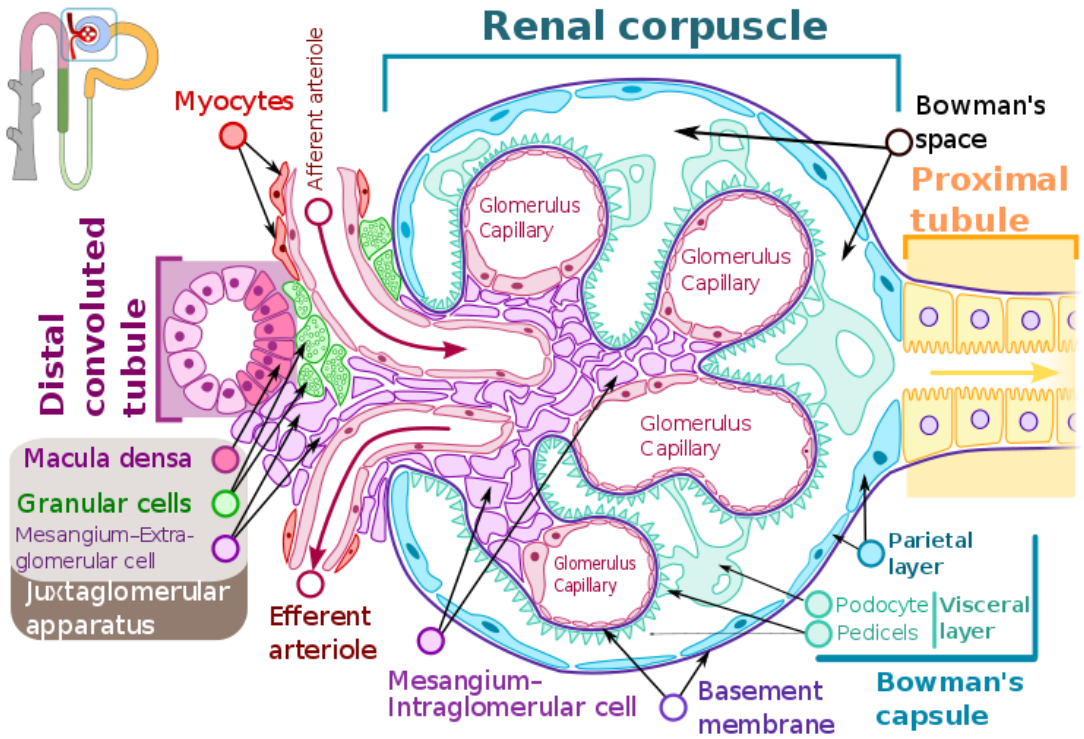
5.3 Glomerular filtration rate (GFR)

GFR is important in the evaluation of renal function and diagnosing chronic kidney disease (CKD) and acute kidney injury (AKI) in children and adults. GFR is also central for monitoring treatment efficacy, disorder progression, as well as for drug dosing and monitoring of drug toxicity (10, 11). GFR can not be directly measured, however, methods for clearance measurement of an exogenous filtration marker or formulas estimating GFR based on endogenous markers, can therefore be a substitute.

The plasma ultra filtrate passes through the glomerular filtration barrier. This consists of a capillary wall with small pores, made of endothelium where solutes, proteins and fluid can pass, a basement membrane to prevent loss of larger plasma proteins out of the blood and podocytes functioning as a final filtration barrier. The afferent arteriole delivers blood to the glomerulus and, in addition, the

efferent arteriole provides resistance to blood flow. The net filtration pressure is the blood pressure when entering the glomerulus minus the hydrostatic pressure of the fluid and the colloidal osmotic pressure from the proteins in the capillaries (Figure 2). One important function is the autoregulation to minimize GFR-loss due to changes in blood pressure (6).

Figure 2



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https://commons.wikimedia.org/wiki/File:Renal_corpuscle-en.svg

In an adult, approximately 1 L of blood flows through the approximately two million glomeruli every minute and this implies that 170-200 L is filtered if the renal function is normal. However, the adult total urine volume is approximately 0.4-2.0 L/day because of the tubular reabsorption of water and solutes. Normal urine output in children is defined as 1.5-2 mL/kg/hour in infants and children, and 1 mL/kg/hour in older children and adolescents (12). The composition of the

solutes is changed in the tubule due to component-specific secretion and reabsorption (6).

GFR is a key parameter in measurement of the renal function, and is defined as the ultra filtrate produced in the total amount of glomeruli per time unit. The GFR depends on the blood flow in the renal arteries, and hence absolute GFR is higher in adults than in children, and higher in bigger children than in smaller age-matched children (6). The renal blood flow in healthy adults is approx. 1 L/min = 600 mL plasma, and the GFR approx. 120 mL/min. The GFR is normalized to body surface area (BSA), which correlates with the weight of the kidney, of an average young healthy adult; 1.73 m^2 and expressed in mL/min/1.73m^2 (13). Both the Haycock and the Mosteller formula, in addition to the Du Bois formula, are commonly used for BSA normalization and comparison of GFR in children of different size (14-16). Piepsz et al. published reference values for normal GFR in children in 2006 and showed that GFR increases from a GFR around 50-60 mL/min/1.73m^2 at birth and stabilizes at a mean value of 104 mL/min/1.73m^2 at the age of 2 year (17). Pottel et al. described this further reporting serum creatinine values for children as well as calculation of GFR to BSA based on Belgian Growth Curves from 2009 (10, 18, 19) (Table 1). The serum creatinine values increases during age due to the increased muscle mass (20).

Table 1: GFR values in presumably healthy children, modified from references (17, 21)

Glomerular filtration rate in children		
Age	Mean GFR (mL/min/1.73m^2)	± SD (mL/min/1.73m^2)
≤1.2 month	52.0	9.0
1.2-3.6 month	61.7	14.3
3.6-7.9 month	71.7	13.9
7.9-12 month	82.6	17.3
12-18 month	91.5	17.8
18-24 month	94.5	18.1
3-4 y	111.2	18.5
5-6 y	114.1	18.6
7-8 y	111.3	18.3
9-10 y	110.0	21.6
11-12 y	116.4	18.9
13-15 y	117.2	16.1

The number of functioning nephrons is another important factor determining the magnitude of GFR. New nephrons are formed until week 36 of gestation and the GFR increases rapidly during the process of maturation in the first weeks of life. Extremely premature children have lower renal mass at birth, and may not gain normal number of nephrons. Infants reach 'adult' GFR values around 2 years of age (BSA corrected GFR values) (10). With increasing age after the age of 40 years, the number of nephrons gradually decreases as does the filtration surface (22). This results in a natural decline in GFR with increasing age; approx. 1 mL/min/1.73m^2 /year from the age of 40 years, with even more after age 65 (23, 24). GFR can also be lower during the night and renal plasma flows can be reduced during exercise (25, 26). Intake of meat can increase the glomerular filtration (27). Thus, the nephrons have the possibility to increase its own filtration rate during stress (= the so-called renal reserve), and can account for a 30%

increase in GFR e.g. during pregnancy or hyperfiltration in early stage of diabetes (28). The kidney compensate for loss in nephrons by increasing the GFR in the functioning nephrons, and loss of this "renal reserve" is a part of the pathology in kidney injury (29).

5.4 Kidney disease

5.4.1 Acute kidney injury (AKI)

The AKI definition from KDIGO Clinical Practice Guideline uses plasma creatinine levels and urine output in the classification of AKI, and the pediatric modified RIFLE criteria is commonly used (Table 2) (30, 31). The patients present with acute loss of kidney functions in electrolyte-, fluid- and metabolic homeostasis, due to prerenal (e.g. hypovolemia, sepsis, circulatory failure), renal (e.g. infections, nephrotoxins/drugs, hemolytic uremic syndrome) or post-renal causes (e.g. congenital or acquired obstruction in kidney, ureter or urethra). It is important to follow the volume status, blood pressure, electrolyte derangements and perform ultrasound of the urinary tract to exclude obstruction. In addition, blood samples (e.g. to investigate kidney- and electrolyte function, coagulation, liver function, blood culture/infection status, immunological status) and urine samples for dipstick, microscopy, electrolytes, osmolality need to be obtained (32, 33). However, the creatinine level could be normal or only slightly increased even if the GFR is severely decreased, since there has not been enough time for creatinine accumulation in plasma or due to dialysis (34, 35).

Table 2: Pediatric modified RIFLE (pRIFLE) criteria, modified from Akcan-Arikan et al. (31)

Pediatric modified RIFLE criteria		
pRIFLE	Estimated creatinine clearance (eCCl)	Urine output
Risk	Decrease by 25 %	<0.5 mL/kg/h for 8 h
Injury	Decrease by 50 %	<0.5 mL/kg/h for 16 h
Failure	Decrease by 75 % or eCCl <35 mL/min/1.73m^2	<0.3 mL/kg/h for 24 h or anuric for 12 h
Loss	Persistent failure > 4 weeks	
End-stage	Persistent failure > 3 months	

5.4.2 Chronic kidney disease (CKD)

The KDIGO definition of CKD is a condition with abnormalities of kidney structure or function, present for 3 months or more, with implications for health. Markers of *kidney damage* could be the following: albumin/creatinine ratio ≥ 3 mg/mmol, urine sediment abnormalities, electrolyte and other abnormalities due to tubular disorders, abnormalities detected by histology, structural abnormalities or history of kidney transplantation. Regarding *kidney function* decreased GFR < 60 mL/min/1.73m^2 is defined as CKD alone, in adults. The criterion for duration above 3 months does not apply for children < 3 months of age and the criterion of GFR < 60 mL/min/1.73m^2 does not apply to children less than 2 years of age. Age dependent decision limits should be used, included protein- or albumin/creatinine ratios. KDIGO guideline stages CKD in different stages based on the level of GFR as described in Table 3 (20) .

Table 3: Chronic kidney disease stage 1-5, modified from KDIGO group (20)

CKD stage	GFR (mL/min/1.73m^2)	Description
1	≥ 90	Normal/high GFR+ kidney damage
2	69-89	Mildly decreased GFR + kidney damage
3a	45-59	Mildly-moderately decreased GFR
3b	30-44	Moderately-severely decreased GFR
4	15-29	Severely decreased GFR
5	<15	Kidney failure

CKD affects approx. 10% of the population.

In 2018 in the Norwegian Renal Registry 77 children with CKD stage 5 were included, and 7 received renal replacement therapy (3 transplantations and 4 dialysis).

Patients with CKD also have an increased comorbidity of cardiovascular disorders and death (36). Estimated GFR and albuminuria are independent predictors for progression to end stage renal disease (ESRD) (37). It is important to focus on risk factors in CKD patients, as well as prophylaxis and treatment of complications. Hallan et al. demonstrated that eGFR in combination with urine albumin measurements could detect 2/3 of the patients with progressive kidney disorder (37).

5.4.3 CKD causes and investigations

The most common causes of CKD in children are congenital anomalies of the kidney and urinary tract (CAKUT) (49%), steroid-resistant nephrotic syndrome (SRNS) (10%), chronic glomerulonephritis (e.g. lupus nephritis, Alport syndrome) (8%) and renal ciliopathies (5%) (38). In addition to assessing GFR, other measures are important when investigation the cause of CKD in children: growth curves, blood pressure, urine protein/creatinine ratio and albumin-creatinine ratio, diuresis, ultrasonography and radiologic imaging (DTPA-clearance and MAG3-clearance (tubular marker), DMSA-scintigrafi), specific blood and urinary samples depending on the diagnostic suspicion (e.g. immunological analysis, oxalic acid, purines/pyrimidines, cystine in leukocytes) and also kidney biopsy.

Table 4

When should we screen for CKD in children?
congenital anomalies of urinary tract
known, hereditary disposition for CKD
neurological bladder-dysfunction
recurrent urinary tract infections
hypertension
known, complex cardiovascular disease
known inborn error of metabolism with risk for kidney disease
systemic disorders with risk for kidney disease
treatment with nephrotoxic medications (e.g. cancer treatment)
intoxications (e.g. medications, mushrooms, etc.)

5.5 GFR estimation using endogenous filtration markers

5.5.1 Overview

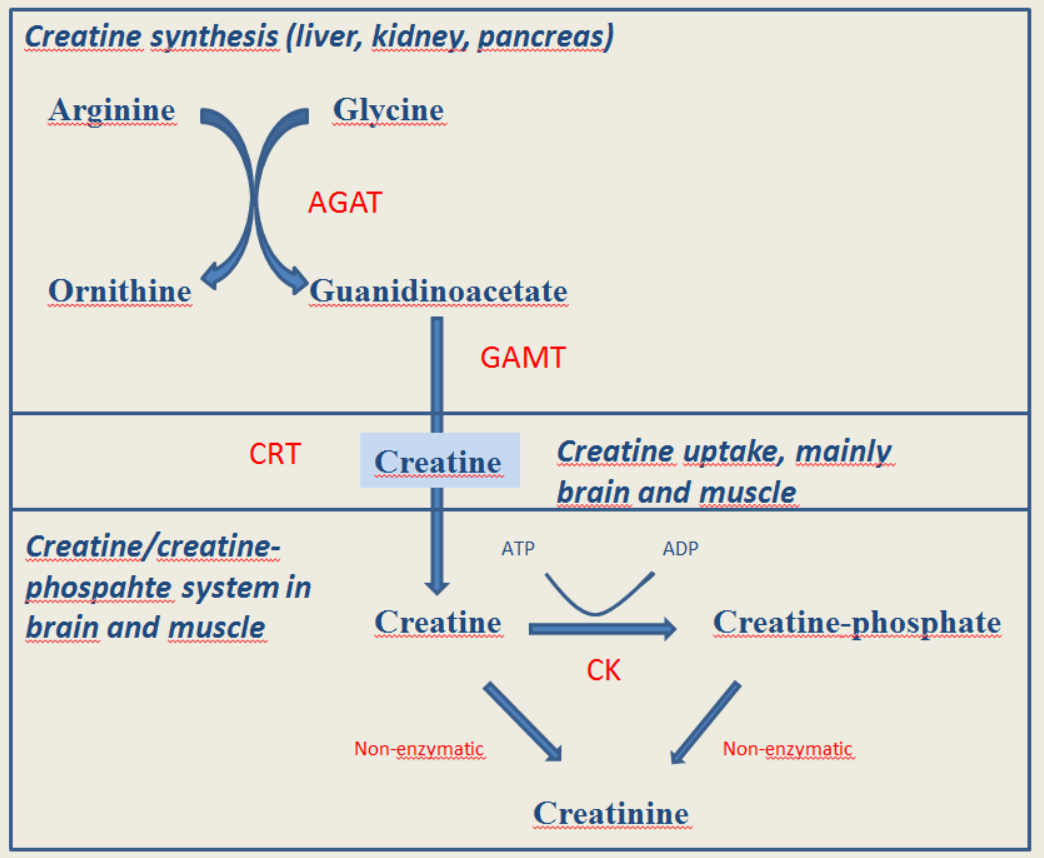
GFR can be estimated using endogenous markers. A perfect marker has a constant production rate, free passage across the glomerular wall, no protein binding, no extra-renal metabolism, no renal tubular secretion or reabsorption and accurate measurement by assays at acceptable cost (39).

5.5.2 Creatinine

5.5.2.1 Biochemistry

Creatine, an organic acid, is produced in the liver, kidney and pancreas from two enzymatic steps (Figure 3) (40). Guanidinoacetate is produced in the first enzymatic step (AGAT enzyme). Creatine, from the second reaction (GAMT enzyme), is transported in blood to muscle and brain, and taken up by the tissue by a creatine transporter. Creatine is then phosphorylated to phosphocreatine (high-energy compound) by creatine kinase. Phosphocreatine and creatine can be non-enzymatically converted to creatinine, and excreted in the urine.

Figure 3: Creatine synthesis, modified from Dunbar et al. (40)



AGAT= arginine:glycine amidinotransferase, GAMT= guanidinoacetate methyltransferase, CRT= creatine transporter, CK=creatine kinase

Plasma creatinine is the most commonly used indicator of kidney function. Creatinine is increased at birth, reflecting the renal function of the mother due to the placental equilibration, and declines during the first weeks, in opposite to the increase in GFR after birth and up to the age of 2 years where adult GFR values correlated to body surface are reached (29). Creatinine is not only influenced by the GFR, but also the muscle mass and ingestion of protein (cooked meat and fish) or creatine supplements (41-44). Creatinine is freely filtered across the glomerular membrane. In addition, creatinine is excreted by tubular secretion (approx. 10%), with increase in secretion when GFR is falling (45, 46). Trimethoprim is a well-known medication that could inhibit tubular creatinine secretion leading to rapid and reversible increase in creatinine, at least in doses above 160 mg (47). This

would lead to falsely underestimation of GFR. Cimetidine, fenofibrate, cisplatin also affects the secretion in the same way. The kidney function could be abnormal even if the creatinine values are in the normal reference range, especially in patients with low muscle mass as creatinine comes from the muscles. This is especially relevant in severely diseased children receiving nephrotoxic chemotherapeutics due to malignant disease.

The GFR can be reduced by 1/3 before the creatinine raises above the reference limits, partly due to the tubular creatinine secretion, a phenomenon called the creatinine-blind range (45). Creatinine is distributed in the total body fluid, and it could take days before a new steady-state is determined, especially at low GFR (48).

5.5.2.2 Analytical method

Previously the Jaffé method from 1886 was widely used, but today the enzymatic method traceable to Isotope Dilution Mass Spectrometry Reference Method (IDMS) is seen as the gold standard (49). The Jaffé method takes advantage of the color change after adding alkaline picrate when creatinine is present (50). The problem is the presence of non-creatinine chromogen, especially in children with low creatinine, where the percentage of error is higher (51). Neonates often have high serum bilirubin because of the hemolysis of fetal erythrocytes and less developed hepatic conjugating system. Creatinine levels can be underestimated in these infants since bilirubin absorbs light in the same spectrum as the chromogen in the Jaffé reaction (52). Biliverdin, oxidized from bilirubin, causes decrease in absorbance at the wavelength of 520 nm used to measure creatinine. The Jaffé method has low costs, and in some countries the method is still used, even if the enzymatic method has less interference and is the preferred method nowadays (51, 53). Neuman et al. investigated the difference of compensated Jaffé method and the enzymatic assay in children, and demonstrated a large intra-patient, age-related difference in serum creatinine values, especially in age 1-5 years (54). The

enzymatic method provided lower creatinine values than the compensated Jaffé method, as described in several papers (55). The consequence will then be different eGFR values and this could lead to inappropriate dosing of renally excreted drugs, e.g. vancomycin in the children (54).

5.5.2.3 Practical use

In Norway we report creatinine in µmol/L (SI unit). Some countries, included USA, still use mg/dL, with the conversion SI x 0.0113 = mg/dL. The reference ranges are age-dependent in pediatrics mainly due to different muscle mass (56). From puberty, around age 14, the male adolescents have higher creatinine than the females, hence creatinine values could be problematic to interpret in patients with very late or very early puberty (56). In children with low creatinine concentration, e.g. children with anorexia nervosa, neuromuscular disease, malignancy, advanced liver disease, amputations or paresis, the reduced GFR could be missed due to falsely normal GFR based on creatinine (57-59).

In daily practice, with known CKD cause, the creatinine measurement is used to control the renal function. Estimated GFR (eGFR) is in general recommended to be reported in addition to the creatinine value (20).

Errors for interpretations:

- Children with low muscle mass, e.g. due to chronic disease, amputations, paresis or failure to thrive, have less production of creatinine and this could give an overestimation of GFR, and the opposite in individuals with increased muscle mass, e.g. athletes (58).
- Children with rapid changes in kidney function, e.g. AKI (60).
- Children receiving medications affecting the secretion of creatinine e.g. trimethoprim, cimetidine (47, 61).
- Eating meat before taking blood sample could influence the creatinine value (43, 44).

- Small changes in creatinine could represent substantial changes in GFR in normal kidney function.
- In uremic patients as much as 16-66 % of the creatinine is excreted via the intestines and could lead to GFR overestimation (62).

5.5.3 Cystatin C

5.5.3.1 Biochemistry

Cystatin C, a protein with molecular weight of 13.3kDa, is one of the proteinase inhibitors in the body (63, 64). It is produced by almost all nuclear cells and is in all body fluids, but not intracellular (65). Therefore, the sensitivity is higher for cystatin C compared to creatinine with total body water as the volume of distribution. Cystatin C is not bound to proteins, and the advantage is the small size and positive charge when passing the glomerular membrane. It is reabsorbed in the proximal tubule by receptor-mediated endocytosis and then catabolized (66). The cystatin C concentration is low in the urine when the tubule is functioning normal, and the marker should only be measured in blood (67). The cystatin C concentration decrease during the first year of life due to maturation of nephrons, and there is little or no placental exchange of cystatin C (68, 69). Cord blood can therefore be used for assessing kidney function in the newborn. Cystatin C is encoded by a houses-keeping gene (65). However, glucocorticoids induce the promotor of the cystatin C gene and increase the cystatin C value in a dose-dependent way (70). The extrarenal clearance of cystatin C in adults is constant (mean ~ 22 mL/min/1.73m^2) and the maximum serum cystatin C value is around 7 mg/L (71). Cystatin C has been used as a GFR marker for more than three decades (69, 72).The endogenous protein cystatin C is an alternative marker for GFR with the advantage that it is less dependent on muscular mass and not affected by the use of medications blocking the tubular creatinine transporter, i.e. Trimethoprim (39). Cystatin C is also a risk marker for cardiovascular disease and death

independently of kidney function, and different theories involving atherogenesis, inflammation and cardiovascular risk factors exist (73, 74).

5.5.3.2 Analytical method

No standardized calibrator was available until 2010, when IFCC-certified ERM-DA471 calibrator was made (75). This is now used by all manufacturers. There are several different methods for measuring cystatin C, and the most common is particle enhanced turbidimetric (PETIA) or nephelometric (PENIA) immunoassays (76, 77). There are still problems regarding uncertainty even after the introduction of IFCC-certified ERM-DA471 calibrator, and just a few systems met the performance criterion in a study by Bargnoux et al. Unacceptable high biases were observed in some reagents and systems (78).

5.5.3.3 Practical use

The cystatin C level is relatively constant from around 1 year of age and until the 40-50s where the age-dependent GFR decrease starts (68, 79). Cystatin C should be analyzed with standardized methods and eGFR based on plasma cystatin C should in addition be reported, not just the absolute value (20). The cost of cystatin C analysis is somewhat higher than creatinine analysis; in Norway per 2019 35 versus 14 NOK, making not all laboratories having the opportunity to analyze cystatin C. Cystatin C is often used for estimating GFR in patients with low muscle mass e.g. children, elderly, patients with anorexia, amputations or paresis, or in patients with high muscle mass, e.g. bodybuilders.

The plasma level of cystatin C could be influenced by the level of:

- Corticosteroids: dose dependent increase (70, 80)
- Thyroid hormone: elevation in hyperthyroidism and decrease in hypothyroidism (81, 82)
- Inflammation: it is not a acute phase reactant (83), but serum values are associated with C-reactive protein , maybe due to micro-inflammation (84, 85)

5.5.4 Urea

5.5.4.1 Physiology

Urea originates from the breakdown of proteins and amino acids in the urea cycle in the liver, to detoxify/neutralize ammonia. It is a small molecule with molecular weight of 60Da, but still the major nitrogen-containing product of protein catabolism. Most of the urea (90%) is excreted in the urine. Urea is filtered freely in the glomeruli, and is partially reabsorbed by the tubules. The reabsorption is correlated to the reabsorption of water. Urea clearance is directly related to urine flow and varies three times between anti-diuresis and urine dilution, so in anti-diuresis state the urea absorption increases (6, 39, 86). Plasma urea increases when GFR is reduced, however the value can be in the normal reference range even if GFR has decreased, if the diuresis is high and protein intake is low (87). Plasma urea is also dependent on the protein load in the body (increases in high-protein diet), catabolism (increase), steroid treatment (increase) and infections and gastrointestinal bleedings (increase) (87-90). The urea pool is rapidly metabolized; <1 day.

5.5.4.2 Analytical method

The direct colorization method, Fearon method, is based on the concept that urea gives a yellow color after addition of diacetyl monoxime, and then turns orange after oxidation (91, 92). The other method is based on an enzymatic reaction where urease breaks down urea to ammonium, which then is measured (93).

5.5.4.3 Practical use

The SI unit of urea is mmol/L. Some laboratories report urea in mg/dL (conversion SI x 6.006= mg/dL) and other report blood urea nitrogen (BUN) in mg/dL (conversion SI x 2.8=mg/dL). Urea is not age-related in the same way as creatinine, but reflects fluid and protein intake as well as renal function (94).

Urea is often used as a marker of the uremic state/acute renal failure, and is an inferior indicator of GFR because of all the non-renal factors mentioned above. Volume depletion increases renal tubular uptake, causing increased serum urea, but in children volume depletion is most often caused by less intake or gastrointestinal losses (95).

Thus, plasma and urine urea are not recommended as a sole marker of GFR. However, urea is a part of some eGFR equations, i.e. CKiD equation (96), and the idea behind this could be the underestimation of GFR from urea-clearance and the overestimation of GFR by creatinine-clearance in the lower GFR range. The intake of meat can also affect the urea level in children with AKI and CKD, leading to normal urea values if not eating meat.

5.6 Equations for estimating GFR based on endogenous markers

The GFR can be estimated (eGFR) using calculation based on single blood sample on natural markers. The advantage of eGFR is the suitability in the daily routine and in need of repeated GFR-evaluations, and its advantage of beeing non-invasive and of low cost is important. Several different formulas for both adults and children have been published, both based on creatinine and/or cystatin C, and also in combination with weight, height, urea, gender, age, ethnicity (39, 76, 96-104). Important parameters when comparing different eGFR equations are the method of reference GFR i.e. measured GFR (mGFR), marker, assay used for marker measurement, mean GFR, age, origin, gender, disease (39).

To have comparable levels, GFR is normalized to regular adult body size; 1.73m^2. eGFR values for adults can be reported in the laboratory information management system as in commonly used equations. Since the issue of KDIGO Clinical Practice Guidelines for Chronic Kidney Disease in 2002 the eGFR is commonly used for identification, classification and treatment for patients with CKD (20). Reporting eGFR based on creatinine in adults has been performed for decades. During the last years several different eGFR formulas have been published based on various parameters. The MDRD formula (Modification of Diet in Renal Disease) was the preferred creatinine-based formula from the mid2000, but the formula was developed from a population of adult CKD patients with low GFR and just a few patients with diabetes, and thus the formula could be misleading and underestimate GFR in patients with normal or slightly decreased GFR (100, 105, 106). Nowadays the recommended international equation for adults is the CKD-EPI creatinine formula from 2009, based on the international creatinine reference analytic method (IDMS) from 2006 (104).

For children, several eGFR equations have been generated based on creatinine. Schwartz et al. published their first formula in children in 1976 based on creatinine and height (107). The formula was modified in 2009 due to the transition to IDMS enzymatic creatinine measurements (99). Tøndel et al. found a huge

overestimation of GFR with the use of the orginial Schwartz formula in children having Fabry disease with a mean bias of 50.6 mL/min/1.73m^2 and P30 of only 27% and P10 of 4%. In contrast P30 and P10 was 87% and 49%, respectively, with the use of the new, modified Schwartz formula in the same cohort (108). This new modified creatinine based Schwartz bedside eGFR-formula, $Schwartz_{bedside}$, was generated from 349 children aged 1-16 years with CKD and mGFR by multipoint iohexol clearance between 16-93 mL/min/1.73m^2 (99). This equation is commonly used in everyday practice at the hospitals; eGFR= 36.5(height/creatinine) [creatinine in µmol/L, height in cm].

The complex CKiD equation ($Schwartz_{CKiD}$) is based on creatinine, cystatin C, urea, height, and gender and generated from the same population as the $Schwartz_{bedside}$ and it is also recommended by KDIGO (96). Several eGFR equations for children use height as a factor in the formulas because height is closely linked to muscle mass in children. These formulas are therefore not reported directly in the laboratory information systems in daily practice. Height-independent formulas also exist, e.g. FAS equations and cystatin C based equations (76, 96-98, 109). Several equations based solely on cystatin C exists, and formulas should be based on standardized cystatin C methods and calibrated to the reference material (75).

Several clinical conditions may give misleading eGFR values with the commonly used creatinine-based equations, and alternative methods need to be considered (58). Creatinine-based formulas may overestimate due to low creatinine in patients with low muscle mass (110). Combined creatinine-and cystatin C formulas could be used instead in these patients (58). On the opposite, eGFR equations do not work well in obese patients either due to the indexation to BSA leading to underestimation of GFR (111). Fetal renal blood flow is low and the kidney function change every day, especially the first 6 weeks after birth during the maturation, thus the GFR is not in steady state. High-protein diet can lead to glomerular hyperfiltration, in contrast to low-protein intake that will decrease the serum creatinine concentration (112). Some studies also show that chronic

changes in homeostasis can occur after changes in diet, for example fasting (decrease GFR) and high-fat diet (113, 114). GFR biomarkers are dependent on gender, and boys have more body mass then girls in the same age (115). Kidney function relates to body mass, and some studies argue that total body water should be used instead of BSA (116). Chemotherapy could decrease renal function and falsely normal eGFR based on low muscle mass could mask the kidney damage and lead to wrongly dosed further treatment (117). GFR should therefore instead be measured by an external marker based mGFR-method in for instance children with cancer (58).

Bias, precision and accuracy are investigated when comparing the performance of the different eGFR equations. Bias is often defined as the mean or median of the difference between eGFR and reference mGFR, precision as the absolute % prediction error and accuracy as the proportion of eGFR results within X% of mGFR, often 10, 20 or 30%. Guidelines state that P30 accuracy should be at least 90%, and several pediatric eGFR equations have P30 around 90% and P10 45% (118). With a mGFR of 100 mL/min/1.73m^2 and a P30 of 90%, there is then 90% probability of eGFR being between 70 and 130 mL/min/1.73m^2. If accurate evaluation of kidney function is needed, GFR should be measured.

5.7 GFR measurement using exogenous filtration markers

5.7.1 Overview

Homer Smith established the creatinine-clearance method in 1951 (119), but this method is not commonly used today due to the substantial variations in the measurements and insufficient accuracy (120, 121). The gold standard method for measured GFR (mGFR) is inulin renal clearance where inulin, a polyfructosan with a molecular weight of 5000Da, is given by continuous intravenous infusion and repetitive and careful timing of blood samples and urine samples from bladder

catheter are collected (119) . This method is however time-consuming and could be problematic especially in children and in patients with vesicoureteral reflux. Also, inulin for i.v. use is hardly available anymore. More common is the use of plasma clearance to measure GFR, i.e. with the administration of ^{125}I-iothalamate, ^{51}CrEDTA, ^{99m}Tc-DTPA, iohexol and iothalamate. ^{51}CrEDTA, ^{99m}Tc-DTPA and ^{125}I -iothalamate are radioactive agents (Table 5). ^{51}CrEDTA and ^{99m}Tc-DTPA are marked with gamma radioisotopes, and the concentration can be determined by a gamma counter. To avoid radioactive substances the non-ionic X-ray contrast agent iohexol, filtered in the glomeruli, can be used instead, see paragraph 5.7.3.

The ideal filtration marker is a substance that is freely filtered and not absorbed or secreted by the tubules. Moreover it should not be toxic, not bound to plasma proteins, not metabolized by the tubules, or excreted in other way than the kidneys, easy to measure in blood and urine, easily available and not too expensive (39).

Table 5: Exogenous markers for measuring glomerular filtration rate, modified from Pottel et al. (10)

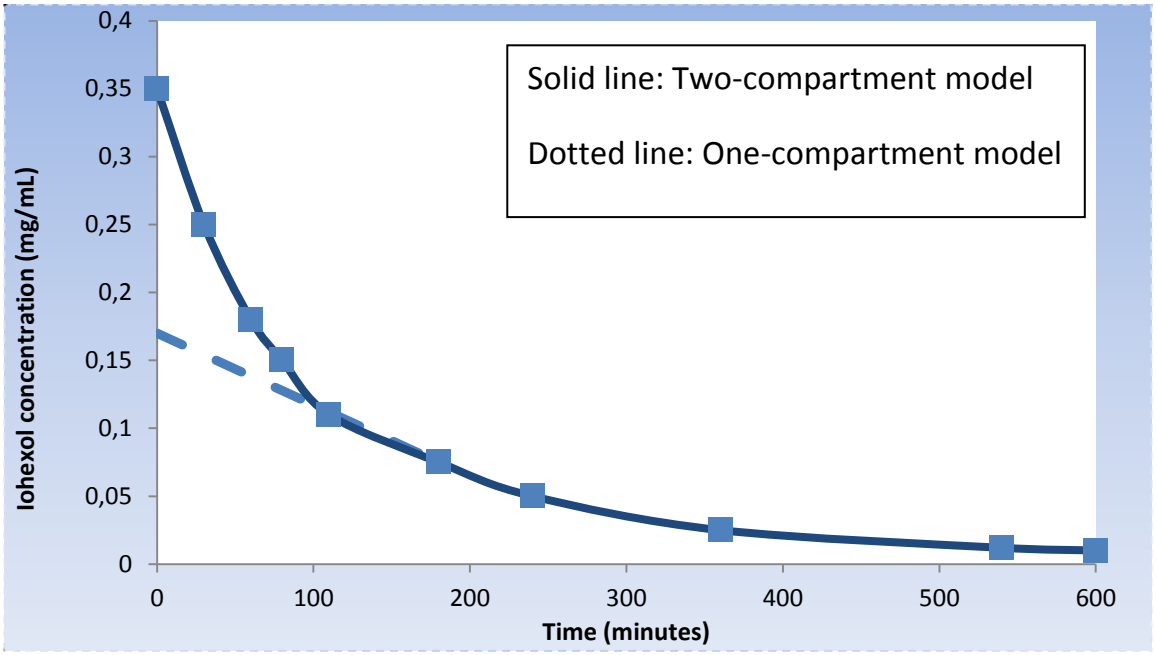
Marker	Strenghts	Limitations
Inulin	Gold standard No side effects	Expensive Not easily available No standardized method for measurement in plasma/urine Difficult urine sampling in patients with vesicoureteral reflux
Iothalamate	Inexpensive Long half-life	Probably some tubular secretion Radioactive substance if ^{125}I is used as tracer Not for use in patients with iodine allergy
Iohexol	Not radioactive Inexpensive Low dose possible Standardized Easy available	Not for use in patients with iodine allergy
^{51}CrEDTA	Inexpensive Accurate measurement Easy available in Europe Long half-life/low dose	Probable tubular reabsorption Must be measured in nuclear medicine departments Not approved by FDA Radioactive material
^{99m}Tc-DTPA	Widely available in USA Accurate measurement Low radiation dose Inexpensive	Radioactive material Must be measured in nuclear medicine departments No standardization Dissociation and protein binding

5.7.2 Renal and plasma clearance

GFR could be measured by either renal clearance or plasma clearance, and the former is the most direct way. GFR (mL/min)= [U] x V/ [P], where U (mg/mL) is the urine concentration of the filtration marker, V(mL/min) is the volume of the timed urine sample and P (mg/mL) is the average plasma concentration in the same time period (21). Several 20-30 minutes urine collections are needed for this procedure, but the procedure is of short duration. It may be difficult to collect urine samples in patients with urine incontinence or retention. The exogenous marker is given either intravenously or subcutaneously (bolus or continuous infusion).

The other alternative, plasma clearance, is easier in everyday clinical practice. The exogenous marker is injected intravenously and the GFR is then calculated from plasma clearance by dividing the injected dose by the area under the plasma-concentration time-curve (AUC). Plasma levels of the marker will decrease, due to the disappearance into the volume of distribution where the tracer leaves the intravascular space (fast component) and due to renal excretion/elimination (slow component) (Figure 4). This decrease in marker concentration can be estimated using either a two-compartment model or a one-compartment model (10).

Figure 4: Iohexol concentration versus time, modified from Pottel et al. (10)



The two-compartment model is the best method for estimating the decline in marker level (10). There is a need for several blood samplings during the first 60 minutes (minimum 2 time-points) as well as from 120 min and forward (2-6 time-points). Filtration markers may need 1-2 hours to mix with the extracellular volume and the fast component is therefore a combination of mixing and clearance and the slow component is the clearance only (Figure 4). On the other hand, the one-compartment model requires only blood sampling from the slow component, and therefore the number of samples needed for estimation is lower; 2-6 samples (122). Both non-linear least square fitting and log transformation to obtain a straight line, expressed by slope and intercept, can be used. The two- and one compartment model will not give exactly the same GFR value because the one-compartment model will underestimate the AUC and thus give an overestimation of the GFR. To overcome this problem, correction factors were generated (123-125), but the most widely used is still the Brøchner-Mortensen formula. In the original Brøchner-Mortensen formula from 1972 for adults, normalization to BSA was done after correction for the distribution phase/calculation of GFR, but in the

modified version for children from 1974 the BSA normalization was done before distribution phase correction , as recommended by guidelines (126).

A simplification from several blood samples to one-sample method is also an option and the Jacobsson formula from 1983 is the most commonly used, based on ^{99m}Tc-DTPA clearance in adults (127). Groth and Aasted published the first 1-point formula in children in 1984, with ^{51}CrEDTA clearance (128). Stake et al. published in 1991 a modification of the Jacobsson formula with sampling point 3 h or 4 h using iohexol clearance compared to ^{99m}Tc-DTPA clearance (129). In 2005 a new 1-sample formula was described by Fleming et al. (130).

However, guidelines from the British Nuclear Medicine Society recommend a one-pool slope-intercept technique with at least two samples (126). There is no consensus regarding optimal sampling time points in children. Some laboratories use 3 h as the optimal time point. By the use of multi-point procedure an outlier can be removed based on elimination curve.

5.7.3 Iohexol clearance

Iohexol (Omnipaque^{TM}) is an iodinated non-radioactive X-ray contrast agent that is filtered in the glomeruli, and probably not reabsorbed or secreted by the tubule. The drug was developed by a Swedish radiologist and introduced in the 1980's and is the most widely used mGFR marker in Europe and many other countries (131-134). There is excellent correlation between iohexol clearance and the gold standard inulin clearance (135-137), in addition to ^{51}CrEDTA clearance (138) . Gaspari et al. published in 2018 a report regarding the safety of iohexol (dose 5 mL) from 1992-2016 and the overall rate of iohexol-related events was extremely low; 0.0066% (139). 5 mL is a small dose compared to the dosage used in X-ray contrast methods. The review article by Carrara and Gaspari also summarize other several important advantages of the use of iohexol clearance, including the well documented reliability, accuracy, easy quantification methods, easy handling and

storage, good stability, low cost (approx.10 euros) and the established international quality proficiency programs (140).

5.7.4 Practical use

Measuring GFR is important in situations where there is need for an accurate GFR result e.g. dosage of toxic medications/nephrotoxic oncological medications, assessment of kidney donor function, diagnosing a resent onset of a kidney disorder, when eGFR based on creatinine and/or cystatin C is not suitable/have discrepancy without known explanation and in children < 1 year (11). The GFR will be overestimated if the patient has edema or ascites due to the expanded extracellular volume which will be misinterpreted as renal clearance or if the blood samples as obtained too early in patients with very low GFR < 15-30 mL/min/1.73m^2 (141).

5.8 Other renal biomarkers

5.8.1 Overview

Beta-trace protein, beta-2-microglobulin, NGAL, L-FABP (liver-fatty acid binding protein), KIM-1 (kidney injury molecule 1), NAG (N-acetyl-glucosaminidase) and interleukin-18, are examples of new biomarkers (142-144). Some of the markers are expressed in the tubule and upregulates during kidney injury, and other are excreted in the urine due to increased filtration and/or reduced tubular reabsorption. The lack of standardization of methods/assay and few studies are a common problem.

5.8.2 Neutrophil gelatinase-associated lipocalin

Neutrophil gelatinase-associated lipocalin (NGAL) is a protein that may be used for early detection of acute kidney injury (AKI) (142, 145). The advantage of this marker is the early increase, before plasma creatinine increases. It is studied in both adults and children after cardiac surgery, in critically ill patients and after contrast agents (146).

5.9 Non-renal biomarkers influenced by renal function

Impaired renal function may affect the level of several disease markers used in daily practice. Inker et al. concluded that lower eGFR was strongly associated with higher odds of multiple laboratory result abnormalities (147). Other biomarkers investigated in our study are the following:

Guanidinoacetate (GAA) and creatine (CRE) are diagnostic markers for creatine deficiency syndromes (CDS)(148). Urinary GAA/creatinine (crn) and CRE/crn are measured in children with unexplained intellectual disability and/or delayed language development. Urinary GAA/crn ratio and blood GAA is elevated in guanidinoacetate methyltransferase (GAMT) deficiency and the CRE/GAA ratio is decreased. The opposite can be seen in arginine:glycine amidinotransferase (AGAT) deficiency. Increased urinary CRE/crn ratio is often seen in X-linked creatine transporter defect (CRT), at least in males. Previously studies have shown low GAA in serum in patients with CKD, based on estimated GFR (149, 150).

Human epididymis protein 4 (HE4) is a tumor marker for ovarian cancer, either used in combination with CA125 or alone (151). Higher plasma HE4 levels have been seen in patients with decreased renal function based on different GFR-estimating formulas or high creatinine (152, 153).

5.10 Proteinuria/albuminuria

5.10.1 Physiology

The definition of proteinuria is increased excretion of protein in the urine. Glomerular proteinuria is mainly due to hyperfiltration or changes in the glomerular filter causing excretion of large protein molecules. On the other hand, tubular proteinuria is due to impaired tubular reabsorption and low-molecular weight proteins will increase in the urine. Albumin is the dominant protein in plasma and normal values are <150 mg protein/24h/1.73m^2 in urine because of reabsorption in the proximal tubule. Newborns and infants have larger loss of protein in the urine due to the immature proximal tubule system. Tamm-Horsfall protein (=uromodulin) is the most abundant protein in urine (50%) in healthy persons, and prevents urinary tract infections (154, 155). In contrast, approx. 20% of the proteins in urine are albumin and 5% immunoglobulin.

5.10.2 Methods

There are different methods for measurements in urine for investigation of kidney disease in children. Urine sticks, microscopy and quantitative urine albuminuria/proteinuria measurements are used in daily practice. Protein/creatinine ratio (PCR) or albumin/creatinine ratio (ACR) in first morning void urine is well correlated with 24h-urine protein analysis (156) and avoid on the same time to include orthostatic proteinuria. Spot-urine measurement of protein (correlated to urinary creatinine) is commonly used. The level could be influenced by exercise, age, gender, BMI, fever and position and also due to the day-to-day variation. Ideally three consecutive first morning void samples are taken to calculate the median value to avoid outliers (157, 158).

5.10.3 Practical use

Albuminuria is associated with endothelial dysfunction and the risk for kidney disease, death and cardiovascular disease in adults, and is commonly used for investigation of kidney disorder, but also hypertension and diabetes mellitus (159, 160). Proteinuria is also an independent risk factor for ESRD and will shorten the time to renal replacement therapy/decline in GFR (161, 162). Albumin/creatinine ratio is very important for investigation early glomerular damage, and in this situation the protein/creatinine ratio could be in the normal range e.g. in diabetic nephropathy. Normally it is <150 mg protein/24h/1.73m^2 and ACR <2.5 mg/mmol and PCR < 20 mg/mmol. The higher the level of albuminuria, less difference between ACR and PCR in glomerular damage (Table 6).

Table 6: Albuminuria staging, modified from reference (20)

Albumin category	Albumin excretion rate (mg/24 h)	Albumin/creatinine ratio (mg/mmol)	Albumin/creatinine ratio (mg/g)	Text
A1	<30	<3	<30	Normal-mildly increased
A2	30-300	3-30	30-300	Moderately increased
A3	>300	>30	>300	Severely increased

6 Aims of the study

The main goal of this project was to investigate and improve methods for measuring and estimating glomerular filtration rate in children, exemplified by its effect on biomarkers in the urine.

Specific aims:

I. to find the best method for estimating GFR by creatinine- and/or cystatin C based eGFR equations in children.
II. to validate multiple formulas and single-point iohexol plasma clearance and find the best method for measuring GFR based on single-point methodology.
III. to investigate if a capillary blood spot method could replace venous blood sampling adequately for measuring iohexol clearance in children.
IV. to investigate how renal function influences different non-renal diagnostic biomarkers in daily routine.

7 Materials and methods

7.1 Materials

96 children with CKD were included in our cross-sectional study; 42 patients from Oslo University Hospital, Oslo, and 54 patients from Haukeland University Hospital, Bergen, Norway (ClinicalTrials.gov Identifier NCT01092260). The patients had stable kidney function and no edema. The distribution in different CKD stages was as follows: 28, 27, 23, 18 in CKD stage 1, 2, 3, and 4-5, respectively. The patients had the following kidney disorders: congenital anomalies of kidney and urinary tract (CAKUT) (n=30), hereditary kidney disorder (n=27), acquired kidney disorder (n=12), glomerulonephritis (n=9), hydronephrosis/vesicoureteral reflux (n=9) and CKD of unknown etiology/miscellaneous (n=9).The median age of the 55 males and 41 females in the study was 9.2 years (range 3 months to 17.5 years), median weight 28.3 (range 6.6-84.6) kg, median height 134 (range 59-177) cm. Median reference GFR based on seven blood sample time points (GFR7p) was 65.9 (range 6.3-153) mL/min/1.73m^2. 32 patients < 6 years were included in a subgroup for analysis of blood spot on filter paper and the median GFR7p was 65 (range 6-122) mL/min/1.73m^2. Exclusion criteria were a history of severe reaction to iohexol and contrast agents given less than 5 days prior to the survey. The study was approved by the Regional Ethics Committee of Western Norway, and performed in accordance with the Helsinki Declaration and Good Clinical Practice. Informed consent form was signed by all patients and/or their designees.

7.2 Laboratory methods

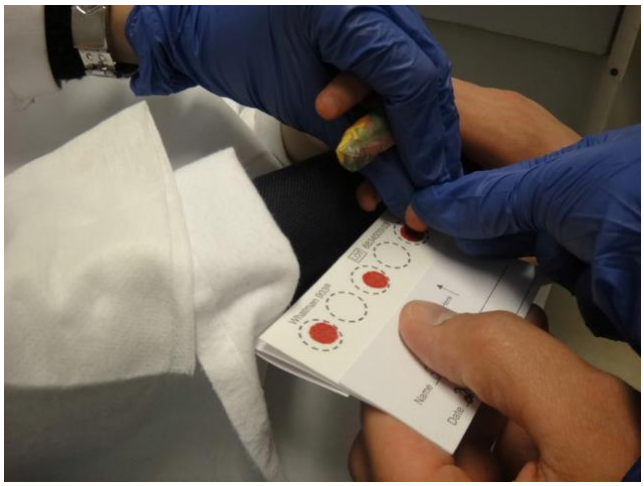
7.2.1 Iohexol clearance

We collected 3 mL serum (whole blood in a vial without anticoagulant) at baseline for measurement of different biomarkers; as described in paragraph 7.2.2, and to exclude interfering substances with the iohexol assay, before injection of iohexol (Omnipaque®, 300 mg iodine/mL, GE Healthcare; i.e.647 mg iohexol/mL). Iohexol was given via an intravenous cannula in doses according to the patients weight as follows: <10 kg; 1 mL, 10-20 kg; 2 mL, 20-30 kg; 3 mL, 30-40 kg; 4 mL, >40 kg; 5 mL. The syringe with iohexol was weighed before and after iohexol injection (accuracy of the weight given in 0.01 g). The dose of iohexol (in mg) was calculated by multiplying the difference in syringe weight by the concentration of iohexol (647 mg/mL) and then divided by its density at room temperature (1.345 g/mL). After the injection of iohexol the intravenous access was flushed with 15 mL physiologic saline. Venous blood samples of 0.5 mL were drawn from a vein of the contralateral arm of the iohexol injection at 10, 30, 120, 180, 210, 240 and 300 minutes after the injection of iohexol. This seven time point GFR (GFR7p) was defined as the reference GFR in the study. In 29 of the 96 patients the second blood sample was drawn after 60 min instead of 30 min. Exact blood sampling time was recorded. The blood samples were centrifuged after 30-60 min at 1000-1300 *g* for 10 min. Serum samples from baseline was stored at -70°C at Oslo University Hospital and serum samples for iohexol measurements were stored for a short period of time at -20°C before analysis at Haukeland University Hospital. Samples collected were shipped on dry frozen ice. Serum for iohexol measurements (50 µL) was vortexed in 30 sec with 200 µL perchloric acid and then analyzed by high performance liquid chromatography (HPLC) system (Agilent 1100) by calculating the area under the largest iohexol peak compared to internal calibrators. Phenosphere 5µ ODS (2) 80 A column with a gradient mobile phase (phosphoric acid and acetonitrile) with flow 1.5 mL/min, flushing phase (methanol), and detection wavelength 245 nm was used. The method is accredited

by the Norwegian Accreditation and complies with the requirements of NS-EN ISO 15189, and the total coefficient of variations (CV) was 4.1% at 10 mg I/L, 3.8% at 25-290 mg I/L and 3.3% > 290 mg I/L.

Capillary blood spots on filter paper (Whatman Protein Saver Card 903, GE Healthcare) were obtained using a lancet (MedLance 2.0mm), at the same time as the venous samples at 120,180, 210, 240 min after the injection of iohexol. One blood droplet was applied on filter paper without touching the area with the finger and using the same procedure used in the National Newborn Screening in Norway (Figure 5). One punch of 3.2 mm (3 µL) was treated with 170 µL 5% perchloric acid, vortexed for 3 min, ultrasonicated for 15 min, kept in room temperature for 30min and centrifuged at 14,000 *g* for 10 min, based on the method by Niculescu-Duvaz et al (163). The same HPLC method as described above for serum iohexol measurement was used.

Figure 5



7.2.2 Analysis of renal biomarkers in serum and urine

Renal biomarkers were analyzed in the sample obtained at baseline, before injection of iohexol. Serum creatinine was measured by an enzymatic colorimetric

method (reagents from Roche Diagnostics®), isotope dilution mass spectrometry (IDMS) traceable, with a total CV ≤ 3.7% and limit of detection of 5 μmol/L. Serum cystatin C was measured by a turbidimetric immunoassay traceable to the ERM-DA471/IFCC reference material, with reagents from Gentian, Moss, Norway. The CV was ≤ 5.0% and limit of detection was 0.03 mg/L. A kinetic UV method was used to measure serum urea. Urine protein and creatinine from morning void urine were analyzed by an immunoturbidimetric method (CV ≤ 9%) and by an enzymatic colorimetric method (CV ≤ 3.7%), respectively. NGAL in serum and urine was measured using immunoassay with reagents from Gentian®, Moss, Norway. All biomarkers were analyzed on Modular P8000 (Roche Diagnostics®).

7.2.3 Analysis of non-renal biomarkers in serum and urine

Hemoglobin (n=16) and hematocrit (n=8) were measured on the project day (n=24) or close to the day of iohexol clearance (n=7) (paper III). CRE and GAA concentration in serum and urine were determined using the method by Bodamer et al., with a LC-MS/MS method (164). HE4 in serum and urine were analyzed using the Elecsys HE4 assay on Cobas e601 (Roche Diagnostics®) (paper IV).

7.3 Calculation of mGFR

7-point GFR was calculated ad modum Sapirstein (two-compartment model) (107, 165). The first part of the curve was calculated after 10 and 30/60 minutes and the second part from 120-300 minutes. GFR was normalized to body surface area by the formula of Haycock et al (15). Six different one-point formulas were included in paper II (Table 1). The one-pool clearance model by Jødal and Brøchner-Mortensen from 2009 was used both in Paper II and III (166), and the following

formula was used to give serum iohexol concentration: blood spot iohexol clearance/(1-venous hematocrit).

7.4 Formulas for eGFR

The following eGFR equations were evaluated in paper I: $Schwartz_{bedside}$ (99), $Schwartz_{CKiD}$ (96), $Schwartz_{cysC}$ (96), FAS_{crea} (98, 167), FAS_{cysC} (97), FAS_{combi} (97), FAS_{height} (167), CAPA (76), LM_{REV} (109), and the LM_{REV}-CAPA (mean of LM_{REV} and CAPA) (Table 2, paper I).

7.5 Formulas for calculation of single-point mGFR

The following formulae for mGFR were evaluated in paper II:
Fleming formula (130), Ham and Piepsz formula (168), Stake formula (129, 169), Grothand and Aasted formula (128), Jacobsson formula (127) and a modification of Jacobsson's formula (170, 171) (Table 1, paper II).

7.6 Statistics

Microsoft Excel (version 2010), SPSS (version 21), R (version 3.3.2 and 3.4.0) and ANOVA (analysis of variance) were used for statistical analysis. For comparing different methods we evaluated bias, precision and accuracy. Bias was defined as the absolute or relative (mean or median) difference between the eGFR or GFR1p or blood spot measurements and sevenpoint-mGFR (GFR7p). As a measure of precision the IQR or SD of the differences and limits of agreement were calculated. Accuracy was defined as the percentage of eGFR results or GFR1p between 5 and 30% of reference GFR7p (from P5 to P30). Bland-Altman

plots, McNemars test with Holm-Bonferroni method for multiple comparisons for accuracy and t tests were used. Multiple linear regression analysis were performed to examine the influence of mGFR, sex and age on the different diagnostic markers in paper IV, and the Gowans' criterion was used to evaluate the biological significance (of the effect size) (172).

7.7 Ethical considerations

The study was approved by the Regional Ethics Committee of Western Norway (REK) and Local Ethical Research Committees at Oslo University hospital and Haukeland University Hospital. Written informed consent was obtained before inclusion in the study, and in accordance with the Helsinki Declaration and Good Clinical Practice. (ClinicalTrials.gov, Identifier NCT01092260, REK 2010/741). A few more blood tests were obtained in the study compared to the routine measurement for iohexol clearance. However, the venous sample volumes were at a minimum (0.5mL). No additional venipunctures were performed. A subgroup of 32 patients < 6 years had four finger sticks with blood sampling on filter paper in addition, but this is hardly very painful, and it is the same method used for measuring blood sugar in patients with diabetes mellitus.

8 Results

8.1 The best way to estimate GFR based on creatinine and/or cystatin C

The $Schwartz_{cysC}$ equation had the lowest median bias 3.27 mL/min/1.73m^2 and best accuracy with P10 of 44% and P30 of 85%, in the group with GFR <60 mL/min/1.73m2, amongst the evaluated different creatinine – and cystatin C based estimating GFR equations in children. However, in the group with GFR > 60 mL/min/1.73m^2, the $Schwartz_{CKiD}$ had the lowest bias of 3.41 mL/min/1.73m^2 and P10 and P30 of 62 and 98%, respectively. For the whole GFR group the $Schwartz_{cysC}$ equation had the lowest bias of -1.49 mL/min/1.73m^2. Overall, P30 was 90% in both these Schwartz formulas. The $Schwartz_{cysC}$ equation also performed relatively well compared to the other equation in a subgroup of patients with proteinuria and in age group < 2 years.

The best creatinine-based equation was the LM_{REV} equation with a P30 of 72%. (Figure 1-2 and Table 3-4, paper I). Several, but of course not all published eGFR formulas were investigated, and the commonly used formula in the daily routine, the $Schwartz_{bedside}$ formula, did unfortunately not perform well in our study, with a P30 of 53%.

8.2 The best way to perform single-point mGFR

Paper II demonstrated that the Fleming formula with sampling at 3 h showed the best performance with P10 of 92% in children with GFR > 30 mL/min/1.73m^2 (n=78).This is not significant different from 2-point measurement with formula of Jødal and Brøchner-Mortenson (P10 =96%). In the whole group, the P10 was 82% with the Fleming formula at 3 h sampling time. In the group with GFR < 30 mL/min/1.73m^2 lower performance was observed for all single-point-formulas.

The highest P10, in the group with CKD 4-5, was 67% with Fleming formula at 5 h. However, P10 was 100% with JBM 2-point methodology in this subgroup (Table 3-4 paper II). 2-point sampling is the preferred method, especially if GFR is < 30 mL/min/1.73m^2.

8.3 Blood spot for measuring GFR with iohexol clearance

In the subgroup of CKD patients < 6 years (n=32) the bias (mean relative) between 2-point blood spot and 7-point venous reference GFR was 7.2%, and 2.3% in the patients with GFR < 60 mL/min/1.73m^2 (n=13), i.e. the capillary blood spot method overestimated the venous GFR. P10 and P30 were 59 and 97%, respectively, for the whole group (n=32) for the 2-point blood spot, and P10 of 77% and P30 of 100% in the group with GFR level below 60 mL/min/1.73m^2 (n=13). (Table 2 and figure 2, paper III).

Table 7 Comparisons of accuracy [a] paper I, II and III

	CKD stage 1-5		CKD stage 3-5	
	P10 (%)	P30 (%)	P10 (%)	P30 (%)
2-point blood spot (2-4h) (paper III)	59	97	77	100
2-point plasma (JBM) (2+5h) (ref:(166))	96	-	100	-
1-point plasma (Fleming)(3h) (paper II)	82	-	91 (CKD 3) 39 (CKD 4-5)	-
eGFR Schwartz$_{cysC}$ (paper I)	44	90	44	85
eGFR Schwartz$_{bedside}$ (paper I)	13	53	17	39
eGFR LM$_{REV}$ (paper I)	27	72	12	51

[a] Accuracy shown in P10 and P30; the percentage of patients within ±10 and 30% of the 7-point reference method, respectively.
JBM: Jødal and Brøchner-Mortensen formula, LM_{REV} = Lund-Malmø revised
- = not calculated

8.4 Effect of GFR on non-renal diagnostic biomarkers

In paper IV we show that GFR significantly affected both S-GAA and U-GAA ($p=2 \times 10^{-4}$ and 5×10^{-11}), so the GAA values decreased in renal impairment and the value of GAA could then be below the lower reference limit. The same effect was not observed for creatine in serum and urine. Age influenced the level of S-GAA, S-CRE and U-CRE/crn ($p<0.001$), with increased S-GAA values and decreased S-CRE and U-CRE/values in older children. The level of GFR significantly affected HE4 in serum ($p=4 \times 10^{-31}$) and U-HE4/S-HE4 ratio ($p=2 \times 10^{-21}$), demonstrating that patients with decreased kidney function showed

increased S-HE4 and decreased U-HE4/S-HE4 ratio. GFR also affected the value of NGAL with increasing S-NGAL with low GFR ($p= 2 \times 10^{-19}$). GFR did not affect U-NGAL to the same extent (p=0.045). Age influenced NGAL in both urine and serum (p=0.012 and 0.007, respectively). (Figure 1,3-4 in paper IV). Renal impairment affected the values of measured biomarkers, and this could lead to misinterpretation of the biomarker.

9 General discussion

9.1 Methodological considerations

9.1.1 Formulas for eGFR

We chose to evaluate ten different eGFR equations in paper I, based on cystatin C (n=3), creatinine (n=4) or a combination of these two (n=3). There are several published eGFR equations in the literature, and the number is growing. We wanted to focus on the most commonly used in daily practice at children's departments in Norway, i.e. $Schwartz_{bedside}$, $Schwartz_{CKiD}$, but also the relatively new FAS equations and the equations used in Sweden; CAPA and $LM_{REV.}$ Sweden has long experience with GFR estimation formulas and the "Lund Model" by Grubb et al. is based on the simultaneous use of cystatin C based- and creatinine based GFR estimation in adults. If the two GFRs agree, i.e. $eGFR_{CysC}/eGFR_{creatinine}$ ratio between 0.8-1.2, the mean value is used. If there is a discrepancy, clinical data is investigated to identify the cause of the difference and the reason for skipping one of the two equations (e.g. abnormal low/high muscle mass, treatment with glucocorticoids etc.). If the GFRs do not agree without any obvious reason, an invasive mGFR determination is recommended (29, 76, 173). Comparing $eGFR_{cystatin\ C}$ and $eGFR_{creatinine}$ could help to identify pediatric patients with the so-called Shrunken Pore Syndrome, characterized by $eGFR_{cystatin\ C}$ being less than 60% of $eGFR_{creatinine}$ (174, 175). The glomerular filtration of 5-40 kDa molecules is selectively decreased compared to that of molecules <0.2 kDa (e.g. creatinine)(176).

Height is a common term of several eGFR equations, but the laboratory information system is not usually designed for reporting height and thus eGFR based on height for the moment being becomes impracticable. Nevertheless, height-independent eGFR formulas should be interesting to implement in order to

report eGFR in the daily routine for the laboratory information systems, although height in a child can vary widely in the same age group.

9.1.2 Iohexol clearance

Our reference method for measurement of GFR is the multipoint iohexol clearance method based on seven sample time points after injection of iohexol. The choice of iohexol clearance was based on its good agreement with the gold standard inulin clearance, it is an easy and available method in daily practice, not radioactive and has few side effects (11, 137, 139, 141). The 'gold standard' inulin renal clearance method is time-consuming , careful timing of blood samples and urine samples from bladder catheter are needed, and importantly, inulin is hardly available anymore (119). Schwartz et al. published a pilot study with 29 children in 2006, and a 9-point iohexol clearance (time point from 10 min – 360 min post-injection) method was used. In the study 4-point sampling (time point from 10 – 300 min) correlated well with the 9-point sampling method, and also 2-point sampling (120 + 300 min) demonstrated good correlation with the 9-point sampling, with the use of Brøchner-Mortensen one-compartment model (107, 123, 124). To avoid multiple blood sampling in the daily routine due to practical reasons, a one-point sampling would be attractive, however, the method needs to have an adequate level of accuracy. Different single-point GFR methods have been published (126, 127, 129, 130, 170). Delanaye et al. recently published a comparison between single- and multiple point sample plasma clearance in adults, and showed that 240 min was the best time point in their study for single-point sampling and GFR > 50 mL/min (not BSA corrected). They recommend sampling at 300 min or later post-injection, if GFR < 50 mL/min (177). This study did not correct for BSA, nevertheless, they showed that late sample is necessary if low GFR, and this is in accordance with our new daily routine. In the routine, the laboratory at Oslo University Hospital recommends a late sample at 8 hour if eGFR 10 - 40 mL/min/1.73m^2, or 24 hour if eGFR < 10 mL/min/1.73m^2. Iohexol

clearance method would probably not be accurate enough if the presence of edema or ascites, and this was an exclusion criterion in our study (141).

There are uncertainty regarding the differences in iohexol measurements, HPLC versus LC-MSMS methods, and how this would affect GFR results, but it is well-known that intra-individual variation exists, combining the biological variation and the errors in GFR measurement (141). With an intra-individual variation of 4.2-10%, the GFR must increase or decrease by 5-15% in order to be clinically relevant (40). We used the most common method, HPLC method, in our study.

A limitation of our study is the low number of patients under 2 years (n=9) and under 1 year (n=1). The total number of patients is 96 which is relatively high in the pediatric age range, and few studies have reported multipoint mGFR in such a large cohort of children with CKD (141, 178). Also, we did not obtain blood samples at 8 hour, and this would have been preferred in the patients with eGFR < 40 mL/min/1.73m^2. The study focused on sampling during a normal work day at the outpatient clinic.

9.1.3 Blood spot method

Several biomarkers in medical biochemistry can be extracted and measured from dried blood spots (DBS) on filter paper. This method has been used in daily routine in the newborn screening programs in Norway since 1960-70s (179). All newborns in Norway have now the opportunity to be screened for 25 different treatable disorders, including inborn errors of metabolism, endocrine disorders, severe combined immune deficiency and cystic fibrosis. Apart from this different enzyme tests, gene tests, pharmacological markers, immunological markers or microbes can also be measured from filter cards (180). The advantage is the stability during transport by mail from different hospitals and homes to the laboratory. In addition it is easy to perform by a finger prick which may be less painful than venous samples, and only a small amount of blood is needed (50 - 80

µl/spot). The long term stability on filter paper during storage in freezer is in general considered to be good (180, 181). We also demonstrated good stability in paper III during 1 year at 4 ^{0}C and - 70 ^{0}C. Niculescu-Duvaz et al. published a blood spot method for mGFR with iohexol clearance in 2006, for an adult cohort (163). It is more time-consuming than the regular measurement in serum. Iohexol is not distributed in red blood cells, and hematocrit should be measured in addition (182). A limitation to our study regarding the blood spot method is the lack of hematocrit measurement in all the patients at the study day as hemoglobin or hematocrit were only measured in 24/32 patients on the study day. Hemoglobin values (g/dL) were multiplied by 2.95 to give the hematocrit values. The factor was the mean (=median) hematocrit/Hb ratio calculated from 13875 routine samples from patients 1-18 years from Oslo University Hospital. Another limitation is the lack of repeated measurements of hematocrit during the study day. Fluid intake during the day was recommended, but the fluctuation in hematocrit would probably be small, but might be higher than described in the literature due to the fluid intake (183). An error in the hematocrit could lead to inaccurate iohexol value used in the formulas and hence also in the GFR (182).

Luis-Lima et al. published in 2018 a DBS comparison for mGFR with iohexol clearance based on both a volumetric method and the non-volumetric method (used in our paper III)(184). The non-volumetric method is based on whole blood directly placed on filter paper and a partial sample is punched out. In contrast, volumetric sampling is based on a fixed volume deposited by a capillary pipette on filter paper, and then the whole blood spot is extracted and analyzed. They used the same extraction method as us, but with a modification based on adding iopamidol as internal standard. They found that the volumetric DBS had acceptable agreement with the plasma reference method. Low hematocrit can lead to reduced blood viscosity, and hence give increased diffusion of blood and increasing the diameter of the blood spot. So, the volume of blood is variable in the non-volumetric DBS method, and this could make it less accurate and precise. In vivo testing with the volumetric method with iopamidol as internal standard gave a P10 of 95.6%, and in the following 16 adults the volumetric DBS method

showed an average error <10%, in contrast to the non-volumetric method with error ranging from 11.9 to 27.6 % (difference between mGFR in plasma and DBS) (184). Unfortunately, there is no external quality control for calculation of iohexol clearance based on DBS, as in plasma. Recently, new capillary blood sampling methods have been published; VAMS (volumetric absorptive microsampling) and HemaPEN (185, 186). VAMS is a plastic handle with a tip that is put into the desired specimen material to absorb a fixed volume of e.g. blood, urine and oral fluid, according to tip size (10-30 µL). One of the advantages is the hematocrit independency. However, the price is as yet higher than for DBS. The complete device is used for analysis, and this is an disadvantage when left-over material might have to be used for other purposes, e.g. further diagnostic tests: either biomarkers, genetic tests or microbiological analysis (185). HemaPEN is a retractable pen that integrates four capillaries for blood collection coupled to four DBS filters, storing an accurate volume of blood (2.74 µL per DBS filter) (187). Ion et al. investigated and developed a sample preparation method for iohexol determination using VAMS and HemaPEN (187). Further studies in patients with CKD and different patient cohorts need to be investigated, and compared to the plasma GFR iohexol determination.

9.1.4 Statistical methods

In paper II and III (a subgroup of 32 children < 6 years in paper III) the mean bias was used for comparing multiple single-point mGFR-formulas and DBS-GFR with the multipoint reference iohexol clearance. In contrast, in paper I and IV, it was focused on the analyzes of different endogenous biomarkers, both biomarkers used in different eGFR equations and several non-renal biomarkers, and the median biases were reported, based on normality testing. Different biomarkers and different number of included children were investigated in paper I-IV, and based on normality testing different statistical methods were used. For comparison of equations, the bias, precision and accuracy were calculated. Accuracy is a

combination of bias (=systematic error) and precision (=random error), and is often reported as P10 or P30, i.e. percentage of estimates within +/- 10 or 30% of mGFR (188). Root mean square method is another way to investigate the accuracy, but this method is not frequently used in daily practice and therefore not included in our papers (189). Root mean square error is dependent on the range of measurements and outliers have large effect. If a child has mGFR of 60 mL/min/1.73m^2, then a P30 interpretation means that the eGFR value could be between 42-78 mL/min/1.73m^2. Lima et al. recommend P10 of 90%, this means that 90% of the estimations should be within a 10% difference limit from the reference method (190). Other studies state that P30 of 90% is "good enough" (118, 191). It can be argued that the P10 should be the accuracy measurement in evaluation of eGFR instead of P30, and P10 of 90% would be meaningful to secure sufficient accuracy. It needs to be a balance between what is practically possible in daily practice and good enough quality of the method. It is difficult to obtain P10 above 90%, and in clinical situations where this could be especially relevant, an easy mGFR method should be the preferred method (191). Bland & Altman plots were used to demonstrate the difference between the methods and the GFR differences were plotted against the mean of the reference and test method in paper I and III (192). Bland and Altman have shown why the preferred method for comparing two methods is to plot the difference against the average, not to "standard method" only (193). When comparing 1-point and 7-point reference mGFR in paper II, the reference mGFR was plotted on the x-axis. Some papers argue that the reference method, i.e. the gold standard method, should be plotted on the x-axis, so it exists different opinions regarding this point (194). In paper IV different biomarkers were investigated, and transformed to remove skewness. Gowans`criterion was used to evaluate the biological significance of a statistical significant value of a marker difference (effect size) (172). In all four papers the two-sided p-value was set to ≤ 0.05. The power was set to 90%, with the effect size selected above and at least 32 patients had to be included in the subgroups..

9.2 Discussion of the results

9.2.1 The best way to estimate GFR based on creatinine and/or cystatin C

The $Schwartz_{cysC}$ and $Schwartz_{CKiD}$ equations presented with lower bias and higher accuracy than the other eight formulas in paper I. The advantage of the $Schwartz_{cysC}$ equation is the lack of height measurement in the formula, and thus it can be reported directly by the laboratory information system. Equations with height measurement can not be reported by the daily laboratory information system today without including a demand of height information when ordering creatinine in children. The $Schwartz_{bedside}$ equation is often used, but our study showed a disappointing low P30 of 53%, a P10 of 13% and median bias of 15.5% mL/min/1.73m^2.

The $Schwartz_{cysC}$ and $Schwartz_{CKiD}$ equations had P30 > 90% in our study, and none of the other equations met this requirement. However, all the equations had P30 < 90% in the group with GFR < 60 mL/min/1.73m^2. The highest P10 value was 62% in the group with GFR ≥ 60 mL/min/1.73m^2 and 48% in the total group, with the use of the $Schwartz_{CKiD}$ equation. eGFR equations, as expected, have lower accuracy than simplified mGFR-methods like singlepoint-GFR, and therefore mGFR should in general be the preferred method for investigation GFR if possible. The 1-point GFR method with Fleming formula in paper II demonstrated a P10 of 82% in the whole group. On the other hand eGFR is needed in the everyday follow-up as an intervention that is needed for mGFR (injection of external marker etc.) cannot be redone very often.

It is well known that an eGFR formula has the best performance in a comparable cohort as the cohort from it was generated. Also, the combination of several markers, as with creatinine and cystatin C, increases the performance of the equations and makes it more robust (96, 195).

We published in 2017 a study of evaluation of different eGFR equations in adult kidney recipients, where we also generated a new formula that performed very well in that adult cohort. The formula is based on both creatinine and cystatin C, and gave relatively small bias and high accuracy with a P30 of 91% and P15 of 73%, in contrast to 74% and 50% with the use of CKD-EPI creatinine formula, in the group with GFR < 60 mL/min/1.73m^2. The MDRD equation was the most accurate of the creatinine-equations with P30 of 85%, however, the combined CKD-EPI$_{creatinine+\ cystC}$ equation performed well in the middle and higher GFR-range, and the P30 was 92%. At higher GFR-levels the cystatin C equations as well as the combined equations performed better than the MDRD equation with a smaller bias, as in accordance with paper I, where both cystatin C-based and combined formula (Schwartz$_{cysC}$ and Schwartz$_{CKiD}$ equations) performed well (101). External validation in other cohorts is needed.

The CKD-EPI equation is recommended by the KDIGO guidelines from the age of 18 years (103). Pottel et al. recently published results from the transition from pediatric care to adult care, in >5700 patients age 10-30 years. They demonstrated a poor precision of CKD-EPI equation in the age group 18-20 years with an overestimation and "jump" of GFR from adolescent to adult age with a positive bias of 21 mL/min/1.73m^2 and P30 of 60%. This demonstrates the problem with sudden change in formula at the age of 18 years. The FAS-Height equation and the weighted average of CKiD and CKD-EPI equation gave smaller changes around the time of transition, and in addition, the LM$_{REV}$ equation (109) demonstrated a P30 of 94% in patients with normal kidney function, i.e. GFR ≥ 75 mL/min/1.73m^2 (196). Selistre et al. discuss this, and the explanation for the poor precision of the CKD-EPI equation could be the correction factor regarding the age-related GFR decline. This decline usually does not happen before the age of 40-50 years (197). We should therefore be aware when eGFR interpretation is done in the transition period. An eGFR value in a 16 year old well-trained boy could be abnormal with the Schwartz$_{bedside}$ equation and normal with the CKD-EPI equation. The Schwartz$_{bedside}$ equation from 2009, or Schwartz$_{CKiD}$ equation, does

not include sex as a parameter. An idea could be to use different formulas and models as described above.

KDIGO guidelines recommend reporting the eGFR value automatically when measuring creatinine and cystatin C in serum (20). It is recommended to report $eGFR_{creatinine}$ with the use of CKD-EPI formula (104), but this is only applicable for adults. Height measurement cannot be directly reported in most laboratory information systems, and due to the height measurement needed in several eGFR equations in children, the clinicians often use eGFR calculation on internet websites or application on mobiles. An alternative is the use of height-independent formulas as $Schwartz_{cysC.}$ One should also report eGFR based on cystatin C values, not just the absolute cystatin C value, but there are still different formulas used by different laboratories, and it is important that it is clear which formula and method that has been used. The price for one creatinine analysis is approx. 14 NOK and cystatin C 35 NOK (same as HbA1c), at Oslo University Hospital. More and more laboratories in Norway include cystatin C in their repertoire. But still, internationally, price is an important factor and creatinine is commonly used worldwide.

The $Schwartz_{bedside}$ equation is commonly used in the daily routine at pediatric departments in Norway, but the formula did not perform well in our study. Despite that, creatinine measurement and the $Schwartz_{bedside}$ equation are easy to perform and have practical advantages in the daily routine. Studies demonstrate different results regarding the performance of the $Schwartz_{bedside}$ equation (29). Staples et al. showed a good agreement and a mean bias of 5.84 mL/min/1.73m^2, i.e. an underestimation, between this equation and iothalamate renal clearance in a population of non-CKD children (198). Another study from Gao et al. demonstrated a good agreement when GFR was between 15-107 mL/min/1.73m^2, but not in the higher GFR range(199). Studies vary in method of measurement, creatinine method and population (size and disorders), so conflicting results might not be surprising. Different results regarding the performance of the formula were observed in our subgroup analysis depending on age (Online Resource 2, paper I).

The $Schwartz_{bedside}$ equation gave better results if age ≥ 13 years with a P10 and P30 of 26 and 84%, respectively, compared to 10 and 50% if age between 2 and 13 years. The children in our cohort have CKD and probably lower muscle mass compared to healthy children on the same age. However, the subgroups are small, and it must be interpreted with caution.

9.2.2 The best way to perform single-point mGFR

Paper II demonstrated a good performance of single-point mGFR when the Fleming formula was used and blood sampling was done 3 hours after iohexol injection. P10 in children with GFR ≥ 30 mL/min/1.73m^2 was 92%, in contrast to 82% in the complete cohort. Stake et al. recommended in 1991 the use of 1-point iohexol measurement in children based on a modification of the Jacobsson formula in a cohort of 143 Norwegian children (127, 129). He recommended the use of one single sample at 24 hours when very low GFR based on a study including 11 patients with CKD 4-5 (200). The relatively few children with low GFR included in the study limits the value. McMeekin et al. compared GFR1p-Fleming formula with sampling at 3 hour with a multi-point method in adults and children, with accuracy levels as in our study (201). When GFR ≤ 30 mL/min/1.73m^2, we recommend based on our findings, two-point sampling for reaching optimal accuracy, in accordance with other studies (177). Delanaye et al. compared single- versus multiple sample method in > 5000 adults. They found that single-sample GFR was similar to multiple sampling GFR except in patients with high BMI (>40 kg/m^2) and with GFR < 30 mL/min (177). There were only 32 patients with GFR < 30 mL/min with multiple samplings, but a systematic bias was observed between the single- and multiple methods, resulting in higher values in single sample GFR. The last sampling point in single-point GFR-method was 240 min post injection, and this could be too early when the GFR is < 30 mL/min (177). Single-point mGFR has advantages, especially in children, i.e. with cancer, where it could be difficult to obtain blood samples and samples are taken

frequently (202). Also, it is less expensive than multi-point mGFR. When we evaluated two-point-methodology in the same cohort, we found that blood sampling at 2 and 5 hours using the formula of Jødal and Brøchner-Mortensen from 2009 gave the best results with P10 of 95.8% (122, 166, 203). It is important not to take the last sample too early. The iohexol elimination phase needs to reach the slow phase for calculating an accurate slope. GFR could be overestimated if sampling is done too early (200), as described in Table 5, paper II. This is most relevant at lower GFR levels (204). 18 patients in our study had CKD stage 4-5, and sampling time at 24 hours is relevant to investigate in these children. GFR based on 8-sampling points including 24 hours sampling was performed in the same cohort, and analyzes shows that single-point GFR gives inaccurate results with P10 ranging from 0-68% using different formulas, in contrast to P10 of 96% with 2-point sampling (205). Another issue is the implementation of 24 h sampling in daily practice. Also, mathematical weight of the 24 h sample in the GFR calculation could be a problem, since a long interval from the previous 2 h sampling is used in the calculation (141).The risk of outliers is crucial when using 1-point sampling, and the test therefore sometimes has to be redone. An outlier in a multiple sampling test is easier to handle, due to the removal of only 1 point after interpreting the elimination curve. The procedure for BSA normalization in the GFR calculation is also of high relevance, and the topic is discussed in several papers (206, 207). The original Brøchner-Mortensen (BM) formula is based on BSA normalization after correction for the distribution phase, but the modified BM version for children is based on BSA normalization before correction for the distribution phase. Even if not recommended by guidelines, several studies still use the original BMadult without early normalization in children. Based on our findings, normalization to BSA should be performed before correction of the distribution phase (122).

9.2.3 Blood spot for measuring GFR with iohexol clearance

In paper III we found a good agreement between 2-point dried blood spot on filter card and 7-point venous reference GFR. However, the blood spot method overestimated the venous GFR by a mean of 7.2% when blood samples were obtained after 2 and 4 hours after iohexol injection. The relative bias was lower in the group with GFR < 60 mL/min/1.73m^2; 2.3% and the accuracy also better with P30 of 100%, in contrast to 97% in the whole group. There was not any further better performance of extending the blood spot method to 3- or 4 samples times, thus we concluded that a 2-point blood spot was satisfactory. This is in agreement with other studies (208). The blood spot method could be an alternative to venous sampling for mGFR iohexol clearance in children. Staples et al. published in 2018 a pilot study comparing venous and finger stick methods in 41 patients between 1-21 years with CKD (209). Iohexol clearance was used and they found a good correlation between 2-point venous GFR and dried blood spot (DBS) (2+5 hours after iohexol injection), and no significant bias. 80% of the DBS measurements were within 10% of the venous 2-point GFR (n=29) and 67% of the DBS within 10% of the venous 4-point GFR (n=24). That low figure was probably due to two outliers in the 4-point group. The authors also demonstrated a slight overestimation of GFR by DBS, but the difference was not clinically significant (bias -3.3 mL/min/1.73m^2 on the group with GFR < 60 vs.-2.9 in all subjects) (209). We did not have a 5 hour DBS sample in our study, and we found a mean absolute bias of 1.5 mL/min/1.73m^2 in the group with GFR < 60 vs. 5.7 in all subjects. Luis-Lima et al., however, published in 2018 a DBS comparison for mGFR with iohexol clearance based on both a volumetric method and the non-volumetric method (used in paper III and ref. (209))(184). As described in the Method section, the non-volumetric method is based on whole blood directly placed on filter paper and a partial sample is punched out. In contrast, volumetric sampling is based on a fixed volume deposited by a capillary pipette on filter paper and the sample is completely included, and therefore the whole blood sample is analyzed (184). They discuss that the volume of blood will be variable

depending on the hematocrit and then the diffusion of blood on the filter paper. The in vitro non-volumetric sampling method showed low precision and accuracy with a P10 of 59%, in contrast to the volumetric method where P10 was 82% (P30 not reported). However, the best choice was the volumetric method with iopamidol as internal standard, demonstrating a P10 of 98% in vitro, compared with the reference mGFR method based on one-compartment model iohexol clearance and 7-point sampling (120-480 min post-injection) if eGFR ≤ 40 mL/min or 5-point sampling (120-240 min) if eGFR > 40 mL/min. In vivo testing showed mean difference/bias of -0.7 mL/min between the GFR values from dried blood spot and plasma samples (184). Our non-volumetric method in paper III demonstrated a P10 of 59% (in the whole GFR group), as the non-volumetric method mentioned above. An interesting study would be to further explore the volumetric method in children with CKD.

9.2.4 Effect of GFR on non-renal diagnostic biomarkers

In Paper IV we demonstrate that several diagnostic biomarkers could be influenced by the renal function, and this could affect the decision limits regarding different disorders. Methylmalonic acid and homocysteine are markers that are well-known to be influenced by the kidney function (210). Renal function should be taken into account when interpreting test results because decreased renal function might change the marker level below or above the decision limits. We examined four non-renal biomarkers, and this study is a model to investigate other disease markers.

GAA and creatine

A group of inherited metabolic diseases, namely the creatine deficiency syndromes, is characterized by low creatine in the brain. The diagnostic biomarkers, U-GAA/crn and U-CRE/crn, are used as first tier screening, and second tier S-GAA and S-CRE could be performed if U-GAA/crn is above or

below the reference interval. This is part of the normal metabolic screening program in patients with unexplained intellectual disability and delayed language development. U-GAA/crn and S-GAA are elevated in GAMT deficiency, and the opposite is seen in AGAT deficiency (148). The third creatine deficiency syndrome, X-linked creatine transporter defect, is characterized by increased U-CRE/crn ratio, at least in males. In females, the values can be borderline/high-normal (40, 148). In patients with renal impairment we observed low S-GAA and U-GAA/crn values. This could then lead to misinterpretation and false negative results for GAMT deficiency screening and false positive results for screening of AGAT deficiency. The same problem was not seen for U-CRE/crn ratio, the diagnostic marker for creatine transporter defect.

When a patient is suspected to have an inborn error of metabolism (IEM), a frozen urine sample should be sent to a specialized laboratory. Laboratory for Inborn errors of metabolism in Norway is localized at Oslo University Hospital Rikshospitalet. Several of the urine disease markers are calculated as marker/ creatinine ratios, e.g. glycerol/crn, mucopolysaccarides/crn, amino acids/crn, purines/crn, pyrimidines/crn. The effect of decreased GFR on these biomarkers has scarcely been reported, and this would be an interesting field to explore, since exact marker values are important for diagnosing these rare disorders. Several IEMs are treatable and early recognition is therefore important. According to our findings we have changed the routine to include plasma creatinine when analyzing for creatine deficiency disorders. We also have the metabolomics platform at our laboratory, and untargeted metabolomics could be a method for further investigations in the CKD cohort. Hallan et al. recently investigated metabolomics profile panel in blood and urine and gene expression in non-diabetic CKD adult patients (211). They found altered metabolites consistent with reduced citric acid cycle activity, reduced fatty acid oxidation and increased ketone body metabolism, and that could suggest decreased mitochondrial function. The use of proteomics is also a promising alternative in CKD patients for the risk assessment and to understand disease pathology (212).

HE4

HE4 is a tumor marker for ovarian cancer in adults. Regarding the interpretation of HE4 we know that several factors can alter its value, and one factor is GFR. We demonstrated in our study that the level of HE4 increased, also above the reference limits, with a modest decrease in GFR, hence the kidney function should be taken into account when interpreting the test results. Creatinine levels could be difficult to interpret in elderly patients, but cystatin C and eGFR equations recommended in the elderly population could be an alternative in routine interpretation of HE4 values. Today, standard comments are used when reporting HE4 results to the clinicians, and with an age above 75 years, the decline in kidney function could be the cause of an elevated value. Hence HE4 is of less diagnostic value in these age cohort. This is not a marker commonly used in pediatric patients, but illustrates the principle regarding misinterpretation if the renal status is not known.

NGAL

The small molecule NGAL has the advantage of early elevation, after 2 h after kidney injury, compared to S-creatinine which increases after 1-3 days after injury. In our study low GFR was associated with elevated S-NGAL, but GFR did not influence U-NGAL to the same extent. In another study from children after heart operation U-NGAL (mean value) increased 2 hours postoperative, 15 x from the initial value, and to a maximum value 4-6 h postoperative (25 x) (213). The NGAL value was normalized after 72 h.

Different cut-offs are published, due to different study protocols, patient cohorts, sampling materials, sampling time and some urine samples are corrected by U-creatinine. In addition, there are several platforms and assays for measurement of NGAL. Some assays only measure the monomeric form of NGAL that is secreted by the kidney epithelial cells and found in the urine of AKI patients, while other methods measure multiple forms of NGAL (214). Infections, inflammation and

malignancy can also increase the NGAL values, which make the interpretation more difficult (215).

NGAL measurement is not a routine marker in daily practice in Norway. We have done a pilot study (unpublished data) on the performance of S-cystatin C, S-NGAL and U-NGAL in adults after cardiac surgery as an early predictor of AKI. The preliminary results demonstrate that S-NGAL and cystatin C may be early biomarkers of AKI, but mean S-creatinine did not raise above the reference limit before 4 h post-operative, and then just above the upper reference limit. S-NGAL and S-Cystatin C were elevated 4 and 12 h postoperatively in AKI patients, compared to the non-AKI patients. The same effect was not seen with U-NGAL in this pilot study. Some published studies have reported U-NGAL as a better biomarker for CKD in children than S-NGAL, but GFR was estimated, and not measured as in our study (216). A biomarker combination was investigated in a recent study in 178 critically ill children, measuring U-NGAL and S-creatinine, on admission, to predict day 3 AKI severity phenotypes. Four combination groups of U-NGAL and S-creatinine were investigated, and the children with U-NGAL elevation without S-creatinine had a form for subclinical AKI with worse outcome compared to injury biomarker negative patients (217). This demonstrates that the use of several biomarkers in combination could be an alternative for diagnosing and risk stratification of AKI patients.

10 Conclusion and future perspectives

The optimal choice for GFR determination is measurement with iohexol clearance with two blood samples in general, at 2 and 5 hours after injection. This should be done at least once a year, however, in everyday practice and for serial measurement during the year, the use of $Schwartz_{cysC}$ or the $Schwartz_{CKiD}$ equations for estimation of GFR in children should be recommended. If GFR> 30 mL/min/1.73m^2, one point sampling at 3 hours with the Fleming formula could be sufficient, and might be an alternative in clinical practice, e.g. in children with cancer and clinical trials. The recommended daily routine for iohexol clearance measurement in children at Norwegian laboratories was changed from 1-point sampling with Stake-methodology to 2-point sampling using the JBM-methodology as a result of the low accuracy for the Stake-methodology found in our research project (218). Our recent finding on 1-point sampling at 3 hours with the Fleming formal is an option with acceptable accuracy. It is important to be aware of the great discrepancies between the different single-point methodologies in children. Further studies with higher number of patients are warranted. One should especially be looking at the mGFR methods and optimal sampling times in patients with severely reduced renal function.

So far, we demonstrated acceptable accuracy and precision between capillary blood spot and venous iohexol clearance measurements, but more studies in larger cohorts of children with a capillary volumetric method is needed, and also with the use of other capillary sampling methods, i.e. microsampling. Several diagnostic biomarkers could be affected by the renal function, and therefore renal function should be taken into account when interpreting biomarker test results. We studied a limited set of four non-renal biomarkers, but this study is also a model to investigate a variety of other disease markers. We would like to examine other markers used for screening of IEMs, e.g. in the diagnostics of several disorders in the pathways of synthesis and salvage of purines and pyrimidines, and whether the diagnostic marker will decrease or increase in concentration due to renal

dysfunction. These markers are measured in urine samples and their concentrations are divided by the U-creatinine. In a running study we are evaluating plasma and urine levels of Growth Differentiation Factor 15 in children with CKD and after renal transplantation and investigating the association with renal function. In addition, several new interesting biomarkers should be measured in the same CKD cohort. Untargeted metabolomics could also be an interesting alternative, to further explore alterations of metabolic pathways in children with CKD.

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ORIGINAL ARTICLE

Iohexol plasma clearance in children: validation of multiple formulas and single-point sampling times

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Abstract
Background The non-ionic agent iohexol is increasingly used as the marker of choice for glomerular filtration rate (GFR) measurement. Estimates of GFR in children have low accuracy and limiting the number of blood-draws in this patient population is especially relevant. We have performed a study to evaluate different formulas for calculating measured GFR based on plasma iohexol clearance with blood sampling at only one time point (GFR1p) and to determine the optimal sampling time point.
Methods Ninety-six children with chronic kidney disease (CKD) stage 1–5 (median age 9.2 years; range 3 months to 17.5 years) were examined in a cross-sectional study using iohexol clearance and blood sampling at seven time points within 5 h (GFR7p) as the reference method. Median GFR7p was 66 (range 6–153) mL/min/1.73 m^2. The performances of six different single time-point formulas (Fleming, Ham and Piepsz, Groth and Aasted, Stake, Jacobsson- and Jacobsson-modified) were validated against the reference. The two-point GFR (GFR2p) was calculated according to the Jødal and Brøchner–Mortensen formula.
Results The GFR1p calculated according to Fleming with sampling at 3 h ($GFR1p_{3h}$-Fleming) had the best overall performance, with 82% of measures within 10% of the reference value (P10). In children with a GFR ≥ 30 mL/min/1.73 m^2 ($n = 78$), the $GFR1p_{3h}$-Fleming had a P10 of 92.3%, which is not significantly different ($p = 0.29$) from that of GFR2p (P10 = 96.2%). Considerable differences within and between the different formulas were found for different CKD stages and different time points for blood sampling.
Conclusions For determination of mGFR in children with CKD and an assumed GFR of ≥ 30 mL/min/1.73 m^2 we recommend $GFR1p_{3h}$-Fleming as the preferred single-point method as an alternative to GFR2p. For children with a GFR < 30 mL/min/1.73 m^2, we recommend the slope-GFR with at least two blood samples.
Clinical Trial Registration: ClinicalTrials.gov, Identifier NCT01092260, https://clinicaltrials.gov/ct2/show/NCT01092260?term=tondel&rank=2

Keywords Glomerular filtration rate · Children · Chronic kidney disease · Renal function

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Introduction

The low accuracy of formulas for estimating glomerular filtration rate (GFR) in children has long been a major challenge,

with studies showing that less than 50% of the GFR levels estimated (eGFR) using formulas based on serum cystatin C, creatinine and/or urea are within ± 10% of the gold standard GFR measurement [1]. In pediatric nephrology care, more accurate determinations of kidney function are therefore needed with a feasible measured GFR (mGFR) methodology based on the plasma clearance of an exogenous GFR marker. Since the 1980s, GFR has been increasingly measured using the non-ionic agent iohexol [2–7]. To avoid extended examinations with multiple blood samples for measuring GFR, many centers have chosen to use the one-pool slope-intercept technique with a minimum of two blood samples [8–11]. Numerous single-point GFR (GFR1p) methods have been developed, and especially in pediatric care, it is clearly beneficial to reduce the number of blood draws from two or three to a single sample, provided an adequate level of accuracy can be preserved [11–14]. However, current guidelines from the British Nuclear Medicine Society (BNMS) do not endorse the routine use of a GFR1p method and recommend a one-pool slope-intercept technique requiring at least two samples [11]. The GFR1p methodology was introduced in adult patients by Fisher and colleagues in 1975 based on ^{51}Cr-EDTA clearance [15], and an improved concept was described by Groth and colleagues in 1981 [16]. In 1983, Jacobsson published a formula for GFR1p which takes into account different distribution volumes and different sampling time points in adults based on ^{99}TC^{m}-DTPA clearance [12]. The Jacobsson formula has been widely used for GFR1p with different markers. Confusingly, modified versions of the Jacobsson formula have also been used but *reported* as being Jacobsson's original formula [14, 17–19]. Here we report results for Jacobsson's original adult single-point formula [12] and for the modified, non-iterative formula [14, 17], which does *not* include Jacobsson's correction for non-uniform distribution. Groth and Aasted published the first pediatric GFR1p formula in 1984 in which they used ^{51}Cr-EDTA clearance with a sampling point at 2 h [20]. In 1991, Ham&Piepsz published a new formula for GFR1p in children, also with sampling at 2 h and based on ^{51}Cr-EDTA clearance. A modification of the Jacobsson formula for pediatric use was published the same year by Stake and colleagues; these authors recommended a sampling point at 3 h based on ^{99}TC^{m}-DTPA clearance [3, 21]. In 2005, Fleming and colleagues described a new formula for GFR1p which they developed from a cohort of 100 children and 225 adults; this formula provided GFR values consistent with those obtained by the slope-intercept technique [22]. Although the Fleming formula first and foremost was suggested as a quality control method for the slope-intercept technique [22], a recent study [19] reports results arguing for the GFR1p-Fleming as a potential stand-alone formula for pediatric nephrology care.

The aims of our study were to: (1) assess the accuracy of the different formulas for GFR1p determination by comparison with the reference iohexol seven-point plasma clearance measurements (GFR7p) and (2) determine the optimal single time point for blood sampling for GFR1p within a feasible time frame (i.e. blood sampling not later than 5 h after injection).

Patients and methods

Patients

Ninety-six children with chronic kidney disease (CKD) were recruited in a cross-sectional study (ClinicalTrials.gov Identifier NCT01092260) which has evaluated the two-point methodology [23]: 54 children at Haukeland University Hospital, Bergen, Norway, and 42 children at Oslo University Hospital, Oslo, Norway. The median age of the included children (55 males, 41 females), was 9.2 years (range 3 months to 17.5 years), the median weight was 28.3 (range 6.6–84.6) kg and the median height was 134 (range 59–177) cm. Median reference GFR based on seven blood sample time points (GFR7p) was 66 (range 6–153) mL/min/1.73 m^2. The individual GFR measurements were divided between the different GFR stages, namely, from 28, 27, 23, and 18 patients in CKD stage 1, 2, 3 and 4–5, respectively.

Methods

Iohexol was administered as Omnipaque®300 mg I/mL (647 mg iohexol/mL; GE Healthcare Technologies Norway AS, Oslo, Norway) in a dose adapted to body weight. Blood samples were drawn at 10, 30 or 60, 120, 180, 210, 240 and 300 min after injection. Additional details are provided in an earlier study published on the same cohort with a focus on two-point methodology [23].

Calculations and statistics

The GFR7p was calculated according to Sapirstein, as described by Schwartz et al. [3, 24] (Tables 1, 2). A two-compartment model was fitted using linear regression of the log concentration values. For three patients the two-compartment slope-intercept method could not be used due to negative values after the slow component of the curve was removed; for these three patients, we fitted the two-compartment model directly using non-linear least squares. GFR was normalized to 1.73 m^2 body surface area (BSA) by the ratio 1.73/BSA, using the formula of Haycock et al. [25]. The GFR1p was calculated with six different formulas: the Fleming formula [22], the Ham and Piepsz formula (Ham&Piepsz; [26], the Stake formula [13, 21], the Groth and Aasted formula (Groth&Aasted; [20]), the Jacobsson formula [12] and a modification of Jacobsson's formula

Table 1 Methodology of glomerular filtration rate calculations

Name of method/reference[a]	Formula[b]
Reference GFR (GFR7p)	
Two-compartment model	$C(t) = Ae^{-\alpha t} + Be^{-\beta t}$
Absolute GFR7p (mL/min)[b]	$Cl = I/(A/\alpha + B/\beta)$
BSA-normalized GFR7p (mL/min/1.7 3 m^2)	$Cl_{BSA} = Cl \times 1.73/BSA$
Single-point GFR (GFR1p)	
GFR1p-Fleming [11]	$V_{app}(t) = \frac{I}{C(t)} \times 1.73/BSA$ $A = -11297 - 4883 \cdot BSA - 41.94 \cdot t$ $B = 5862 + 1282 \cdot BSA + 15.5 \cdot t$ $Cl'_{BSA} = \frac{A + B \cdot \ln\frac{V_{app}(t)}{1000}}{t}$ $Cl_{BSA} = \max\left(Cl'_{BSA}, 0\right)$
GFR1p-Ham&Piepz [26]	$C_{120} = C(t) \cdot \exp[0.008 \cdot (t - 120)]$ $V_{120} = I/C_{120}$ $Cl = 2.602 \cdot V_{120}/1000 - 0.273$ $Cl_{BSA} = Cl \cdot 1.73/BSA$
GFR1p-Groth&Aasted [16]	$A = -72.295 \cdot \ln(t) + 425.41$ $B = -553.124 \cdot \ln(t) + 3236.76$ $x = \ln\left(\frac{I}{C(t) \cdot BSA \cdot 10^7}\right)$ $Cl_{BSA} = A \cdot x + B$
GFR1p-Stake [13, 21]	$V' = 231 \cdot weight + 1215$ $Cl' = \ln\left(\frac{I}{V' \cdot C(t)}\right) / \left(\frac{I}{V' \cdot C(t)} + 0.0016\right)$ $Cl'_{BSA} = Cl' \cdot 1.73/BSA$
GFR1p-Jacobsson [12]	$Cl_{BSA} = 180 - 14.1\sqrt{[133 - \min(Cl'_{BSA}, 133)]}$ $V = 246 \cdot weight$ $Cl_v = \ln\left(\frac{I}{V \cdot C(t)}\right) / \left(\frac{I}{V \cdot C(t)} + 0.0016\right)$ $m = 0.991 - 0.00122 \times Cl_v$ $V' = V/m$
GFR1p-Jacobsson-mod. [17]	$Cl = \ln\left(\frac{I}{V' \cdot C(t)}\right) / \left(\frac{I}{V' \cdot C(t)} + 0.0016\right)$ $Cl_{BSA} = \mathrm{Cl} \cdot 1.73/\mathrm{BSA}$ $V = 246 \cdot weight$ $Cl = \ln\left(\frac{I}{V \cdot C(t)}\right) / \left(\frac{I}{V \cdot C(t)} + 0.0016\right)$ $Cl_{BSA} = \mathrm{Cl} \cdot 1.73/\mathrm{BSA}$

See Table 2 for additional formulas and an example

[a] GFR, Glomerular filtration rate; GFR7p, reference GFR based on seven blood sample time points; GFR1p, GFR value based on blood-draw at one time point

[b] *I*, the dose of iohexol in mg; *C(t)*, the concentration in mg/mL at *t* min after injection; BSA, body surface area in m^2, calculated according to Haycock [25]; *V* and *V′*, estimated volume of distribution; *Cl*, unadjusted GFR; Cl_{BSA}, BSA-adjusted GFR estimate

[c] See Patients and methods sections for additional information on calculation of the GFR7p value

(GFR1p-Jacobsson-mod.) [14, 17] which is based on performing only the first step in Jacobsson's three-step GFR calculation. Tables 1 and 2 show the formulas used in the calculation of the GFR values, along with numerical examples. One patient had an obviously incorrect value measured for the 3.5-h sample, and this value was therefore removed before the analyses; otherwise the data were complete, with no missing values.

Table 2 Example data, with additional information on calculations

Examples	Value	Units	Calculation/comment
Product			
Omnipaque	300	mg I/ml	
Product density	1.345	g/ml	Product density at room temperature.
Iohexol density	647	mg/mL	
Injected dose			
Omnipaque, weight	2.8	g	
Omnipaque, volume	2.08	mL	2.8 g/1.345 g/mL
Iohexol, weight	1346.9	mg	2.08 mL × 647 mg/mL
Example patient			
Sample time	180	min	3 h × 60 min/h
Concentration	0.100	mg/mL	100 μg/mL
Body weight	13	kg	
Body height	90	cm	
BSA	0.574	m^2	$0.024265 \times height^{0.3964} \times weight^{0.3964}$ $=0.024265 \times 90^{0.3964} \times 13^{0.5378}$
GFR1p values (BSA-adjusted)			
GFR1p-Fleming	72.9	mL/min/1.73 m^2	See Table 1
GFR1p-Ham&Piepz	64.6	mL/min/1.73 m^2	See Table 1
GFR1p-Groth&Aasted	61.8	mL/min/1.73 m^2	See Table 1
GFR1p-Stake	76.5	mL/min/1.73 m^2	See Table 1
GFR1p-Jacobsson	75.7	mL/min/1.73 m^2	See Table 1
GFR1p-Jacobsson-mod.	74.9	mL/min/1.73 m^2	See Table 1
GFR7p calculations			
Measured concentrations at all time points:			
Time point	Time (min)	$C(t)$ (mg/mL)	$C^*(t)$ (mg/mL)
1	10	0.464	0.169
2	30	0.343	0.082
3	120	0.156	–
4	180	0.100	–
5	210	0.084	–
6	240	0.072	–
7	300	0.051	–

Two-compartment model: $C(t) = Ae^{-\alpha t} + Be^{-\beta t}$ = fast part + slow part

Regression of $\ln(C(t))$ on t for the slow part (time point 3–7):

(Intercept) $\ln(B) = -1.16 \Rightarrow B = 0.31$

(Slope) $-\beta = -0.0061 \Rightarrow \beta = 0.0061$

$C^*(t)$ is the concentration after removing the slow part of the curve:

$C^*(t) = C(t) - Be^{-\beta t} = C(t) - 0.31e^{-0.0061t}$

Regression of $\ln(C^*(t))$ on t (time point 1–2):

Intercept: $\ln(A) = -1.42 \Rightarrow A = 0.24$

Slope: $-\alpha = -0.036 \Rightarrow \alpha = 0.036$

AUC for fast part: $\frac{A}{\alpha} = \frac{A}{\alpha} = 6.7$

AUC for slow part: $\frac{B}{\beta} = \frac{B}{\beta} = 51.1$

Total AUC = 51.1 + 6.7 = 57.8

Unadjusted GFR**: $Cl = \frac{I}{AUC} = \frac{I}{AUC} = 23.3$

Glomerular filtration rate (GFR) adjusted for body surface area (BSA): $Cl_{BSA} = GFR \cdot 1.73/BSA = 70.3$. Note that the final calculations are based on more decimals than are shown in the intermediate calculations

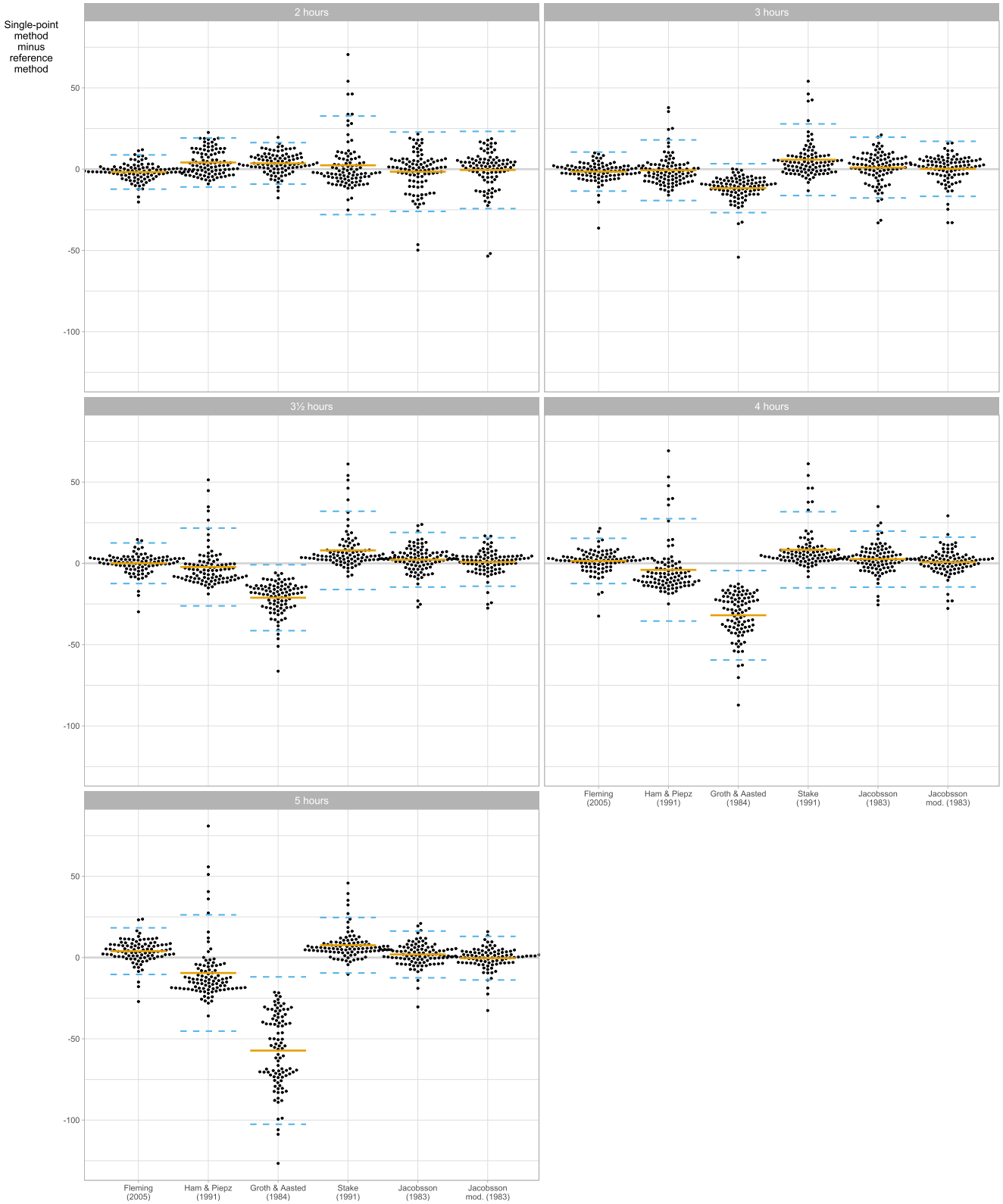
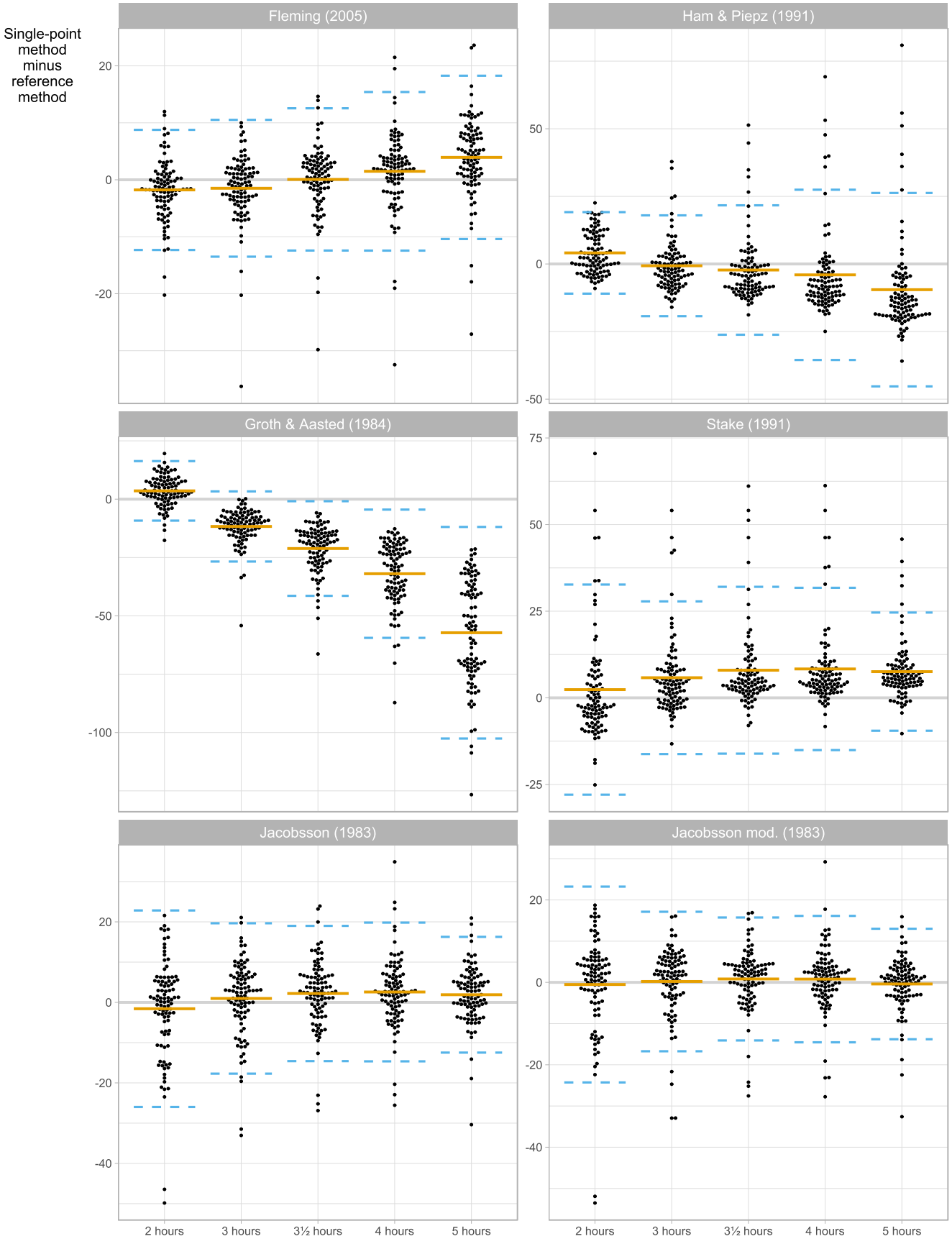


Fig. 1 Plot of estimation error versus the estimation method for glomerular filtration rate (GFR) calculated by six single-sample formulas [12, 13, 17, 20, 22, 26], stratified by sampling time point (n = 96 children). The y-axis shows the difference between the single-point GFR and a reference GFR based on seven sampling time points. Each point corresponds to a combination of patient, estimation method and sample time. The solid horizontal line is the bias, i.e. the mean difference between the single-point GFR estimate and the reference GFR. The dashed lines are limits of agreement, i.e. bias ± two standard deviations of the differences. The figure can be used to compare different methods *within* each sampling time point


Single-point method minus reference method
Fleming (2005)
Ham & Piepz (1991)
Groth & Aasted (1984)
Stake (1991)
Jacobsson (1983)
Jacobsson mod. (1983)
2 hours
3 hours
3½ hours
4 hours
5 hours

◀ **Fig. 2** Plot of estimation error versus time point for glomerular filtration rate (GFR) calculated at five time points, stratified by estimation method (n = 96 children). The y-axis shows the difference between the single-point GFR [12, 13, 17, 20, 22, 26] and a reference GFR based on seven sampling time points. Each point corresponds to a combination of patient, estimation method and sample time. The solid horizontal line is the bias, i.e. the mean difference between the single-point GFR and the reference GFR. The dashed lines are limits of agreement, i.e. bias ± two standard deviations of the differences. For each estimation method, the figure can be used to compare the performance of the single-point GFR estimates at different sampling time-points

To compare the GFR1p methods and the reference method, we calculated the difference between the various GFR1p and the reference GFR for each patient, along with estimated bias (mean difference) and limits of agreement (bias ± twice the standard deviation of the differences). The data are presented as difference plots comparing: (1) different methods within a single sampling time point (Fig. 1), (2) different sampling time points for each method (Fig. 2), and (3) the bias for different GFR values

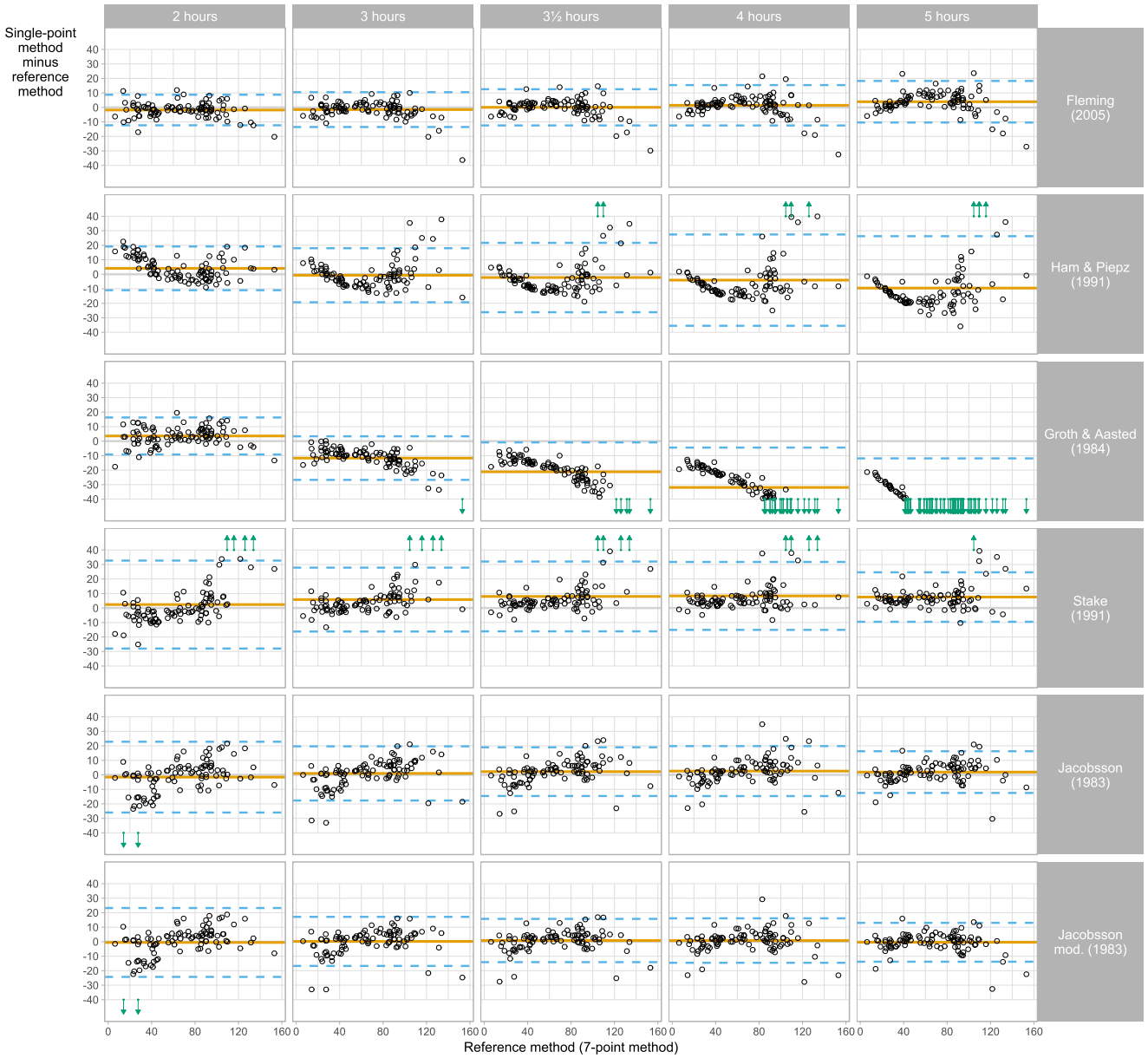


Fig. 3 Plot of estimation error versus reference glomerular filtration rate (GFR) for GFR calculated by six single-sample formulas [12, 13, 17, 20, 22, 26] and at five sampling time points (n = 96 children). The y-axis shows the difference between the single-point GFR estimate and a reference GFR based on seven sampling time points. The x-axis shows the reference GFR. Each point corresponds to a combination of patient, determination method and sample time. The solid horizontal line is the bias, i.e. the mean difference between the single-point GFR and the reference GFR. The dashed lines are limits of agreement, i.e. bias ± two standard deviations of the differences. Large determination errors, i.e. errors outside the displayed range, are indicated by arrows. The figure can be used to examine patterns in how the estimation errors of the different estimation methods vary with GFR for each method and sampling time

for each method (Fig. 3). We also present the corresponding numerical estimates for the time points recommended in the original publications (Table 3) and for various subgroups (Table 5) according to age (< 10 years and ≥ 10 years), BSA group (< 1.0 m^2 and < 1.45 m^2) and stage of CKD (< 30 mL/min/1.73 m^2, 30 to < 60 mL/min/1.73 m^2, 60 to < 90 mL/min/1.73m^2, ≥ 90 mL/min/1.73m^2).

To further quantify the performance of the GFR1p-methods, we calculated the number of GFR values that were within 5%, 10%, 15% or 20% of the reference method for each formula, labeled as P5, P10, P15 and P20, respectively, along with confidence intervals based on the recommended Wilson method [27] (Tables 3, 4, and 5; Fig. 4). Differences between methods and between time points for these 'P*x*' values (*x* = 5, 10, 15 or 20) were evaluated using the McNemar test with mid-*P* correction.

For comparison, subanalyses for data on the best available two-point methodology (GFR2p) (Jødal and Brøchner-Mortensen [23, 28, 29]) at 2 and 5 h (i.e. GFR2p-JBM) are also included in Tables 3, 4, and 5.

We used R version 3.4.0 for Windows for all statistical analyses and figures [30]. Statistical significance is defined as *P* values ≤ 0.05, using two-sided tests, not adjusted for multiple comparisons.

Results

The performances of six different formulas for GFR1p determination [12, 13, 20, 22, 26] compared to the reference method are shown in Tables 3, 4, and 5 and Figs. 1, 2, 3, and 4. The results of different time points of blood sampling in Table 3 are limited to the recommended time points given in the respective original publications. The formula of Fleming with sampling at 3 h ($GFR1p_{3h}$-Fleming) showed the best performance, with 82% of the GFR values falling within 10% of the reference method (P10). For the samplings at 2, 3, and 3.5 hours, the results with the Fleming formula were also significantly better than all the other tested formulas for P10 (Table 3). A comparison between the performances of all tested

Table 3 Effect of different formulas at their recommended time points

Formula	Mean bias (GFR1p − GFR7p)				Proportion of measures within *x*% of reference method (95% CI)[a]								*P* value[a]
	Time (h)	*r*	Bias	Limits of agreement	P5		P10		P15		P20		
					% of measures	95% CI	% of measures	95% CI	% of measures	95% CI	% of measures	95% CI	
GFR1p-Fleming	2	0.99	− 1.8	− 12.3 to 8.8	54	44–64	79	70–86	86	78–92	90	82–94	0.42
	3	0.98	− 1.5	− 13.5 to 10.5	56	46–66	82	73–89	89	81–93	91	83–95	N/A
	3.5	0.98	0.1	− 12.4 to 12.5	44	35–54	81	72–88	87	79–93	89	82–94	0.51
	4	0.98	1.5	− 12.4 to 15.4	41	31–51	75	65–83	90	82–94	92	84–96	0.10
GFR1 p-Ham&Piepz	2	0.98	4.1	− 11.0 to 19.2	42	32–52	64	54–72	73	63–81	76	67–83	< 0.001
GFR1 p-Groth&Aasted	2	0.98	3.6	− 9.2 to 16.3	35	27–45	65	55–73	75	65–83	81	72–88	< 0.001
GFR1p-Stake	3	0.97	5.8	− 16.2 to 27.8	33	25–3	66	56–74	78	69–85	82	73–89)	0.002
GFR1p-Jacobsson	2	0.97	− 1.6	− 26.0 to 22.8	32	24–42	58	48–68	69	59–77	78	69–85	< .001
	3	0.98	1.0	− 17.7 to 19.7	34	26–44	58	48–68	74	64–82	81	72–88	< .001
	3.5	0.98	2.2	− 14.6 to 19.0	27	19–37	64	54–73	75	65–82	82	73–89	< .001
	4	0.98	2.6	− 14.6 to 19.8	32	24–42	65	55–73	76	67–83	88	79–93	< .001
	5	0.98	1.9	− 12.5 to 16.3	43	33–53	72	62–80	85	77–91	91	83–95	0.04
GFR1 p-Jacobsson-mod.	2	0.97	− 0.5	− 24.3 to 23.2	33	25–43	61	51–71	70	60–78	78	69–85	< 0.001
	3	0.98	0.2	− 16.7 to 17.1	35	27–45	69	59–77	75	65–83	84	76–90	0.003
	3.5	0.98	0.8	− 14.1 to 15.7	41	32–51	65	55–74	80	71–87	88	80–93	< 0.001
	4	0.98	0.8	− 14.5 to 16.1	42	32–52	71	61–79	85	77–91	91	83–95	0.02
	5	0.98	− 0.4	− 13.8 to 13.0	50	40–60	74	64–82	90	82–94	94	87–97	0.08
GFR2p-JBM	2 and 5	0.99	− 1.7	− 9.4 to 6.1	73	63–81	97	97–99	100	96–100	100	96–100	< 0.001

Evaluation of optimal time for blood sampling was investigated using five different sampling time points after iohexol injection, namely 2, 3, 3.5, 4 and 5 h. glomerular filtration rate (GFR) (mL/min/1.73m^2) was estimated by one-point methods at time points recommended in the original publications ($n = 96$ for all time points except for 3.5 h, where $n = 95$) and by the reference method (GFR7p). Mean bias, 95% limits of agreement and correlation (*r*) with reference method are shown calculated. For comparison the two-point method of Jødal Brøchner Mortensen (GFR2p-JBM) was added

N/A, Not applicable

[a] Estimated accuracy is shown as P5, P10, P15 and P20, namely, the percentage of patients within ± 5, 10, 15 and 20% of the reference method, respectively, along with 95% confidence intervals (CI)

[b] Comparison with Fleming P10 at 3 h

GFR1p formulas and slope-intercept methodology revealed that the GFR2p-JBM methodology was significantly better than all GFR1p formulas in the entire cohort of children with CKD 1–5 (Tables 3, 4).

With respect to the effect of sampling time on the performance, the Fleming formula gave results for sampling at 2, 3.5 and 4 h (i.e. time frame recommended by Fleming) which were not significantly different from the results at 3 h (Table 3; Figs. 2, 4). However, when blood was drawn at 5 h (i.e. outside the time frame recommended by Fleming), this formula showed a significantly lower performance, with a P10 of 55% ($P < 0.01$) (Table 4; Figs. 2, 4). When sampling at 4 h, the Fleming and Jacobsson-mod. formulas performed significantly better (P10 was 75 and 71%, respectively) than the formulas of Ham & Piepsz, Groth & Aasted and Stake (Figs. 1, 4). For blood sampling at 5 h, the two Jacobsson formulas had the best performance, with a P10 of 74 and 72%, respectively, which was significantly better ($P < 0.01$) than the performance of all other tested formulas at 5 h (Figs. 1, 4; Tables 3, 4).

All GFR1p formulas studied showed large bias when blood was drawn outside the time frames originally described for the respective formulas (Fig. 1). Nevertheless, the formulas of Fleming and Jacobsson gave relatively good GFR1p determinations for the entire 2- to 5-h range (Figs. 2, 3). The GFR1p formulas also showed non-constant bias (and, to a lesser degree, variance) over the GFR range (Fig. 3), especially outside their recommended time frames. However, the Fleming and Jacobsson formulas at their best-performing time points (3 and 5 h, respectively) had an approximately constant bias and variance as a function of GFR (Fig. 3).

Subgroup analysis revealed that in children with CKD 1–3, $GFR1p_{3h}$-Fleming scored very well, with a P10 of 92%, which was significantly better than those of all other GFR1p formulas investigated, and not significantly different from the P10 of GFR2p-JBM, which was 96% ($P = 0.29$) (Table 4). In those patients with a GFR < 30 mL/min/1.73 m^2, much lower performances were found for all GFR1p formulas. In this subgroup, the highest P10 was 67% when the GFR1p-Fleming formula was used with blood sampling at 5 h ($GFR1p_{5h}$-Fleming). However, the performance of $GFR1p_{5h}$-Fleming was not significantly better than that of the $GFR1p_{5h}$-Jacobsson which had a P10 of 44% ($P = 0.23$). In contrast, the GFR2p-JBM scored 100% for P10 ($P < 0.0001$) in the patients with GFR < 30 mL/min/1.73 m^2 (Tables 4, 5).

Age and BSA did not seem to influence the scores of GFR1p-Fleming, whereas GFR1p-Ham&Piepsz, GFR1p-Groth&Aasted and GFR1p-Jacobsson all had better scores in the smaller children (Table 5).

Discussion

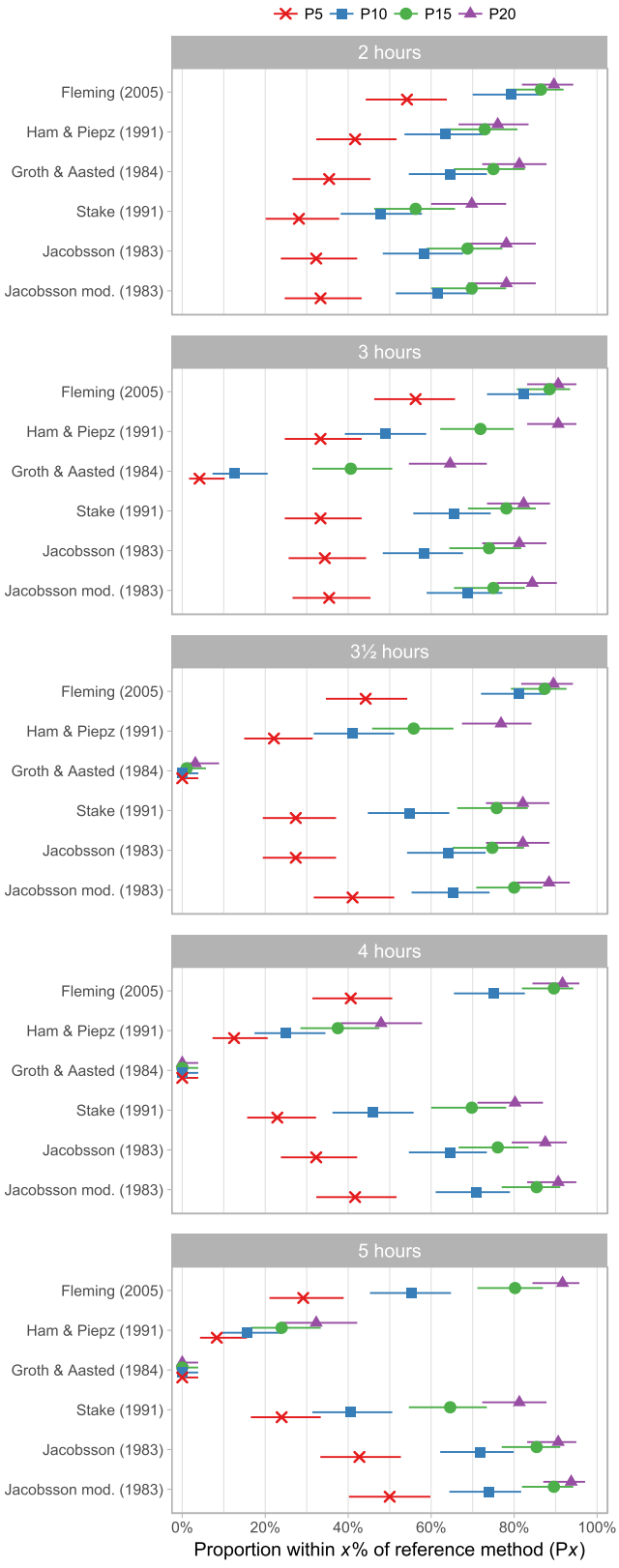
The results of our iohexol plasma clearance study of a cohort of 96 children with CKD 1–5 shows that GFR1p measurements reached acceptable precision in patients with CKD 1–3. The best formula for single-point measured GFR in children was the GFR1p-Fleming, which showed a significantly better performance than the GFR1p-Ham&Piepsz, GFR1p-Groth&Aasted and GFR1p-Stake formulas [13, 20–22, 26] at all tested time points (Table 3; Fig. 1). GFR1p-Fleming was also significantly better than GFR1p-Jacobsson [12] when blood samplings were done after 2, 3 and 3.5 hours, whereas no significant difference was found between these formulas at 4 h (Table 4; Fig. 4). For blood sampling at 5 h, GFR1p-Jacobsson was significantly better than all other single-point formulas (Table 4; Fig. 1). Comparison with the two-point methodology showed that GFR2p-JBM, with a P10 of 97%, was significantly better ($P < 0.001$) than all single-point methods investigated in this study when all CKD stages were included in the analysis. However, an interesting finding was evident from the subgroup analysis, which showed no significant difference between the best single-point method, $GFR1p_{3h}$-Fleming, and GFR2p-JBM in children with CKD 1–3 (Tables 4, 5). The scores for all single-point formulas were low in children with CKD 4–5, with the best P10 of 67% compared to 100% with GFR2p-JBM ($P < 0.001$) (Tables 4, 5). McMeekin and colleagues recently compared the $GFR1p_{3h}$-Fleming with a multi-point reference method in a combined cohort of children and adults, with a total of 411 tests (247 pediatric and 164 adult tests) [19]. These authors found that 92.7% of measures [95% confidence interval (CI) 90–95%] were within 20% of the reference. This is in accordance with the results from our cohort showing a P20 of 91% (95% CI 83–95%). Our results also support the discrepancy between formulas reported by McMeekin and colleagues who found lower P20 for $GFR1p_{2h}$-Ham&Piepsz, $GFR1p_{2h}$-Groth&Aasted, $GFR1p_{3h}$-Stake and $GFR1p_{4h}$-Jacobsson compared to $GFR1p_{3h}$-Fleming in their cohort [19].

Our study clearly demonstrates the importance of using the optimal blood sampling time points adapted to each formula. This is especially evident in the methods described by Ham&Piepsz and Groth&Aasted [11, 26], where the performance scores of all time points outside the recommended are low (Figs. 2, 3; Table 4). Furthermore, variable performance across GFR levels has to be taken into account since both these formulas scored fairly well in children with CKD 1–2, whereas the scores were unacceptably low in children with CKD 3–5 (Table 4). As the GFR1p-Ham&Piepsz formula has been a recommended single-point method in guidelines [11] and was developed from a high number ($n = 657$) of GFR measurements [26], a higher general score should be expected. Interestingly, in our study, the P10 of GFR1p-Ham&Piepsz was very high in the group of children with CKD 2, with a

Table 4 Percentage of GFR1p measures within 10% of reference stratified by GFR-levels

Formula	Time (h)	Proportion of measures within 10% (P10) of reference method (95% CI)											
		CKD 1		CKD 2		CDK 3		CKD 1–3		CKD 4–5		CKD 1–5	
		% of measures	95% CI	% of measures	95% CI	% of measures	95% CI	% of measures	95% CI	% of measures	95% CI	% of measures	95% CI
GFR1p-Fleming	2	97	83– 99	88	71–96	78	58–90	88	80– 94	39	20–61	79	70–86
	3	90	74–96	96	81–99	91	73–98	92	84–96	39	20–61	82	73–89
	3.5	83	65–92	96	81–99	91	73–98	90	81–95	41	22–64	81	72–88
	4	79	62–90	85	66–94	78	58–90	81	71–88	50	29–71	75	65–83
	5	69	51–83	46	29–65	39	22–59	53	42 –63	67	44–84	55	45–65
GFR1p-Ham&Piepz	2	79	62–90	96	81–99	57	37–74	78	68–86	0	0–18	64	54–72
	3	62	44–77	54	35–71	39	22–59	53	42–63	33	16–56	49	39–59
	3.5	66	47–80	35	19–54	13	5–32	40	30–51	47	26–69	41	32–51
	4	41	26–59	35	19–54	0	0–14	27	18–38	17	6–39	25	17–35
	5	34	20–53	19	9–38	0	0–14	19	12–29	0	0–18	16	10–24
GFR1p-Groth&Aasted	2	79	62–90	81	62–91	57	37–74	73	62–82	28	12–51	65	55–73
	3	14	5–31	12	4–29	4	1–21	10	5–19	22	9–45	12	7–21
	3.5	0	0–2	0	0–13	0	0–14	0	0–5	0	0–18	0	0–4
	4	0	0–12	0	0–13	0	0–14	0	0–5	0	0–18	0	0–4
	5	0	0–12	0	0–13	0	0–14	0	0–5	0	0–18	0	0–4
GFR1p-Stake	2	48	31–66	81	62–91	35	19–55	55	44–66	17	6–39	48	38–58
	3	45	28–62	85	66–94	91	73–98	72	61–81	39	20–61	66	56–74
	3.5	45	28–62	77	58–89	65	45–81	62	50–72	24	10–47	55	45–64
	4	55	38–72	58	39–74	52	33–71	55	44–66	6	1–26	46	36–56
	5	55	38–72	62	43–78	22	10–42	47	37–58	11	3–33	41	31–51
GFR1p-Jacobsson	2	66	47–80	77	58–89	48	29–67	64	53–74	33	16–56	58	48–68
	3	62	44–77	73	54–86	61	41–78	65	54–75	28	12–51	58	48–68
	3.5	76	58–88	77	58–89	61	41–78	72	61–81	29	13–53	64	54–73
	4	79	62–90	65	46–81	70	49–84	72	61–81	33	16–56	65	55–73
	5	86	69–95	77	58–89	70	49–84	78	68–86	44	25–66	72	62–80
GFR1p-Jacobsson-mod	2	69	51–83	81	62–91	52	33–71	68	57–77	33	16–56	61	51–71
	3	83	65–92	85	66–94	65	45–81	78	68–86	28	12–51	69	59–77
	3.5	79	62–90	77	58–89	61	41–78	73	62–82	29	13–53	65	55–74
	4	79	62–90	81	62–91	83	63–93	81	71–88	28	12–51	71	61–79
	5	72	54–85	96	81–99	74	54–87	81	71–88	44	25–66	74	64–82
GFR2p-Jødal-Brøchner-Mortensen	2 and 5	90	74–96	100	87–100	100	86–100	96	89–99	100	82–100	97	91–99

CKD Chronic kidney disease; *GFR* glomerular filtration rate; *CI* confidence interval



◀ **Fig. 4** Percentage plot showing the determination accuracy of six single-sample determination methods [12, 13, 17, 20, 22, 26] at five sampling time points (n = 96 patients/children). Each symbol, labeled Px (P5, P10, P15 and P20), shows the calculated proportion of single-sample glomerular filtration rate (GFR) within x% of the reference method. The horizontal lines show the corresponding 95% confidence intervals

P10 of 96%, but only 57% in those with CKD 3, and no patient was within 10% of the reference with a GFR of < 30 mL/min/1.73 m^2 (Table 4). A plausible explanation could be that the reference method used in the Ham & Piepsz study was not a multipoint-method, and the development of the formula was based on GFR measurements mainly in the normal range [26].

The Groth & Aasted formula was developed in a cohort with a broader distribution of GFR [16], which could explain why the single-point scores with this formula were more evenly distributed across the different CKD groups in our study (Table 4). The cohort of Groth & Aasted was, however, considerably smaller, and their five-point reference GFR had the last time point set early (2 h) [20], which probably explains the low scores in general for GFR1p-Groth&Aasted. The fairly good scores for GFR1p-Stake in children with CKD 2–3 at 3 h in contrast to the low scores for those with CKD 1 and CKD 4–5 (Fig. 2; Table 4) are probably due to the fact that the Stake-formula was developed in a cohort of 100 children mainly with CKD 2–3 and with a two-point$_{3h,4h}$ iohexol-GFR as reference method [13].

Both the Fleming and the Jacobsson formulas have distribution volume and time-point adaption included in the respective formulas. This gives a lower vulnerability in terms of time-point variability for blood sampling, as long as the true sampling time is used in the formula. The GFR1p$_{3h}$-Fleming scored significantly better (P < 0.05) than all other formulas on their recommended time points, except for GFR1p$_{5h}$-Jacobsson-mod. (P = 0.08) (Table 3) in the cohort as a whole, and it was not significantly different from GFR2p-JBM in the subgroups CKD 1, CKD 2 and CKD 3. The subgroup analysis also showed that age and body size did not significantly influence the scores of GFR1p-Fleming. Importantly, when a child is expected to have CKD 4–5, our study shows that a single-point methodology with blood sampling up to 5 h is not recommended and that at least two blood samples should be collected (Table 5). Calculation of the eGFR [1], despite its limitations, can be helpful in making the decision to take more than one blood-sample or not, i.e. with 30 ml/min/1.73 m^2 as the cutoff value.

Iohexol has been increasingly used as a marker for GFR measurements in recent decades. It is a nonradioactive substance, safe, inexpensive, has low inter-laboratory variation and is stable and easy to use [4, 31, 32]. Although the GFR1p-Fleming formula was originally developed using a radioactive marker in adults and children [21],

Table 5 Subgroup analysis

Patient group	Formula	Time (h)	*n*	*r*	Mean bias (GFR1p − GFR7p)		Percentage of measures within *x*% of reference method (P*x*)[a]				*P* value[b]
					Bias	Limits of agreement	P5	P10	P15	P20	
Age < 10 years	GFR1p-Fleming	3	52	0.99	− 0.5	− 9.7 to 8.8	65	83	88	92	N/A
	GFR1p-Ham&Piepz	2	52	0.97	2.3	− 13.3 to 17.8	48	73	83	87	0.15
	GFR1p-Groth&Aasted	2	52	0.99	2.0	− 10.7 to 14.7	40	69	81	87	0.02
	GFR1p-Stake	3	52	0.97	7.3	− 14.1 to 28.6	29	62	75	79	0.008
	GFR1p-Jacobsson	5	52	0.98	2.0	− 11.1 to 15.1	52	79	90	92	0.55
	GFR1p-Jacobsson-mod.	5	52	0.98	0.1	− 12.8 to 13.0	58	81	90	94	0.77
	GFR2p-JBM	2 and 5	52	0.99	− 2.0	− 10.2 to 6.3	60	96	100	100	0.02
Age ≥ 10 years	GFR1p-Fleming	3	44	0.98	− 2.7	− 17.1 to 11.6	45	82	89	89	N/A
	GFR1p-Ham&Piepz	2	44	0.99	6.3	− 7.1 to 19.6	34	52	61	64	0.001
	GFR1p-Groth&Aasted	2	44	0.99	5.4	− 6.6 to 17.3	30	59	68	75	0.02
	GFR1p-Stake	3	44	0.98	4.1	− 18.5 to 26.6	39	70	82	86	0.11
	GFR1p-Jacobsson	5	44	0.98	1.8	− 14.1 to 17.7	32	64	80	89	0.04
	GFR1p-Jacobsson-mod.	5	44	0.98	− 1.0	− 15.0 to 13.0	41	66	89	93	0.02
	GFR2p-JBM	2 and 5	44	1.0	− 1.4	− 0.5 to 5.8	89	98	100	100	0.02
BSA < 1.0 m^2	GFR1p-Fleming	3	47	0.99	0.0	− 9.5 to 9.6	68	81	87	91	N/A
	GFR1p-Ham&Piepz	2	47	0.97	2.9	− 13.0 to 18.7	43	68	79	83	0.09
	GFR1p-Groth and Aasted	2	47	0.99	2.1	− 11.3 to 15.5	45	64	79	85	0.01
	GFR1p-Stake	3	47	0.98	7.6	− 12.1 to 27.3	23	57	72	77	0.008
	GFR1p-Jacobsson	5	47	0.97	1.5	− 12.4 to 15.4	57	83	89	89	0.73
	GFR1p-Jacobsson-mod.	5	47	0.98	− 0.2	− 13.6 to 13.3	60	83	91	94	0.75
	GFR2p-JBM	2 and 5	47	0.99	− 1.9	− 10.1 to 6.3	60	96	100	100	0.02
BSA < 1.45 m^2	GFR1p-Fleming	3	77	0.99	− 0.4	− 9.6 to 8.9	60	84	90	92	N/A
	GFR1p-Ham&Piepz	2	77	0.97	3.4	− 11.7 to 18.4	42	65	77	81	< 0.001
	GFR1p-Groth&Aasted	2	77	0.98	3.1	− 9.5 to 15.6	39	68	78	86	0.001
	GFR1p-Stake	3	77	0.97	7.2	− 15.6 to 30.0	30	64	75	81	< 0.001
	GFR1p-Jacobsson	5	77	0.98	2.6	− 10.8 to 15.9	48	75	87	92	0.10
	GFR1p-Jacobsson-mod.	5	77	0.98	0.6	− 11.5 to 12.6	55	77	91	96	0.17
	GFR2p-JBM	2 and 5	77	0.99	− 1.7	− 9.7 to 6.3	69	96	100	100	0.01
CKD stage 1	GFR1p-Fleming	3	29	0.83	− 4.1	− 21.9 to 13.8	59	90	93	97	N/A
	GFR1p-Ham&Piepz	2	29	0.93	2.6	− 12.6 to 17.8	59	79	97	100	0.34
	GFR1p-Groth&Aasted	2	29	0.91	4.3	− 8.7 to 17.3	48	79	97	100	0.34
	GFR1p-Stake	3	29	0.82	14.9	− 15.4 to 45.1	21	45	66	72	0.001
	GFR1p-Jacobsson	5	29	0.82	2.6	− 17.0 to 22.3	45	86	86	93	0.69
	GFR1p-Jacobsson-mod.	5	29	0.81	− 2.9	− 21.5 to 15.7	55	72	97	97	0.03
	GFR2p-JBM	2 and 5	29	0.92	− 1.7	− 15.3 to 9.1	59	90	100	100	0.81
CKD stage 2	GFR1p-Fleming	3	26	0.93	0.4	− 6.8 to 7.7	73	96	100	100	N/A
	GFR1p-Ham&Piepz	2	26	0.91	− 0.7	− 9.5 to 8.0	62	96	96	96	0.75
	GFR1p-Groth&Aasted	2	26	0.91	5.5	− 3.5 to 14.5	42	81	92	96	0.06
	GFR1p-Stake	3	26	0.89	3.9	− 7.1 to 15.0	38	85	92	96	0.13
	GFR1p-Jacobsson	5	26	0.93	4.6	− 4.3 to 13.5	42	77	96	100	0.03
	GFR1p-Jacobsson-mod.	5	26	0.94	2.1	− 5.5 to 9.7	58	96	96	100	0.75
	GFR2p-JBM	2 and 5	26	0.96	− 2.0	− 7.6 to 3.7	69	100	100	100	0.50
CKD stage 3	GFR1p-Fleming	3	23	0.97	0.0	− 4.8 to 4.9	61	91	100	100	N/A
	GFR1p-Ham&Piepz	2	23	0.77	3.7	− 6.2 to 13.6	30	57	70	78	0.01
	GFR1p-Groth&Aasted	2	23	0.78	1.9	− 9.5 to 13.3	35	57	65	74	0.004
	GFR1p-Stake	3	23	0.96	0.6	− 5.0 to 6.2	48	91	100	100	1.00

Table 5 (continued)

Patient group	Formula	Time (h)	*n*	*r*	Mean bias (GFR1p − GFR7p)		Percentage of measures within *x*% of reference method (P*x*)[a]				*P* value[b]
					Bias	Limits of agreement	P5	P10	P15	P20	
	GFR1p-Jacobsson	5	23	0.90	2.1	− 7.0 to 11.3	43	70	96	96	0.03
	GFR1p-Jacobsson-mod.	5	23	0.90	2.0	− 6.3 to 10.3	48	74	96	96	0.06
	GFR2p-JBM	2 and 5	23	0.98	− 0.7	− 3.7 to 2.4	87	100	100	100	0.25
CKD stages 4–5	GFR1p-Fleming	3	18	0.83	− 2.0	− 11.7 to 7.6	22	39	50	56	N/A
	GFR1p-Ham&Piepz	2	18	0.75	14.0	5.3–22.8	0	0	6	6	0.008
	GFR1p-Groth&Aasted	2	18	0.77	1.7	−15.3 to 18.7	6	28	28	39	0.45
	GFR1p-Stake	3	18	0.75	0.6	− 11.4 to 12.6	28	39	50	56	0.84
	GFR1p-Jacobsson	5	18	0.73	− 3.4	− 14.7 to 7.8	39	44	56	67	0.75
	GFR1p-Jacobsson-mod.	5	18	0.75	− 2.9	− 13.9 to 8.0	33	44	61	78	0.75
	GFR2p-JBM	2 and 5h	18	1.00	− 0.3	1.2–1.0	83	100	100	100	< 0.001

Evaluation of bias and accuracy for blood sampling for various patient groups and time points after iohexol injection. GFR (mL/min/1.73m ^{2}) was determined by one-point methods and by the reference method (GFR7p). Mean bias, 95% limits of agreement and correlation (*r*) with reference method is shown calculated

GFR glomerular filtration rate; *CKD* chronic kidney disease; *BSA* body surface area

[a] Estimated accuracy is shown as P5, P10, P15 and P20, the percentage of patients within ±5%, 10%, 15% and 20% of the reference. For comparison, the two-point method of Jødal Brøchner Mortensen (GFR2p-JBM) was added

[b] Comparison with Fleming P10 at 3 h

our iohexol study has shown that this formula gives an accurate mGFR determination in children with CKD 1–3.These findings are of great clinical value. For the follow-up of children with cancer treated with nephrotoxic substances, as well as for children with renal and urologic diseases and mild and moderate kidney dysfunction, it is clearly beneficial to reduce the number of blood draws from two to three to a single sample. The risk of outliers is an issue in all tests, and in a single-point procedure it is necessary to redo the test if a result is surprising, whereas using a multi-point GFR procedure it is possible to remove the outlier based on examination of the elimination curve.

A limitation of this study is the lack of inulin-based gold standard analyses, but the continuous intravenous infusion and timed urine collections necessary in inulin clearance is cumbersome, and inulin is nowadays difficult to obtain. In addition, the number of patients in our study was limited to 96 children, which reduces the power of subgroup analysis. The last time point of iohexol measurement at 5 h may limit the value of the study in patients with severely reduced kidney function. However, the validity of our study is strengthened by our comparisons of a high number of blood samples at different time points and with multiple formulas.

Conclusion

Determination of GFR in children at all ages with CKD stage 1–3 based on iohexol plasma clearance and single-point sampling at 3 h analyzed with the Fleming formula achieved the same level of performance as the two-point method. All other tested single-point formulas had a considerably lower performance. When the GFR is lower than 30 mL/min/1.73 m^2, a procedure with at least two blood-samples is recommended for mGFR.

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Compliance with ethical standards

Financial disclosure The authors have no financial relationships relevant to this article to disclose.

Approval The study was approved by the Regional Ethics Committee of Western Norway and an informed consent form was signed by all patients and/or their designees. The study was performed in accordance with the Declaration of Helsinki.

Conflict of interests The authors declare that they have no conflict of interest.

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III

IV