Markers of nephropathy in young Fabry disease patients; role of kidney biopsies and functional measurements

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Contents

Scientific environment

This thesis emerged from the Renal Research Group, Institute of Medicine, Faculty of Medicine and Dentistry, University of Bergen. Main supervisor was professor Einar Svarstad and co-supervisors were professor Bjarne M. Iversen and professor Bjørn Egil Vikse.

The collaboration with the Institute of Gade and the Institute of Clinical Medicine, University of Bergen and the Department of Paediatrics, Department of Medicine, Department of Pathology, Laboratory of Clinical Biochemistry and Centre for Medical Genetics and Molecular Medicine, Haukeland University Hospital was of great importance.

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Abstract

Backgound: Fabry disease is an X-linked disease affecting glycosphingolipid metabolism due to deficiency of the lysosomal enzyme α-galactosidase. The natural course of the disease is related to progressive accumulation of globotriaocylceramide (GL3) in various cell types and causes premature death from stroke, renal failure or cardiac disease. Modern therapies in many common kidney diseases, as well as in a rapidly increasing number of rare diseases, include potentially toxic and expensive drug interventions. As a consequence, it becomes important to use accurate methods for investigation of kidney disease.

Aims: The aims of this thesis were to evaluate early Fabry nephropathy by means of morphologic and exact functional methods in children and young adults and to validate the safety of kidney biopsies in general.

Materials and methods: Renal biopsies from thirteen young patients with Fabry disease (mean age 17.3 ± 7.5, range 7-33 years) were examined before and after long-term (5 years) enzyme replacement therapy (ERT). Comparisons of different formulas for estimation of GFR (eGFR) and measured GFR (mGFR) were performed in 42 children with Fabry disease (mean age 12.3 ± 3.6, range 2-17 years). Safety evaluation of kidney biopsies was done in 715 paediatric (mean age 12.0 ± 4.9, range 0.04-17.9 years) and 8573 adult (mean age 50.6 ± 17.7, range 18.0-94.4) cases registered from 1988 to 2010 in The Norwegian Kidney Biopsy Registry.

Results: The baseline biopsies of Fabry disease patients showed disease specific potentially progressive morphologic changes in glomerular, tubulointerstitial and vascular compartments. In the follow-up biopsies complete clearance of glomerular endothelial and mesangial GL3 deposits was found in all patients and linear regression analysis showed a significant correlation between podocyte GL3-clearance and cumulative agalsidase dose (r=0.804, p=0.002). Simultaneous eGFR and mGFR analysis showed that the widely used original Schwartz formula overestimated mGFR

by an average of 50.6 ml/min/1.73 m^2 in our cohort with normal mGFR. Nationwide renal biopsy registry data showed that major complications after kidney biopsy were rare both in children and adults (blood transfusion 0.9 % and surgery/angiographic embolization 0.2 %), the most important risk factors for major complications were lower GFR and smaller centre size.

Conclusions and consequences: Long-term ERT clears glomerular endothelial and mesangial GL3 deposits across all dosing regimens of agalsidase. The reduction of Fabry disease specific damage in podocytes is dose-dependent, suggesting that podocyte damage may be a promising biomarker in Fabry nephropathy. Kidney biopsy is a low risk procedure and we recommend a baseline biopsy in the assessment of early renal damage in Fabry disease. A follow-up biopsy after 5 years ERT is valuable in the evaluation of progression and reversibility of kidney damage. Several GFR formulas show low accuracy in the normal GFR-range. The new abbreviated Schwartz formula (2009) has the better performance and is recommended in the routine follow-up of children with Fabry disease. Additional mGFR is recommended when exact measurements are needed; e.g. when ERT is initiated.

Abbreviations

ACR – albumin-creatinine-ratio

CD – critical difference [CD (95% CI) = 2.77 x $\sqrt{(CVi^2 + CVa^2)}$]

CKD – chronic kidney disease

CKD-EPI – the Chronic Kidney Disease Epidemiology Collaboration equation

Cr-EDTA – Chrome-Ethylene Dinitrilo Tetra Acid

CI – confidence interval

CVa – analytical imprecision

CVi – intra-individual biological variation

DS3 – Fabry disease severity scoring index eGFR – estimated glomerular filtration rate

EMA – European Medical Assosication

ERT – Enzyme Replacement Therapy

ESRD – end stage renal disease

FDA – Food and drug administration

FIPI – Fabry international prognostic index

FOS – Fabry Outcome Survey

FSGS – focal segmental glomerular sclerosis

GFR – glomerular filtration rate

GLA – The human alfa-galactosidase A gene GL3 – globotriaocylceramide

IDMS – the Isotope Dilution Mass Spectrometry

ISGFN – International Study Group of Fabry Nephropathy

K/DOQI – the Kidney Disease Outcomes Quality Initiative LVM – left ventricular mass

Lyso-GL3 – lyso-globotriaocylceramide

MRI – magnet resonance imaging

MDRD – Modification of Diet in Renal Disease Study equation

mGFR – measured glomerular filtration rate

MSSI – Mainz Severity Scoring Index

NKBR – Norwegian Kidney Biopsy Registry

OR – odds ratio

PIP – pediatric investigation plan

PCR – protein-creatinine-ratio

pGL3 – plasma globotriaocylceramide

RRT – renal replacement therapy

99mTc-DTPA – Technetium-99m-Diethylene Triamine Pentacetic Acid

uGL3 – urine globotriaocylceramide

List of publications

Paper I: Monitoring renal function in children with Fabry disease: comparisons of measured and creatinine-based estimated glomerular filtration rate. Camilla Tøndel, Uma Ramaswami, Kristin Moberg Aakre, Frits Wijburg, Machtelt Bouwman, Einar Svarstad: Nephrol Dial Transplant 2010; 25(5): 1507-1513

Paper II: Renal biopsy findings in children and adolescents with Fabry disease and minimal albuminuria. Camilla Tøndel, Leif Bostad, Asle Hirth and Einar Svarstad: Am J Kidney Dis 2008; 51(5): 767-776

Paper III: Agalsidase benefits renal histology in young Fabry disease patients. Camilla Tøndel, Leif Bostad, Kristin Kampevold Larsen, Asle Hirth, Bjørn Egil Vikse, Gunnar Houge and Einar Svarstad: J Am Soc Nephrol, 2013; 24(1):137-148

Paper IV: Safety and complications of percutaneous kidney biopsies in 715 children and 8573 adults in Norway 1988-2010. Camilla Tøndel, Bjørn Egil Vikse, Leif Bostad, Einar Svarstad: Clin J Am Soc Nephrol 2012; 7(10):1591-1597

1. Introduction

1.1. Fabry disease

1.1.1. Definition and history

Fabry disease is an X-linked lysosomal storage disease due to absent or deficient activity of alfa-galactosidase A resulting in progressive accumulation of the neutral glycosphingolipid globotriaocylceramid (GL3) in many cell types, including endothelial cells in glomeruli and capillaries, renal cells (podocytes, mesangial and tubular epithelial cells), cardiomyocytes and nerve cells (1). Renal biopsies in Fabry patients typically show GL3 deposits in various kidney cells developing from early age (2-5). Importantly, the disease is a multisystemic disorder, and the natural course of the classic phenotype leads to premature death from renal, cardiac or cerebral disease, usually at the age of 40-50 years (6, 7). The overall life expectancy is reduced by about 25 years for males and about 10 years for females (8). However, over the later years it has become increasingly clear that Fabry disease is a heterogenous disease, even within the same family, with a wide spectrum of manifestations in both genders including seemingly asymptomatic patients (9, 10). The disease was first described in 1898 by two independently working dermatologists, Johannes Fabry in Bonn and William Andersson in London, describing the classical angiokeratomas in the skin (11, 12). Christian de Duve first described the lysosome in the 1950s, and was later awarded the Nobel Prize in Physiology for this achievement, and proposed the concept of enzyme replacement therapy (ERT) for lysosomal storage diseases (13, 14). In 1965, observations by electron microscopy of "extremely overcrowded lysosomes" in perivascular cells, endothelial cells, fibrocytes and smooth muscle cells in skin biopsies were reported as likely of genetic origin, and lack of lysosomal enzymes were postulated (15). Brady et al. identified the deficiency of the agalsidase enzyme in 1967 (16).

The human alfa-galactosidase A gene *(GLA)*, located on the X-chromosome (Xq22.1) was structurally described in the 1980s (17). About 500 disease-associated *GLA* mutations have been reported in The Human Gene Mutation Database (18, 19). However, it is increasingly recognized that not all the mutations are pathogenic (10). The mechanisms for this variability are largely unknown, but random X-inactivation may explain why some women are asymptomatic whereas others may have similar progressive disease phenotypes as hemizygous males (20-22).

1.1.2. Incidence

The incidence of the disease is ranging from 0.015 to 80 per 100 000, the highest numbers found by neonatal screening in Italy and in Taiwan (23-30). In the latter population the majority (86 %) had a mutation found in later-onset cardiac phenotype patients (29, 31). In Western Norway the prevalence is estimated to 1:17 000 (32).

1.1.3. Symptoms and organ involvement

Initial symptoms in Fabry disease are normally seen in the first decade of life, earlier in boys than girls (33). The first symptoms are usually acroparesthesias which are seen in more than half of classically affected boys and girls (burning pain in hands and feet), abdominal complaints (diarrhoea, pain, bloating), and hypohidrosis (reduced sweating) (33). With increasing age the gradual development of renal, cardiac and cerebrovascular disease is observed, and has been reported as early as in the teens (26, 34, 35). Hearing disturbances are common and sudden deafness may occur even in childhood (26, 36). Most patients have symptoms and progressive disease in multiple organ systems, and different disease severity scoring systems have been developed to estimate the total disease burden in individual patients; e.g. Mainz severity score index (MSSI), Fabry disease severity scoring index (DS3) and the Fabry international prognostic index (FIPI) (37-39).

1.1.4. Enzyme replacement therapy

Before the era of enzyme replacement therapy (ERT) in Fabry disease, the treatment was essentially symptomatic such as alleviating the neuropathic pain, treating cardiac and renal disease or palliative, like dialysis or renal transplantation, not interfering with the other multi-organ manifestations. Enzyme replacement therapy was for the first time described by Brady et al. in 1973 in two Fabry patients using ceramidtrihexosidase purified from human placental tissue (40). Alfa galactosidase A from spleen and plasma was a few years later given to two Fabry patients by Desnick et al (41). Two brands of recombinant alfagalactosidase preparations were developed; agalsidase alfa (Replagal®, TKT 5S, later Shire) from transfected human fibroblasts and agalsidase beta (Fabrazyme®, Genzyme) produced in a widely used hamster ovarian cell line. Early phase clinical trials performed in the late 1990s showed effect on tissue in humans: In patients treated with agalsidase alfa reduction of GL3 deposits was shown in liver biopsy as well as in sheds of tubular epithelial cells in the urine sediment (42). Biopsies from Fabry disease patients treated with agalsidase beta showed dose-dependent clearance of GL3 in liver, kidney and endomyocardium (43).

ERT was licenced in Europe in 2001 for the two different preparations, agalsidase alfa and agalsidase beta (44, 45). Both products are licensed for intravenous infusion every other week (eow), agalsidase alfa in the dosis 0.2 mg/kg/eow and agalsidase beta in the dosis 1.0 mg/kg/eow and both treatments may be given as home infusion (46). In the United States, only one preparation was licensed in line with their regulations for stimulating research on orphan diseases (44, 47). The Food and Drug Administration's choice of agalsidase beta in 2003 as the licensed preparation was primarily due to evidence of short-term clearance of GL3 from glomerular endothelial and mesangial cells in phase III trials (44, 48). Studies on the effect of ERT on the kidney after six months of ERT (44) show clearance of GL3 in endothelium cells and mesangium cells with agalsidase beta 1.0 mg/kg/eow, and decrease of mesangial widening with agalsidase alfa 0.2 mg/kg/eow (45). Partial GL3-clearance of the podocytes (terminally differentiated cells with slow turnover

rates) has been shown in long-term (54 months) treatment with agalsidase beta 1.0 mg/kg/eow (49). There are no published studies that include evaluation of renal biopsies after long-term treatment with agalsidase alfa 0.2 mg/kg/eow. Lubanda et al. performed a renal biopsy study in 21 adult male patients (median age 35.7 years, range 19.9-55.3 years) treated with agalsidase beta 1.0 mg/kg/eow for 6 months with subsequent dosereduction to 0.3 mg/kg/eow for another 18 months (50). They reported persisting complete clearance of GL3 deposits in the endothelium cells in 90 % of the patients treated with reduced dose. The podocytes were not completely cleared in this short-term study, but partial clearance (scored as 0-3) was found in 3 of 19 patients after 6 months of 1.0 mg/kg/eow and in 9 of 17 patients after another 18 months of 0.3 mg/kg/eow compared to the baseline biopsy. Up to now, no clinical trials including renal biopsies in ERT-treated children have been published.

1.1.5. Dosing of agalsidase alfa and agalsidase beta

In vitro studies have shown similar effect of agalsidase alfa and agalsidase beta per mg substance (51, 52). After more than 10 years of ERT an important and still unresolved issue is the question of clinical equipotency of the two available enzymes (53, 54). No sufficiently powered randomized controlled trials have compared the efficacy of the two licensed drugs. A Dutch study was the first randomized controlled clinical trial comparing the two preparations of ERT, equal drug doses were tested, i.e.0.2 mg/kg/eow, and showed no difference between agalsidase alfa and beta after12 months and 24 months in measurements of left ventricular mass (LVM) reduction or clinical adverse events (55). Comparison with the higher dose of agalsidase beta (1.0 mg/kg/eow) showed a greater decline in urinary GL3 and reduction of LVM of the higher dose. Antibodies against alfagalactosidase A were frequent in male patients and may interfere negatively with the effect of ERT given at 0.2 mg/kg/eow, whereas infusion of 1.0 mg/kg/eow seemed more robust regarding neutralizing effects of antibodies (56). Interestingly, a recent study from the same group has demonstrated that antibodies induce a rise in plasma and urine lyso-GL3 (a promising surrogate

biomarker), a finding that may be associated with an increase in cardiac hypertrophy (57). Another study indicates that switch from agalsidase beta 1.0 mg/kg/eow to a lower agalsidase beta dose (0.5 mg/kg/eow) or agalsidase alfa 0.2 mg/kg/eow did not alter the clinical event rate over an observation period of about one year, although an increase in lyso-GL3 was observed. The clinical impact of these observations needs to be evaluated over a longer time-interval (58). There is also an on-going Canadian study comparing the clinical outcomes in patients randomized to either of the two drugs in their licensed doses (59).

The majority of comparisons between the two agalsidase preparations are indirect comparisons of clinical trials performed by the two pharmaceutical companies Shire and Genzyme as well as separate reports from the two observational databases Fabry Outcome Survey (FOS) (supported by Shire) and Fabry Registry (supported by Genzyme). Both drugs have numerous small reports demonstrating beneficial effects on stabilization or attenuation of progressive kidney disease and cardiac disease in patients with early organ affection, i.e. eGFR above 60 ml/min/1.73 m^2 and proteinuria below 0.5 – 1.0 g/day. However, several small studies indicate that agalsidase beta 1.0 mg/kg/eow may be somewhat superior to agalsidase alfa 0.2 mg/kg/eow (53, 54). Due to different analytical methods, inhomogenous patient groups, and insufficient sample sizes and outcome measures, it is not possible to draw any firm and evidence based conclusions as to the equipotency of the drugs. Moreover, the registry studies are hampered by serious selection bias (53). All patients treated with ERT should be followed in a close and standardized way to achieve sufficent robust conclusions regarding long-term effect and equipotency. There is still not enough data to conclude that ERT can effectively prevent progressive kidney damage in the long run, and the need for a continuous update of unbiased treatment data in a central registry is mandatory (60).

1.1.6. Biomarkers

Specific biomarkers in Fabry disease are GL3 in plasma and urine. Urinary GL3 and plasma GL3 are not ideal markers, and may be overlapping with normal levels especially in heterozygote women (61). The new biomarker lyso-GL3 is promising and correlates with disease activity in some patients, but not in all (e.g. some patients with late onset and in heterozygotes) (57, 62, 63). Measurement of GL3 deposits in tissue biopsies from kidneys and skin is an invasive procedure, and therefore many centres refrain from biopsies. Semiquantitative scoring has been the method of preference in the evaluation of renal biopsies from Fabry patients (38-40, 43, 54, 55). The most comprehensive and systematic attempt to establish a standardized scoring system of potentially prognostic markers in Fabry nephropathy was undertaken by the International Study Group of Fabry Nephropathy (ISGFN) in a group of 59 patients (mean age 39.4 ± 13.2 years) (35 males) with mild to moderate nephropathy in CKD stage I and II, the mean eGFR was 81.7 ml/min/1.73 m^2 and urine protein to creatinine ratio was 122.0 mg/mmol). Both disease-specific (lipid depositions) and unspecific general lesions of progression (fibrosis and sclerosis) were defined and showed a surprisingly wide spectrum of appearance even in clinically mild disease. It was anticipated that the scoring system would be useful for longitudinal assessment of prognosis and responses to therapy for Fabry nephropathy (5). Recently, a more quantitative and unbiased method has been introduced using stereologic morphometry (64), but all morphologic scoring attempts are hypothesis generating up to now and need confirmatory studies and assessment of follow-up studies in untreated and treated patients across several CKD stages and phenotypes.

In many centers MRI of the brain and heart, echocardiography, audiometry and measurements of thin fiber neuropathy are objective measurements and part of routine assessments of the individual total disease burden, but many of these examinations are relatively insensitive and often normal in the young Fabry disease population.

In the regular follow-up of enzyme treated and untreated patients clinical assessment and anamnesis is important, and visual analogue scale questionnaires (quality of life, intensity of pain and gastrointestinal symptoms) have been used systematically in the routine examinations and in many studies. However, such questionnaires should be evaluated with care as only few studies in adults have been placebo controlled (44, 45). Measurements of kidney function and general clinical assessments are usually done simultaneously and are complementary parameters in the routine assessments of Fabry disease patients. In most centers kidney function measurements are routinely based on serum creatinine and creatinine based estimated GFR, as well as different methods for assessment of albuminuria/proteinuria. Creatinine based methods are subject to caveats and are insensitive when it comes to the diagnosis of early progressive kidney dysfunction, the so-called "creatinine-blind window", a phenomenon that is particularly relevant in Fabry disease where ERT probably should be initiated before progressive dysfunction occur. Most follow-up protocols therefore recommend yearly examinations or 6 months intervals as a standard in otherwise stable patients. In line with all kidney disease patients, such standard follow-up routines are subject to variation according to the patient`s disease symptoms.

1.1.7. Research in orphan diseases

The use of potent and potentially toxic drug intervention is increasing in many common as well as rare renal diseases. In fact, many biotechnology companies prioritize development of orphan drugs where modern research are subject to strict surveillance imposed by detailed legislation, also in children (65).

The number of rare disorders is estimated to be 6000-8000 affecting about 55 million people in Europe and the US (66). Rare disease in the US is defined as a disease affecting less than 200 000 inhabitants in addition to being life threatening and chronically debilitating, e.g. 6.5 patients per10 000 inhabitant, and in the EU 5

patients per 10 000 inhabitants (47). In Norway, a rare disease is defined as affecting less than 1 per 10 000, i.e. less than 500 cases per disease. Special regulations stimulating research on orphan diseases were introduced in the US in 1983, in Japan in 1993 and in the EU in 2000 (47, 67). The number of orphan drugs approved for use in children has been increasing (68).

The Food and drug administration (FDA) and the European Medical Association (EMA) have recently presented guidelines that highlight the importance of meticulous safety and follow-up control of tolerance and side effects of new medical treatment also in children. Since 2007 new regulations from EMA have instructed the drug providers to make a pediatric investigation plan (PIP), and high quality research on therapeutic drugs to children has recently been focused (69). Instead of previous general reluctance to allow childrens participation in clinical trials, children should now be protected through clinical trials and should not be regarded “small adults”. Hence, this fundamental change of attitude should also be reflected in the use of relevant clinical methods subjected to evidence based risk and safety evaluation, also when it comes to indication for functional measurements and renal biopsies.

1.2. Evaluation of chronic kidney disease

The key parameter in the assessment of chronic kidney disease is the measurements of glomerular filtration rate (GFR), and quantitative definitions (CKD-stageing and albumin-creatinine ratios in urine samples) have greatly improved diagnosis and treatment of kidney diseases. Supplementary information about structural (e.g. ultrasonogaphic or radiologic imaging and kidney biopsies) and functional (e.g. hematuria, proteinuria and GFR) kidney damage is necessary in the characterization of early kidney disease (CKD stage 1-2), and is of paramount importance in the diagnosis and risk stratification of individual CKD-patients (e.g. magnitude of proteinuria).

Chronic kidney disease is defined as kidney damage (structural abnormalities or damage markers in blood or urine) or renal failure defined as GFR) reduced to < 60 ml/min/1.73 m^2 lasting for three months or more. A staging of chronic kidney disease in five levels was defined in 2002 by the Kidney Disease Outcomes Quality Initiative (K/DOQI) (70) (Table I).

Table I: Chronic kidney disease stage 1-5 (modified from reference (70))

Stage	**Description**	**GFR (ml/min/1.73 m^2)**
1	Kidney damage with normal or ⇑ GFR	≥ 90
2	Kidney damage with mild ⇓ GFR	60-89
3	Moderate ⇓ GFR	30-59
4	Severe ⇓ GFR	15-29
5	Kidney failure	< 15 (or renal replacement therapy)

This staging has paved the way for the increasing recognition that CKD is an important public health problem affecting 10 – 13 % of the general population, with an increased risk of cardiovascular complications and death (71). During the latter decade much effort has concentrated on defining low- and high-risk groups of CKD patients with respect to risk of many complications (especially cardiovascular events) and progression to end-stage renal disease (ESRD), an example of high-risk patients is co-existent hypertension, cardiovascular disease, diabetes mellitus and especially the combination of lower GFR and proteinuria (72, 73). Estimated GFR and albuminuria have been shown to be independent predictors for progression of kidney disease to ESRD (73), and even low levels of albuminuria is associated with progressive nephropathy in diabetes mellitus patients (74). In fact, the recognition of increasing levels of albuminuria/proteinuria is important both in the diagnosis and as a target of therapy in kidney diseases (75).

The knowledge of the associated high risk of complications in CKD-patients, also including infections and cancer, lead to an updated position statement by the KDIGO Board of Directors in 2007, highlighting the importance of continuous focus on risk factors, as well as prophylaxis and early treatment of complications in the follow-up of CKD patients (72). Reduced GFR without accompanying risk factors often carry a good prognosis, and screening the general population for kidney disease therefore is a controversial issue. However, by combining eGFR and urine albumin excretion Hallan et al. showed that 2/3 of patients with progressive kidney disease could be detected. They stated that urinary albumin excretion is a marker of the rate of kidney disease progression, whereas eGFR is a marker of how advanced the kidney disease is (73).

1.2.1. Glomerular filtration rate (GFR)

GFR is the key parameter in the evaluation the kidney function, and creatinine measurements in serum and urine have been the basis for kidney function

measurements for many decades. The clearance of creatinine was for the first time measured by Rehberg in 1926 and in 1951 Homer Smith established the method and described its potential and limitations as a renal function parameter (76). The gold standard method for measuring GFR is inulin clearance, performed by continuous infusion of inulin and timed collection of urine samples via bladder catheter (77). The method, which was introduced by Earle and Berliner in 1946 (76), is time consuming and not feasible nowadays since inulin is not available. Several new gold standard methods have been adopted, and plasma clearance of adequate filtration markers ad modum Sapirstein (78) and Chantler (79) are based on timed blood samples and have been validated as alternative gold-standard methods. These methods have later been further simplified by Brochner-Mortensen in 1972 (80) and Jacobsson in 1983 (81). The commonly used substances Chrome-Ethylene Dinitrilo Tetra Acid (Cr-EDTA), technetium-99m-Diethylene Triamine Pentacetic Acid (99mTc-DTPA) Tc-DTPA and I-iothalamate are radioactive agents, and repeated use is often avoided due to concern of long-term detrimental consequences (82). The non-ionic radiocontrast agent iohexol is an ideal filtration marker and was introduced in the 1980's (83), and is most widely used in Scandinavia and Italy. The safety of iohexol has been well documented (84-86), and iohexol-GFR-measurements are shown to correlate well with inulin-GFR-measurements (87-89).

Estimated GFR measurements are calculations based on single blood sample measurements mainly of serum creatinine, and different formulas have been constructed for adults and children. The original formulas were primarily developed for adult patients with CKD stage 3-5, where eGFR show better correspondance with measured GFR. In the 1970's and 1980's the majority of formulas for estimating GFR was based on serum creatinine and body length, plus eventually age and sex in children (90-92). Later formulas including body weight have been developed (93), and cystatin C based estimations have been tested both in children and adults (94, 95). However, cystatin C based formulas have not been universally adopted, mainly because of lack of consensus about standardization, and the cost is higher than creatinine-based measurements. Recently, substantial efforts have been reported to

improve and standardize the methods of analyzing creatinine in blood. Especially in children great variability in the broadly used Jaffe method has been demonstrated, and the calibration of both enzymatic serum creatinine analysis as well as the compensation of the Jaffe analysis to the Isotope Dilution Mass Spectrometry reference method (IDMS) results in 10-20 % lower values of serum creatinine compared to the previously widely used creatinine methods in the original methodologic reports of eGFR in children (96-98).

By comparison with iohexol clearance as gold standard method, Schwartz et al recently constructed new formulas and subsequently validated the new and old formulas in children with CKD stage II-V (99). At the same time a new improved equation for GFR in adults was published: the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (100) showing better performances than the broadly used Modification of Diet in Renal Disease Study equation (MDRD) (101), especially in the normal and higher levels of GFR.

In the general CKD population, the eGFR formulas have proved to be valuable tools in the risk stratification for associated complications (especially cardiovascular complications) across all CKD-stages (71). However, it has not been proven whether the same predictive capacity is valid in smaller cohorts with specific diseases, e.g. Fabry disease. Furthermore, it is well known that the precision and accuracy of formula GFR may be lower in patients with high or low body mass index (102, 103), the latter is often the case in Fabry disease.

In Fabry disease, formula based GFR estimation are used in the majority of renal function studies, usually the Modification of Diet in Renal Disease Study equation (MDRD) in adults (104) and the original Schwartz formula in children (91, 92, 105). We have previously shown that these formulas overestimate mGFR in children and in adult males, and these methods may also falsely indicate hyperfiltration in many patients, an observation that was strengthened by simultaneous measurement of normal GFR (102, 106, 107) (Figure I and Table II). These methodologic caveats may lead to the erroneous assumption that GFR is normal or falsely high in patients

where measured GFR discloses a borderline normal or early decline of GFR. Furthermore, serum creatinine usually will be normal in such cases with CKD stage I-II, due to the insensitivity of creatinine and creatinine-based equations (i.e. the "creatinine-blind window") to discover subtle decreases in true GFR.

Figure I: Difference eGFR-mGFR (95 %CI) in children and adults (blue bars: MDRD formula, green bars; Cockcroft-Gault (CG) formula, black bars; original Schwartz (S) formula, purpel bars: Counahan-Barratt (CB) formula) (107):

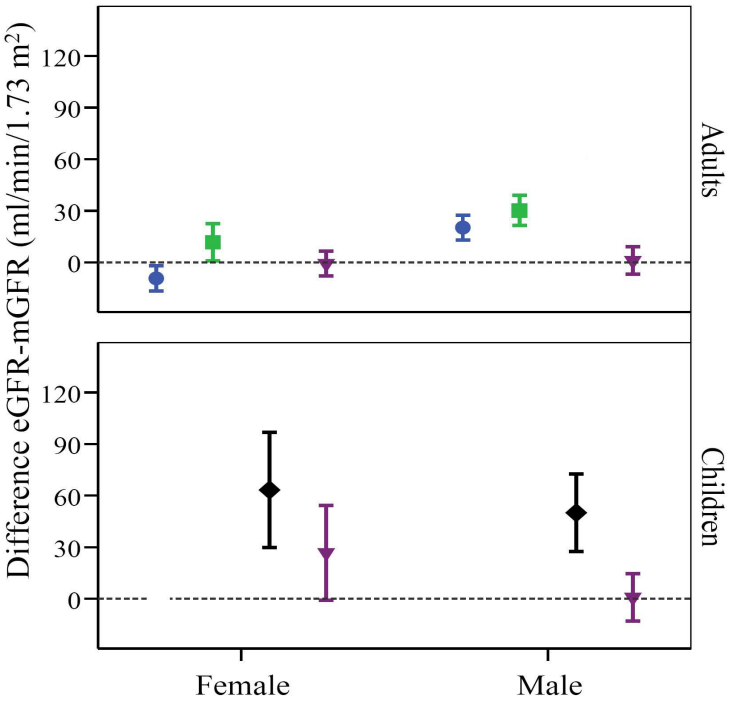


Table II: Comparison eGFR vs mGFR (107):

	Men (n=9)	Women (n=18)	Children (n=12, females 4)
Age (years)	28 ± 12	44 ± 17*	14 ± 3
BMI (kg/m²)	22 ± 3	26 ± 5*	20 ± 4
s-creatinine (μmol/L)	76 ± 14	64 ± 9*	55 ± 13
mGFR (ml/min/1.73 m²)	96 ± 25	95 ± 21	106 ± 12
Albumin creatinine ratio (mg/mmol)	6.5 ± 8.4	7,0 ± 8.6	2.3 ± 1.8
$eGFR_{MDRD}$ – mGFR	17 (CI 5,28)#	-4 (CI -12,6)*	
$eGFR_{CG}$ - mGFR	27 (CI 16,37)#	17 (CI 2,32)#	
$eGFR_{CB}$ - mGFR			10 (CI -3,22)
$eGFR_{S}$ - mGFR			54 (CI 39, 70)#

*Comparison between females and males were done with independent student t-test, * p<0,05.*
Difference eGFR-mGFR (test value 0) was evaluated with one sample student t-test, # p<0.01

1.2.2. Urine tests

Urine dipsticks, urine microscopy and quantitative measurements of albuminuria, proteinuria and classification of proteins of glomerular or tubular origin, are important examinations in patients with various kidney diseases. The methodology in the ascertainment of urinary excretion of proteins have been changing from qualitative to quantitative methods which are important for diagnosis as well as targets for treatment (73, 75).

The use of spot urine samples was introduced in the eighties (108), and protein-creatinine-ratio in morning void urine has been shown to correlate well with 24h-urine protein excretion (109). The use of protein-creatinine-ratio (PCR) and albumine-creatinine-ratio (ACR) was introduced as a routine measurement in Norway ten years ago (110, 111). The definition and implementation of various test methods is highly different in many publications and most clinical studies over the later decades refer to 24 hour protein-excretion. A recent report by Lamb et al. (112) has given a comprehensive overview of various methods and guidelines used for assessing urinary albumin and protein excretion (Table III). They suggest that quantitation of albuminuria should be the preferred method, as has been the case for

many years in the staging and monitoring of progressive diabetic nephropathy (113). The relevance of comparing diabetes mellitus and Fabry disease has recently been highlighted by Alberto Ortiz`s research group, demonstrating similar activation of detrimental and protective signals in the podocytes in models of the two diseases (114, 115).

Table III: Different guidelines for assessing albuminuria/proteinuria (Table modified from reference (112)).

Guidelines	**Diabetes Mellitus (DM)**	**Definition proteinuria in nonDM**
NKF-KDOQI (2002)	ACR	PCR > 23 mg/mmol
CARI (2004)	ACR	u-protein > 150-300 mg/24 h
KDIGO (2005)	Albumin (ACR or albuminstrip)	ACR > 3.4 mg/mmol
SIGN (2008)	ACR	PCR > 100 mg/mmol
NICE (2008)	ACR	ACR > 30 mg/mmol or PCR > 50 mg/mmol

The benefit of regular assessments of albuminuria as a predictive biomarker for various complications in the general population was highlighted in a recent metaanalysis in Lancet. Mutsushita and colleagues showed that albumin-creatinine ratio with a cut off value as low as 1.1 mg /mmol creatinine was an independent predictor of all case mortality as well as cardiovascular mortality (116). The use of micro- and macroalbuminuria in addition to GFR-level improves the prediction of disease progression substantially (73). An albumin-creatinine ratio as low as ≥ 0.75 mg/mmol has been shown to be associated with cardiovascular morbidity in a Norwegian study of the general population (117). Furthermore, increasing levels of

albuminuria assessed by spot urine measurements have recently been shown to predict reduction in life expectancy in a huge population-based study in Canada (118).

There are greater day-to day variations in albumin/protein-excretion estimated by spot urine compared with 24 hour estimates (119). Therefore a collection of three consecutive morning void urines samples are preferred, and the median value is judged to be the representative one (111).

1.2.3. Kidney biopsies

1.2.3.1. History and frequency of kidney biopsies

Renal biopsies have been done for more than a century (120) and the percutaneous methodology was introduced and established in the 1950s (121-123). The technical advancements in imaging methods have evolved from indirect visualization to direct simultaneous real-time ultrasound guidance of the biopsy needle. The construction of automated spring-loaded devices by the end of the1980s (124-126) has replaced the manual aspiration methods and manual true-cut needle procedure. The modern biopsy techniques have improved the precision and efficacy of the procedure, and even more important, have reduced the frequency of procedure related complications (127). Knowledge of the histology from kidney biopsies is shown to alter the treatment in a high percentage of the patients (123, 128, 129), and the acknowledgement of the importance of kidney biopsies was in fact an important factor that lead to the establishment of nephrology as a subspecialty in 1960 (130). Generally, in many renal diseases there is a lack of valid early biomarkers. The role of kidney morphology as an important prognostic and therapeutic guide in common renal diseases has been convincingly demonstrated; e.g. diabetes nephropathy (131), IgA nephritis (132), lupus nephritis (133) and in orphan diseases like Fabry disease (5, 49).

The annual rate of kidney biopsies varies from country to country dependent on the traditions and guidelines for performing a renal biopsy. In Australia there has been a relatively liberal indication for renal biopsy including isolated microscopic hematuria, and the frequency of native kidney biopsies (excluding transplants) has been reported 23 per 100 000. In Italy 3.3 per 100 000 has been reported (1993), and in France 16.2 per 100 000 (1990). In children, Australia has reported a biopsy frequency of 7 per 100 000, and Italy 1.7 per 100 000 (134). In the USA, 7.5 native renal biopsies per 100 000 per year has been reported (personal communication Richard Glassock). In Norway the overall frequency of renal biopsies has been intermediate compared with many other countries, and the average has been largely unchanged (about 11 per 100000) in the period since the establishment of the Norwegian Kidney Biopsy Registry in 1988 (135). A higher threshold in some countries for performing a renal biopsy, especially in children, may be related to general concern over complication rates and legal consequences of complications. On the other hand, it is well known that kidney biopsies are often essential in the diagnosis and follow up of specific renal diseases also in children (136).

1.2.3.2. Indications for kidney biopsies

The variations in frequency of kidney biopsies are partly explained by differences in indications and procedural methods. In addition, indications and kidney disease spectrum have changed over the years, e.g. patients with isolated hematuria were more frequently biopsied in the 1990s than during the later decade according to data in annual reports from the Norwegian Kidney Biopsy Registry. Table IV shows recommended indications for a kidney biopsy (137), and Table V shows the current clinical indications that are reported in the patient data form (“K-skjema”) from Norwegian renal units to the Norwegian Kidney Biopsy Registry (one patient may have several positive tickboxes).

Table IV. Indications for kidney biopsy (137)

Isolated glomerular hematuria
Isolated nonnephrotic proteinuria
Nephrotic syndrome; in adults, in small children when steroid resistent
Acute nephritic syndrome
Unexplained acute renal failure

Table V. Indications for kidney biopsies reported in the patient data form to NKBR

Kliniske indikasjoner for biopsi (kryss for alle aktuelle):

	< 1mnd	1-12 mnd	1-10 år	>10 år
Nefrotisk syndrom (proteinuri >3 gram per døgn, s-albumin <35 og ødemer)	☐	☐	☐	☐
Akutt nefrittisk syndrom (glomerulær hematuri, proteinuri, akutt nyresvikt med eller uten ødem/HT)	☐	☐		
Akutt nyresvikt (akutt kreatinin-stigning mer enn 50%)	☐	☐		
Kronisk nyresvikt (GFR <60 ml/min/1,73m2 i minst 3 mndr)		☐	☐	☐
Proteinuri (> 300 mg/døgn)	☐	☐	☐	☐
Mikroalbuminuri	☐	☐	☐	☐
Hematuri	☐	☐	☐	☐
Hypertensjon (behandlingskrevende)	☐	☐	☐	☐

In general, proteinuria is the most important sign leading to a kidney biopsy, but the definition of proteinuria and biopsy threshold is subject to variations from center to center, and from country to contry. Some centers argue that by isolated proteinuria urinary protein-creatinine-ratio should be > 3.5 g/g (396 mg/mmol) to indicate a renal biopsy whereas others have done general school-screening-programs performing renal biopsy if urinary protein-creatinine-ratio > 0.2 g/g (22.6 mg/mmol). A recent study showed that a renal biopsy may be indicated in isolated proteinuria when urinary protein-creatinine-ratio > 0.5 mg/mg (56.5 mg/mmol) (138).

1.2.3.3 Complications and safety aspects of kidney biopsies

In general, the risk of complications after a kidney biopsy is low when general contraindications (e.g. bleeding diathesis and hypertension) are respected, and the risk has been decreasing after the introduction of automatic springloaded biopsy devices and real-time ultrasound guidance (127). The most serious complications are uncontrolled bleeding necessitating surgical and/or radiologic vascular intervention, and deaths have been reported (139, 140). On the other hand, postprocedural perirenal hematomas are more or less obligate and often asymptomatic, occurring in up to 60-90 % of the procedures (126, 140-142). Most kidney biopsy studies comprise a limited number of patients, and there are few studies in children.

Many kidney biopsy studies are published after 1990, and some metaanalyses are available. In 1991 Parrish reported a mortality rate of 0.12 % and surgery was performed in 0.29 % in a review of more than 10 000 kidney biopsies performed since the 1950s (139). In 2002 Korbet reviewed a high number of newer publications, excluding the patients in Parrish' study (140). Korbet found a rate of blood transfusions of 1.24 %, rate of surgery 0.23 %, and a mortality rate of 0.03 % in about 7500 biopsies (140). An overview (personal review) of publications on safety of renal biopsies the last 20 years, not included in the two reviews above, shows a mean rate

of blood transfusions of 1.22 %, rate of intervention of 0.32 % and a mortality rate of 0.02 % in a total of about 9500 biopsies (Table VI).

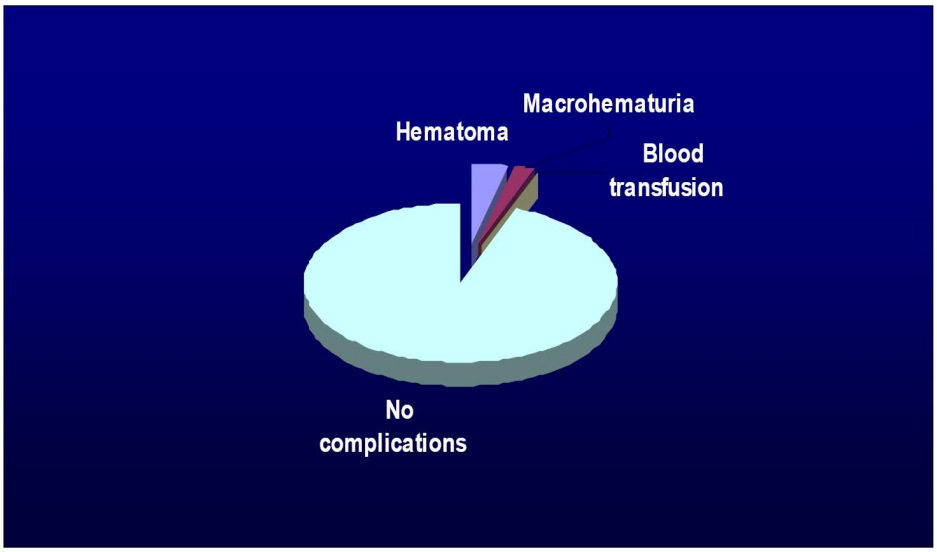
Relative contraindications to performing a renal biopsy are severe hypertension despite adequate treatment, incorrectable bleeding diathesis, active renal or perirenal infection, hydronephrosis, solitary native kidney, small hyperechoic kidneys (indicative of chronic irreversible disease), multiple, bilateral cysts or a renal tumor (137). We have previously presented low kidney biopsy complication rates in children based on data from the Norwegian Kidney Biopsy Registry; 3.7 % of the 464 patients had hematoma (which may not be regarded a complication per se), 2.1 % gross hematuria, and only one patient received blood transfusion. None of the patients needed surgical intervention, and there were no deaths (143) (Figure II).

Table VI: Major complications (intervention, transfusion, death)

First author (year)	Biopsies performed	N (T)	Intervention N (%)	Transfusion N (%)	Death N (%)
Al Rasheed (1990)* (144)	1982-1989	120	2	5	1
Schow (1992) (145)	1985-1990	157 (28)	0	0	0
Riehl (1994) (126)	1986-1992	458 (143)	2	-	0
Richards (1994) (128)	1991	276	0	2	0
Mendelssohn (1995) (146)	1992-94	544 (139)	0	3	0
Bohlin (1995)* (147)		109	0	0	0
Hergesell (1998) (148)	1993-97	1090 (114)	1	3	0
Kersnik Levart (2001)* (141)	1994-1999	88	0	0	0
Whittier (2004) (149)	1983-2002	750	5	38	1
Manno (2004) (150)	1995-2002	471	4	2	0
Eiro (2005) (151)	1997-2001	394	0	0	0
Shidham (2005) (152)	1981-2001	645	4	16	0
Stratta (2007) (129)	1973-2002	1387	3	12	0
Mackinnon (2008) (153)	2000-2007	1120	2	18	0
Soares (2008) (154)	1996-2006	289	5	6	0
Hussain (2010)* (155)	2005	531 (221)	1	4	0
Mishra (2010) (156)	2006-2008	78	0	0	0
Skalova (2010)* (157)	1997-2007	166	0	0	0
Zhang (2011) (158)	2007-2009	280	0	0	0
Torres Munôz (2011) (159)	1998-2008	623	2	2	0
Total	1973-2009	9576	31 (0.32)	111 (1.22)	2 (0.02)

*children only . N; Number of biopsies performed, T; transplant biopsies included in the overall results

Figure II: Complications in children (143):



2. Aims of the thesis

The aims of this thesis was to evaluate functional and morphologic renal disease in children and young adults with Fabry disease with and without enzyme replacement therapy by means of the currently best available routine methods for GFR assessment (ie. measured GFR and various creatinine-based GFR equations) (Study I), and assessment of disease specific and non-specific morphologic changes in kidney biopsies (Studies II and III). The safety and complications of kidney biopsies were evaluated in a large national cohort (Study IV).

3. Material and methods

3.1. Renal evaluation in young Fabry patients

Among the patients with Fabry disease followed at Haukeland University Hospital a total of twenty-one young individuals, thirteen males and eight females, with a mean age of 15.4 ± 6.7, range 5-33 years, were included in one or all of the three Fabry disease studies. In addition, in Study I, fifteen patients from Addenbrookes University Teaching Hospital, Cambridge, United Kingdom and thirteen from Department of Paediatrics, Academic Medical Centre, Amsterdam, the Netherlands, were included. The studies were approved by the Regional Ethics Committee of Western Norway (REK: 154.04. "Pasientoppfølging ved Fabry sykdom". Project leader: professor Gunnar Houge), and were performed in line with the Declaration of Helsinki. All included patients and/or their designees signed informed consent.

In Study I 42 patients, 24 girls and 18 boys, mean age 12.3 ± 3.6, range 2-17 years, from three Fabry centres in Bergen, Cambridge and Amsterdam were included. A total of eighty-two parallel eGFR- and mGFR-values were studied. eGFR was calculated with five different formulas based on serum creatinine values (90-93, 99):

Counahan76: k x height(cm)/serum creatinine(mg/dl), k=0,43 (90)

Schwartz76: k x height(cm)/serum creatinine(mg/dl), k=0,55, boys ≥13 years k=0,70 (91, 92)

Schwartz09: k x height(cm)/serum creatinine(mg/dl), k=0,413 (99)

Leger02: 0,641(weight(kg)/serum creatinine(mg/dl)) + 16,063(height(m)^2/s-creatinine(mg/dl)) (93)

Leger09: 0,542(weight(kg)/serum creatinine(mg/dl)) + 9,948(height(m)^2/s-creatinine(mg/dl)) (93, 99)

Serum creatinine was measured by an IDMS-traceable enzymatic method or by a Jaffe alkaline picrate method calibrated traceable to IDMS. The mGFR was measured by Iohexol single-point method at 4 hours (high performance liquid chromatograpy analysis) in the Norwegian patients, by Cr^{51}-EDTA (three-point method; 2, 3 and 4 hours) in the British patients and by iothalamate (four-point method; 1, 2, 4 and 6 hours) in the Dutch patients. Analytical imprecision (CVa) and intra-individual biological variation (CVi) of the GFR-measurements were evaluated (please find definitions of CVa and CVi in the description of the methodology in Paper I). Urine-albumin excretion was measured as albumin-creatinine-ratio and values > 2.5 mg/mmol creatinine were defined as microalbuminuria.

In Study II and III eleven males and two females, mean age 17.3 ± 7.5, range 7-33 years, had from one to three kidney biopsies performed. Three male patients had already received ERT for 2-3 years before the first kidney biopsy was performed. One female patient did not receive ERT and she was therefore not included in Study III. In addition seven patients without a renal biopsy were included in the baseline study to describe the spectrum of clinical findings in the children cohort followed in our centre.

The kidney biopsies were described by an experienced nephropathologist (light microscopy of paraffin-embedded sections and McDowell fixated sections (toluidine blue, semithin) as well as electron microscopy). For the GL3-accumulation semiquantitative scores (0 to +++) were used (2, 3), and in Study III the biopsies had an additional scoring of the GL3-accumulation in the podocytes based on the scoring system of the International Study Group of Fabry Nephropathy (ISGFN) (160). We also expanded the scoring model of the ISFGN-group by combining the scores of light microscopy of the paraffin embedded sections with the scores of the toluidine blue sections in a “composite score” to obtain an even higher number of scorable glomeruli and increase the representativity of the scoring. Parallel to the kidney biopsies, functional renal evaluation was performed by means of standardized triple urine-tests (albumin-creatinine-ratio and protein-creatinine-ratio) and GFR-

measurements (plasma-clearance of iohexol). Additional clinical parameters were added to describe the spectrum and burden of general Fabry disease manifestations.

For statistical analysis SPSS version 14.0 and 17.0 was used and a p-value of 0.05 was considered statistically significant.Baseline data are given as mean with 95 % confidence interval. Differences between groups were analysed with one way ANOVA in Study I. In Study III differences between the groups were analyzed with unpaired two-sided t-tests or Mann Whitney U-tests, dependent on wether the distribution of the variables followed a normal distribution or not. Equality of variances was tested with Levene's test and corresponding t-tests were used. Differences within groups were evaluated by paired t-test if normally distributed variables and with Wilcoxon matched pairs signed rank sum tests if variables were not following a normal distribution. Linear regression analysis was performed to explore correlations. In Study I bias is given as mean value of eGFR–mGFR for the different equations and as a measure of precision the standard deviation and interquartile range (IQR) for the differences between eGFR-mGFR values were calculated. Percentages of the eGFR results within 30 %, 20 % and 10 % of the mGFR results (P30, P20 and P10) were gives as a measure of accuracy. The concept of critical difference (CD), defined as the minimal difference needed between two consecutive measurements to conclude that the results are truly different, was used to evaluate longitudinal changes in individual consecutive measurements (161).

3.2. Safety and complications of kidney biopsies in the Norwegian Kidney Biopsy Registry

The Norwegian Kidney Biopsy Registry was established by professor Bjarne M. Iversen in 1988 and since April 1988 clinical and histopathologic data have been registered for nearly all patients with a native renal biopsy in Norway, a country with a current population of 5.0 million inhabitants. Local nephrologists from 26 different hospitals report clinical data in a registry notification form, and histopathological data are reported by a limited group of nephropathologists in one centre (Haukeland University Hospital) who examines all the biopsies. The Norwegian Kidney Biopsy Registry is authorized by the Norwegian Data Inspectorate and all included patients or their designees have signed informed consent. In the time- period 1988-2010 clinical data were registered in 9645 cases. In 3.7 % of the reports information regarding biopsy-related complications was lacking and these cases were therefore excluded leaving 8573 adult and 715 paediatric cases (in total 9288 cases) for analysis of the procedure related complications and safety issues in the period from April 1988 through November 2010.

Five different complication alternatives were listed in the report forms; ”hematoma”, “gross hematuria”, “blood transfusion” ,“surgery” and a free text field. When arterial embolization was noted in the free text field the case was included in the “surgery”-group. The term “complications” used in the current study was defined as gross hematuria, blood transfusions and/or surgery/arterial embolization. The term “major complications” was confined to blood transfusions and/or surgery/angiographic embolization. The following variables were explored; age, estimated glomerular filtration rate (calculated with the MDRD formula in adults (104) and the revised Schwartz-formula in children (99), systolic blood pressure, proteinuria (u-protein $\geq$ 0.3 g/d or reported “proteinuria” or “nephritic syndrome”), hematuria (u-dipstick ≥ 1 or reported “hematuria” or “nephritic syndrome”), chronic kidney disease stage 3-5, (estimated glomerular filtration rate < 60 ml/min/1.73 m^2), nephrotic syndrome (u-protein $\geq$ 3.0 g/d and serum-albumin < 35 g/l or clinician marked “nephrotic

syndrome"), acute renal failure, biopsy needle size, number of needle passes, speciality of the doctor performing the biopsy and the hospital size (≥650 and <650 biopsies in the study period corresponding to ≥ 30 or < 30 biopsies per year) .

Statistical analysis was performed using the SPSS 17 package (SPSS Inc., Chicago, IL). A p-value of < 0.05 was considered significant. Student's t-test, Mann–Whitney test and Pearson chi-square test were used for significance testing and all tests were two-tailed. Analysis of risk factors for major complications was performed by logistic regression analysis and unadjusted analyses as well as analyses adjusted for age and categorized glomerular filtration rate were performed.

4. Results

4. 1. Estimated and measured glomerular filtration rate in children with Fabry disease

The results and comparisons of estimated GFR (eGFR) and measured GFR (mGFR) in young Fabry disease patients in our centre showed that eGFR overestimated mGFR substantially (Paper II (2008)), falsely indicating hyperfiltration in several patients (Table VII and Figure III). This issue was further explored in Paper I (2010).

Figure III: Estimated GFR (original Schwartz formula (91, 92)) overestimates GFR compared to GFR measured with iohexol clearance (iGFR) in all patients ($p<0.001$). Measured GFR was normal in all patients, while eGFR suggest hyperfiltration in several patients (Figure presented in poster at ASN Renal Week, 2005) (162).

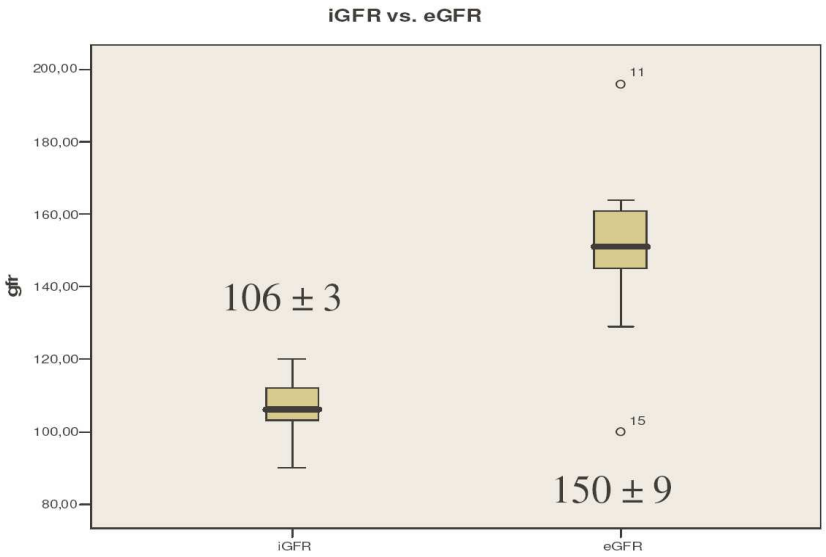


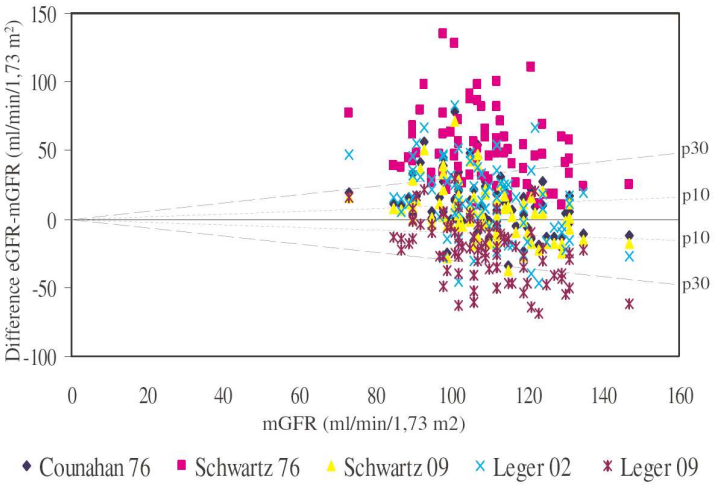
Table VII: Laboratory data in 16 Fabry children. α-Gal: α-GalactosidaseA levels in serum (μkat/kg protein). mGFR: iohexol GFR. eGFR: estimated GFR (Schwartz formula, 1976). *protein concentration (g/L)

Patient	Sex (F/M) Age (yr)	α-Gal	Albumin-ratio (mg/mmol)	Proteinratio (mg/mmol)	Serum creatinine (μmol/L)	mGFR (ml/min/1.73m^2)	eGFR (ml/min/1.73m^2)
1	M7	2,4	4,8	28,3	40	106	151
2	F11	16,8	6,0	<0,15*	45	105	196
3	F14	11,1	1,2	7,0	50	90	158
4	M16	0,7	6,4	11,8	59	107	149
5	M17	2,0	3,0	10,5	61	112	164
6	M18	3,3	11,8	28,4	77	96	138
7	M12	3,8	0,6	9.0	58	103	129
8	M16	8,6	1.3	10,4	64	112	161
9	M11	2,7	3,8	13.0	49	120	145
10	M10	3,0	1,6	12.0	49	-	140
11	F13	17,5	1,9	6.0	62	-	125
12	F14	13,4	1,1	<0,15*	59	91	138
13	F14	13,2	0,8	<0,15*	72	-	109
14	M5	3,0	1,7	<0,15*	57	-	97
15	F16	19,2	2,0	6,0	72	-	112
16	F9	11,5	1,9	15,0	37	-	162

In the 42 children (mean age 12.3 ± 3.6, range 2-17 years) included in Study I, the mean baseline mGFR was 108 ±13.5 ml/min/1.73 m^2, range 87-147 ml/min/1.73 m^2 and 19 % of the patients had microalbuminuria (Table 1, Paper I). Normal body mass index was present in 86 % of the patients. No correlation was seen between albumin-creatinine ratio and GFR, age or gender, and there were no significant differences in body mass index and serum creatinine between the Norwegian, the British and the Dutch cohort. The mean mGFR was higher ($p = 0.01$) in the Dutch patients compared to the British patients.

The mean eGFR overestimated the mean mGFR in four of five formulas: the Schwartz76-formula had a bias of 50.6 ± 26.1 ml/min/1.73 m^2, the Counahan76-formula 9.9 ± 20.0 ml/min/1.73 m^2, the Schwartz09-formula 5.3 ± 19.4 ml/min/1.73 m^2, the Leger02-formula 12.1 ± 27.1 ml/min/1.73 m^2 and the Leger09-formula -21.9 ± 21.7 ml/min/1.73 m^2 (Table 2, Figure 1, Paper I). The findings were similar in all three centres, when the first examination of each patient was analyzed separately (n = 42, data not shown), as well as when repeated examinations were compared for example; first vs second (data not shown). Inter quartile ranges, similar to standard deviations, were large for the mean values of eGFR-mGFR; the Schwartz76-formula had a inter quartile range of 31.5 ml/min/1.73 m^2, the Leger09-formula 31.7 ml/min/1.73 m^2 and the Leger02-formula 35.3 ml/min/1.73 m^2 (the largest variance), whereas the Counahan76-formula had a inter quartile range of 22.4 ml/min/1.73 m^2 and the Schwartz09-formula was the equation with the highest precision and had a inter quartile range of 21.7 ml/min/1.73 m^2. The Schwartz09 formula also showed the best accuracy of all GFR estimates with 87 % of the values within 30 % of the mGFR-value (P30), 79 % within 20 % of the mGFR (P20) and 49 % within 10 % of the mGFR (P10) (Table 2, Figure 1, Paper I and Figure IV).

Figure IV: Difference eGFR-mGFR in 42 children with Fabry disease (163).

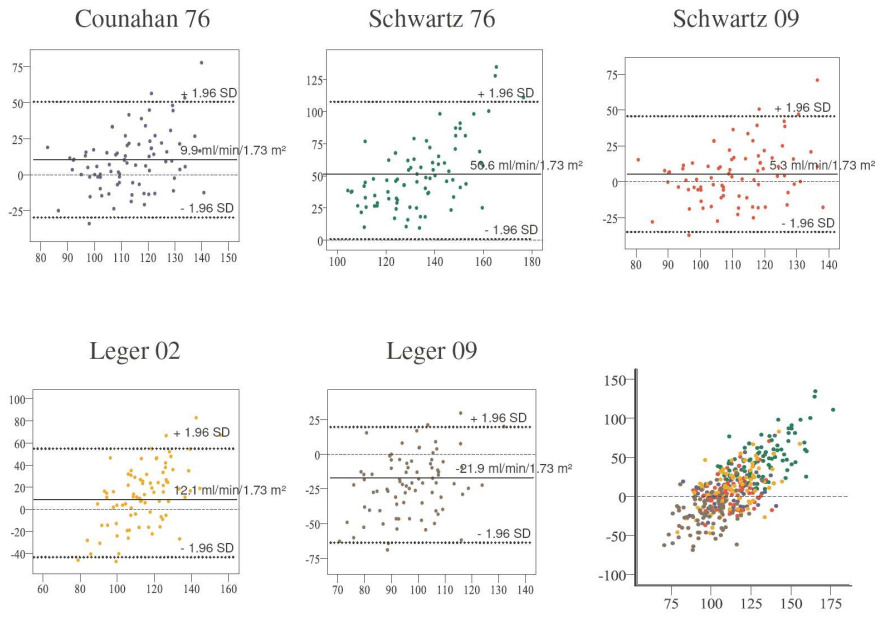


The Counahan76-formula had P30 of 87 %, P20 of 76 % and P10 of 41 %, the Leger02-formula had P30 of 72 %, P20 of 54 % and P10 of 24 % and the Leger09-formula had P30 of 71 %, P20 of 50 % and P10 of 24 %. The Schwartz76 showed very low P30-P10-values of 27 %, 11 % and 4 %, respectively. Bland-Altman plots show the uncertainty for the five different equations (Figure V).

In the thirteen patients with three or more consecutive mGFR measurements no significant deterioration of kidney function was noted. Substantial variability by repeated measurements was found in both mGFR and eGFR (Figure 3, Paper I). The variance of mGFR was within the limits of the defined critical difference in 10 patients, one patient (patient 5) showed a temporary decrease in renal function (probably due to measurement error) whilst two patients (patient 6 and 10) showed improved mGFR. Larger variations was found in eGFR; five patients (patient 1, 2, 7, 8 and 10) showed substantial increases of eGFR compared with the expected values, while two patients (patient 3 and 11) showed deteriorating values. Only one patient

(patient 10) showed concordant changes (increased GFR) of both mGFR and eGFR. In 6 of 13 patients eGFR falsely signalled changes of the renal function (either decrease or increase of GFR outside the limits of critical difference), and did not detect an improved function for one of the two patients that had such findings by mGFR. Patient 3 had a substantial discrepancy between the first mGFR and eGFR, whereas there was a lack of discrepancy at the second and third GFR measurement (Figure 3, Paper I).

Figure V: Bland Altman plots. X-axis: (eGFR+mGFR)/2.Y-axis: mean eGFR-mGFR for the five eGFR equations (ml/min/1.73 m^2). In the lower right panel data from all the equations are included in one figure.



4.2. Kidney biopsies in young Fabry patients

The mean age of all the twenty patients with Fabry disease included in Studies II and III was 15.3 ± 6.7, range 5-33 years (seven females and thirteen males). Eye changes were present in 90 %, acroparesthesias in 85 %, gastrointestinal symptoms in 65 %, hypohidrosis in 60 %, angiokeratomas in 20 %, white matter lesions (cerebral MRI) in 15 % and left ventricular hypertrophy in 10 % (Table VIII, Table 1, Paper II and Table 3, Paper III).

Table VIII: Clinical symptoms and findings

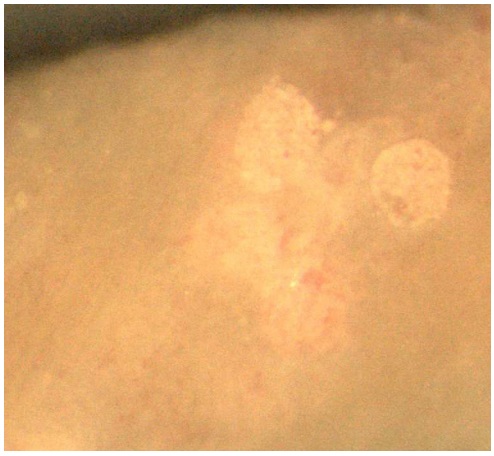
Patient (age/sex)	Eye changes	Acro-paresthesias	Gastro-intestinal symtoms	Hypo-hydrosis	Angio-keratoma	White matter lesions on cerebral MRI	Left ventricular hypertrophy
M5	+	+	+	+	-	-	-
M7*	+	+	+	+	-	-	-
F9	+	-	-	-	-	-	-
M10	-	+	-	-	-	-	-
M11*	+	+	+	+	-	-	-
F11*	+	+	+	-	-	+	-
M12*	+	+	-	+	-	-	-
F13	+	-	+	-	-	-	-
F14*	+	+	-	-	-	-	-
F14	+	+	-	-	-	-	-
F14	+	+	-	-	-	-	-
M16*	+	+	+	+	+	-	-
M16*	+	+	+	+	-	-	+
F16	+	-	-	-	-	-	-
M17*	-	+	+	+	-	-	-
M17*	+	+	+	+	-	+	-
M18*	+	+	+	+	+	+	-
M23*	+	+	+	+	-	-	-
M30*	+	+	+	+	+	-	-
M33*	+	+	+	+	+	-	+

*Renal biopsy performed in study II-III

Among the thirteen patients with renal biopsy in the two studies, seven males and two females were less than 18 years of age at time of baseline biopsy and four patients were young adult males aged 18, 23, 30 and 33 years (mean age 17.3 ± 7.5, range 7-33 years). All male patients had very low alfagalactosidase-levels, and the two female patients slightly decreased levels. Mean alfagalactosidase was 4.9 ± 4.6, range 0.65-16.8 μkatal/kg protein (normal reference 17.7 – 26.4 μkat/kg protein), mean plasma GL3 was 8.3 ± 3.0, range 2.6-13.1 (normal reference 1.6-3.3 μmol/L) and mean urinary GL3 was 4.7 ± 5.7, range 0.3-21.6 mol/mol (normal reference <0.6 mol/mol). Ten of thirteen patients (77 %) had microalbuminuria at baseline with mean albumine-creatinine-ratio 5.1 ± 4.0, range 0.59-13.6 mg/mmol creatinine and mean protein-creatinine-ratio 16.2 ± 8.1, range 7-28.4 mg/mmol creatinine. Mean GFR was 104.6 ± 9.7, range 86-120 mmol/min/1.73 m^2, and all but one 30 years old male patient had normal mGFR (Table 2, Paper II and Table 1, Paper III).

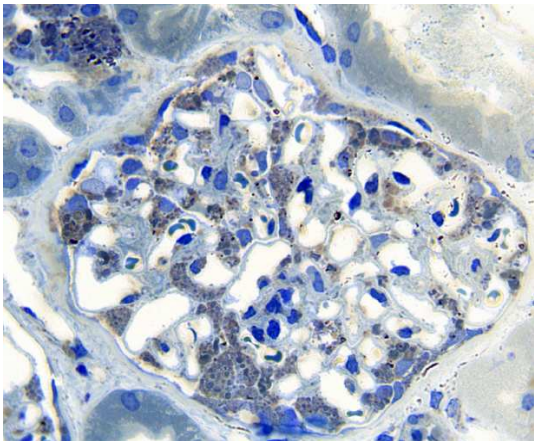
The baseline biopsies showed white looking glomeruli in bedside stereomicroscopy in all patients due to the illumination of sphingolipids (GL3) in the podocytes in the surface-near glomeruli (Figure VI and Figure 1, Paper II).

Figure VI: Stereomicroscopy, renal biopsy from 11 year old boy with Fabry disease



All patients had maximal score of GL3-accumulation in the podocytes when the toluidine blue stained semi-thin sections were examined by light microscopy (Figure VII, Figure 2, Paper II and Table 5, Paper III).

Figure VII: Toluidine-stained semithin section, renal biopsy from 11 year old boy with Fabry disease



In addition, examination with electron microscopy showed segmental foot process effacement in all thirteen patients as well as glomerular endothelial cell and mesangial cell inclusions in all patients except in the three males already treated with ERT before their baseline biopsy (Figure VIII, Table IX, Table 4, Fig 6, Paper II and Table 4, Paper III). Light microscopy of the Periodic-Acid-Schiff (PAS) sections showed vascular, interstitial and/or glomerular changes in nearly all patients (Table 3, Paper II); arteriopathy in 6/13 patients (Fig 3, Paper II), glomerular hyaline in 4/13 patients (Fig 4, Paper II), interstitial fibrosis in 5/13 patients, global glomerular sclerosis in 5/13 patients and early sign of focal segmental glomerular sclerosis (FSGS) was seen in one male and one female patient (Fig 5, Paper II). The scoring of the vacuoles in the podocytes gave full or nearly full score in all patients. (Table 5, Paper III).

Figure VIII: Electron microscopy, renal biopsy from 11 year old boy with Fabry disease

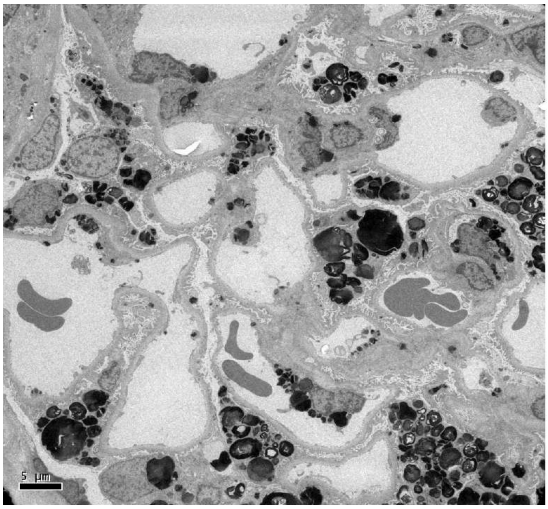


Figure IX: Periodic-acid-schiff section, renal biopsy from 11 year old boy with Fabry disease

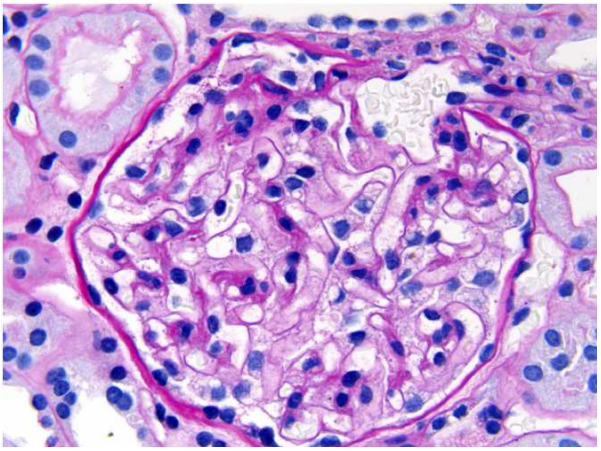


Table IX: Scoring of renal biopsies; light microscopy and electron microscopy

N	Age/ Gender	T (y)	GS	FSGS	Glom Hya-line	Interstit Fibrosis %#	Arterio-pathy	Podocyte composite score (number of scored glomeruli)	Mes. Cell Incl.	Glom Endot Cell Incl	Segmental Foot-Process Effacement	Dist Tub Epith Cell Incl	Mes-angium
1	7	0	0/23	-	+	-	-	7.0 (34)	+	+	+	+	-
	M	5	0/11	-	+	-	-	0.4 (20)	-	-	(+)	-	-
2	11	0	0/24	-	-	-	-	6.9 (52)	+	+	(+)	+	-
	M	3	0/26	-	-	-	-	7.0 (34)	-	-	(+)	+	-
		5	0/16	-	-	-	-	6.8 (19)	-	-	(+)	+	-
3	11	0	0/29	-	-	-	+	7.0 (35)	+	+	+	+	Matrix+
	F	5	0/10	-	-	-	+	2.9 (27)	-	-	(+)	-	-
4	12	0	0/23	-	-	-	+	7.0 (27)	+	+	(+)	+	-
	M	5	0/12	-	-	-	-	6.6 (16)	-	-	+	+	-
5	16	-2											
	M	0*	1/12	-	+	5	+	6.9 (13)	+	-	+	+	Matrix+
		1	1/7	-	-	-	+	3.6 (14)	-	-	(+)	+	-
		5	0/18	-	-	-	(+)	1.8 (22)	-	-	(+)	+	-
6	16	0	0/22	-	-	-	-	6.9 (23)	+	+	(+)	+	-
	M	3	0/15	-	-	-	(+)	7.0 (36)	-	-	(+)	+	-
		5	0/17	-	-	-	(+)	7.0 (21)	-	-	+	+	-
7	17	-2											
	M	0*	1/9	-	-	5	+	7.0 (12)	-	-	+	+	-
		1	0/7	-	-	-	+	7.0 (30)	-	-	+	+	-
		5	0/11	-	-	-	+	7.0 (18)	-	-	+	+	-
8	17	0	0/17	(+)	+	-	+	7.0 (29)	+	+	+	+	Cells +
	M	1	0/33	(+)	+	-	+	7.0 (46)	-	-	-	+	-
9	18	0	1/21	-	-	5	-	7.0 (25)	+	+	+	+	Cells +
	M	1	0/12	+	-	-	-	7.0 (17)	-	+	(+)	+	Cells (+)
		5	0/12	+	-	-	-	4.9 (18)	-	-	-	+	-
10	23	0	0/18	-	-	-	-	7.0 (20)	+	+	+	+	Matrix +
	M	1	0/9	-	+	-	-	7.0 (36)	-	-	+	+	Matrix +
		5	0/13	+	+	-	-	7.0 (17)	-	-	+	+	Matr ix+
11	30	0	1/18	-	+	5	-	6.9 (25)	+	+	+	+	Matrix +
	M	1	1/23	-	+	5	(+)	7.0 (27)	-	-	+	+	Matrix +
		5	0/12	-	+	5	+	7.0 (16)	-	-	(+)	+	-
12	33	-3											
	M	0#	1/15	-	-	5	+	7.0 (42)	-	-	+	+	Matrix+
		5	1/12	-	-	5	+	7.0 (42)	-	-	+	+	Matrix +

T: time of biopsy: 0 is baseline, numbers represent years after baseline. GS: Global glomerual sclerosis (number of GS/total glomeruli), FSGS: focal segmental glomerulosclerosis, Glom: glomerular, Interstit: interstial, Mes. Cell incl: GL3-inclusion in mesangial cells, Glom.endot. Cell incl: GL3-inclusions in glomerular endothelial cells, Dist. Tub. Epith.Cell incl: GL3-inclusions in distal tubular epithelial cells. *2 years of ERT at baseline, #3 years of ERT at baseline. #estimated semiquantitively and scored to nearest 5 %. NA: not available. The GL3-inclusions in the podocytes are given in values 0-7 as composite podocyte scores based on scoring of the GL3-inclusions in toluidine-stained semithin sections (0-4) and scoring of the vacuolization of the podocytes (0-3), the scoring system of the International Study Group of Fabry Nephropathy (5).

Twelve Fabry patients (mean age 17.6 ± 7.7, range 7-33 years) were treated with ERT in the study period for a median of 65, range 13-69 months. Three patients received ERT before baseline biopsy, and the total treatment-time with ERT was 68 ± 22, range 13-99 months (Table 3, Paper III). There were no statistical significant differences between the baseline data in the “low-dose”-group (6 patients, agalsidase 0.2 mg/kg/eow) and the “high-dose”-group (6 patients, agalsidase 0.4-1.0 mg/kg/eow) (Tables 1-2, Paper III).

All patients had stable mGFR throughout the study period with a mean mGFR of 107.3 ± 12.9 ml/min/1.73 m^2 after a mean ERT of 60.3 ± 15.2 months (Table X and Figure 1, Paper III).

Table X: Laboratory assessments

No	Age/ Gender	Time of biopsy (y)	ACR (mg/mmol)	PCR (mg/mmol)	mGFR (ml/min/1.73 $m^{2)}$	pGL3 (µmol/L)	uGL3 (mol/mol)
1	7	0	4.80	28.3	106	7.5	3.5
	M	5	1.00	17.3	97	5.0	0.32
2	11	0	3.80	13.0	120	9.3	1.7
	M	3	1.30	13.5	124	3.9	0.65
		5	1.20	9.2	123	4.6	1.12
3	11	0	6.00	< 0.150 g/L	105	2.6	0.3
	F	5	0.60	9.8	95	3.6	0.09
4	12	0	0.59	9.0	103	8.1	5.0
	M	5	0.60	9.6	110	3.9	0.79
5	16	-2	0.003µg/min	NA	91*	11.3	7.4
	M	0*	6.40	11.8	107	5.1	0.73
		1	1.40	12.6	112	4.2	0.64
		5	1.60	8.9	115	3.2	0.09
6	16	0	1.30	10.4	112	13.4	1.8
	M	3	0.90	19.2	112	5.2	0.24
		5	1.00	11.2	105	7.2	0.71
7	17	-2	0.019 µg/min	NA	93*	9.8	21.6
	M	0*	3.00	10.5	112	7.5	0.83
		1	1.50	11.7	110	4.1	0.36
		5	19.50	28.2	111	7.3	0.15
8	17	0	2.60	8.5	99	9.0	1.0
	M	1	1.30	11.9	107	4.9	0.94
9	18	0	11.80	28.4	96	4.8	4.5
	M	1	5.60	12.7	93	NA	NA
		5	8.90	17.1	97	2.3	0.03
10	23	0	13.60	27.5	113	8.5	3.6
	M	1	6.90	15.0	123	8.4	3.4
		5	12.00	25.0	127	3.5	7.7
11	30	0	7.20	25.1	86	10.2	8.6
	M	1	4.50	14.3	88	NA	NA
		5	7.70	23.7	82	4.1	0.46
12	33	-3	U-dipstick neg	U-dipstick neg	131*	13.1	NA
	M	0#	3.60	15.4	111	5.9	1.9
		5	3.20	17.3	101	5.4	1.07

ACR: albumine-creatinine-ratio, ref <2.5 mg/mmol, PCR: protein-creatinine-ratio ref < 20 mg/mmol, pGL3: plasma-triohexosid, ref 1.6-3.3 µmol/L, uGL3: urine-trihexosid, ref < 0.6 mol/mol * eGFR in child new "bedside"Schwartz formula, in adult CKD-EPI formula.

Normalization of microalbuminuria was found in five patients ≤ 17 years, two remained normoalbuminuric and four patients ≥ 18 years had stable albumin-creatinine-ratio (Table X and Figure 1, Paper III). One 17 years old male (patient 7) had an increase of microalbuminuria and de novo proteinuria (Table X and Figure 1, Paper III) due to combined glomerular and tubular damage shown by protein

classification. Two of four patients that had slightly elevated protein-creatinine ratio (25.1-28.4 mg/mmol) at baseline (Table X and Table 1, Paper III) normalized their proteinuria and the other two stayed in the same level. 41.7 % (5/12 patients) of the urinary GL3-values and 18.2 % (2/11 patients) of the plasma GL3-values normalized on ERT. Anti-alfagalactosidase antibodies were found only in patient 3 and 5; in both cases the titers were just above the cut-off-values and the elevations were transient.

The re-biopsies after 5 years of ERT showed complete clearance of mesangial cell inclusions and glomerular endothelial cell inclusions in all patients (Table 4, Paper III). In seven of eight of the patients who had a repeat biopsy after 1-3 years this finding was also evident at this stage. Almost complete clearance of the podocytes was found in one patient, a male patient aged seven years at start of ERT (Figure 2-4 and Table 5, Paper III and Figure X), and substantial clearance was found in another three patients. Gradual decrease in podocyte inclusions in biopsies after 1 and 5 years ERT was seen in patient 5 (Table IX, Table V, Paper III). Significant correlation of the decrease of podocyte scores was found with cumulative agalsidase dose as well as with change in ACR (Table XI and Figure 2 and 4, Paper III). No significant correlation was found between changes in podocyte scores and duration of treatment, clinical scores, age, or change of podocyte effacement.

Patients in Group 2 (“high dose”) had a significant decrease of podocyte inclusions ($p=0.037$). The four patients with the highest ERT-doses (patients 1, 3, 5 and 9) had a decrease in podocytes-GL3-score of 2.84 ($p=0.010$), in composite podocyte-score of 4.5 ($p = 0.018$) and in ACR of 4.2 mg/mmol ($p = 0.005$) and this change was significant greater compared with Group 1, $p=0.004$, $p= 0.010$ and $p = 0.011$ respectively (Table 5, Paper III). The four patients with the greatest decrease in podocyte-GL3-score showed concomitant reduction of podocyte effacement. Among the patients with nearly no change of podocyte-GL3-score only two patients had less podocyte effacement (patients 8 and 11). The change of urinary albumin-creatinine ratio correlated significantly with the change in podocyte-GL3-score ($r=0.837$, $p=0.001$) as well as with the composite podocyte-score ($r=0.823$, $p=0.002$) (patient 7 was excluded due to mixed glomerular and tubular proteinuria) (Figure 4, Paper III).

Figure X: Stereomicroscopy of Fabry kidney tissue: A; patient 1, B; patient 2, C; patient 7, D; patient 9 (Paper III)

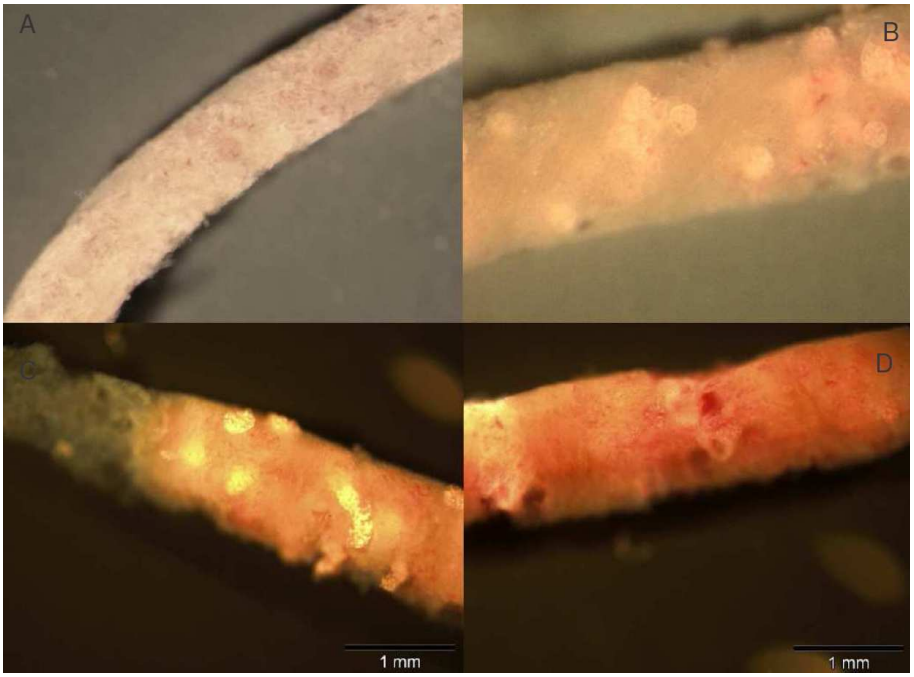


Table XI: Linear regression analysis of change in podocyte-scores vs ERT-dosis and change in albumin-creatinine-ratio after 5 years ERT.

	Change of GL3-inclusion score (toluidine-stained semithin sections)		**Change of vacuolization score (periodic-acid-schiff sections)**		**Change of composite score (inclusions and vacuolization)**	
	r	p-value	r	p-value	r	p-value
Total ERT-dose	0.804	0.002	0.736	0.006	0.783	0.003
Change of albumin-creatinine-ratio	0.837	0.001	0.775	0.005	0.782	0.003

The GL3-inclusions are given in values 0-4, the vacuolization scores are given in values 0-3, the combined inclusion- and vacuolization scores are given in values 0-7. Albumine-creatinine-ratio (ACR), reference <2.5 mg/mmol . (Patient 7 was excluded from the regression analyzis including albumin-creatinine-ration due to mixed glomerular and tubular proteinuria)

GL3-inclusions in the epithelial cells in the distal tubuli were cleared for GL3-inclusions in two patients in the final biopsies, and both patients (patient 1 and 3) were treated for 5 years with agalsidase 1.0 mg/kg/eow. In four of the five patients with global glomerular sclerosis at baseline, no global glomerular sclerosis was found in the re-biopsies, and no de novo interstitial fibrosis emerged. Generally, arteriopathy remained unchanged in the group as a whole.

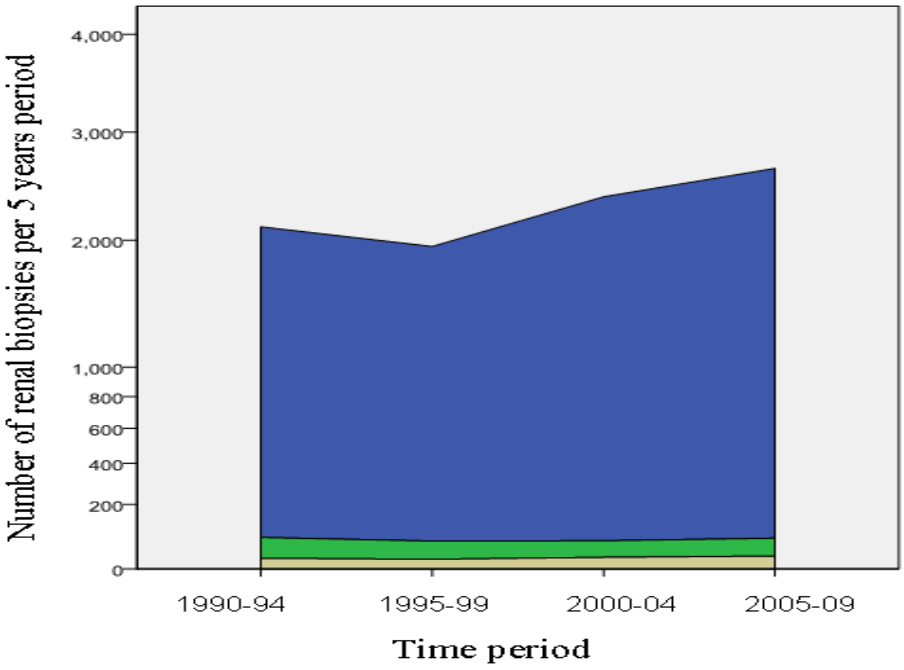
4.3. Safety and complications of renal biopsies from the Norwegian Kidney Biopsy Registry

The mean age of the 715 children included in the study was 12.0 ± 4.9, range 0.04-17.9 years, and in the 8573 adult patients the mean age was 50.6 ± 17.7, range 18.0-94.4 years. The clinical characteristics of the patients at the time of kidney biopsy were proteinuria in 81.0 %, hematuria in 68.8 %, CKD stage 3-5 in 61.6 %, nephritic syndrome in 28.7 %, acute renal failure in 18.6 % and rapidly progressive glomerulonephritis in 3.4 %. Nephrotic syndrome and hematuria were more common in children than in adults ($p<0.001$ and $p=0.001$, respectively), and chronic as well as acute renal failure were more frequent in adults ($p<0.001$) (Table 1, Paper IV).

The frequency of gross hematuria was 1.92 %, of blood transfusion 0.85 % (significantly lower in children, $p= 0.03$) and invasive procedures (surgery or angiographic embolization) 0.19 %. Complications defined as one or more of theese, were present in 2.6 % and serious complications defined as blood transfusion and/or invasive procedures were present in 0.9 % (Table 2, Paper IV). Although the number of biopsies per year increased, the frequency of complications (gross hematuria, blood transfusion and invasive procedures) remained stable throughout the whole study period (Figure XI and Figure 1, Paper IV).

Arteriovenous fistula, urosepsis and ascites leakage were reported in one case each (free text field). Death was not reported in any patients. In 97.9 % of the cases there were no complications.

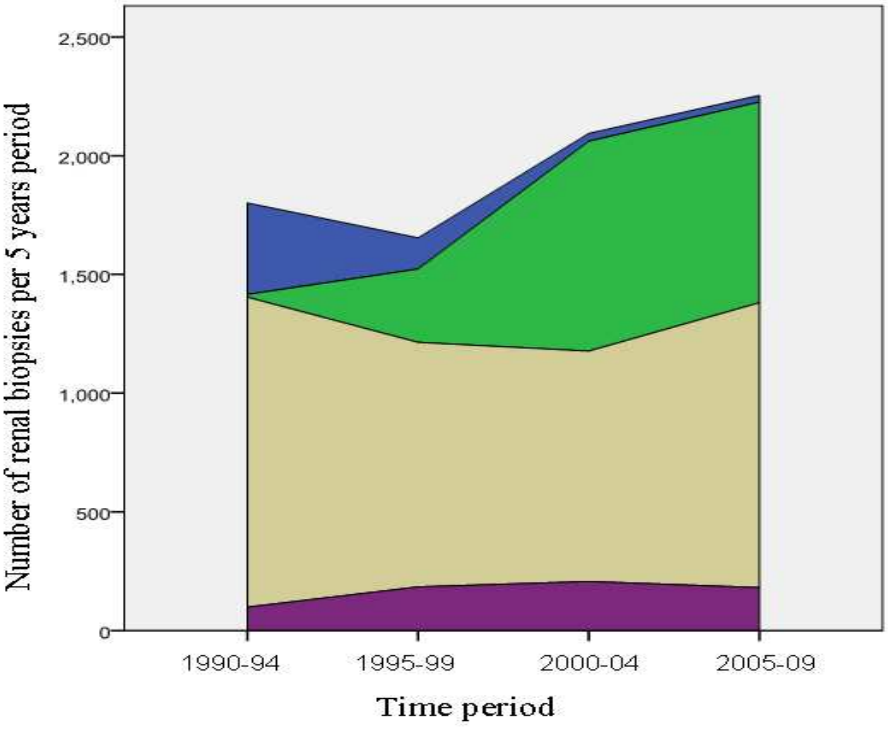
Figure XI: Kidney biopsy comlications (blue: no complications, green: gross hematuria, yellow: blood transfusion, catheterbased embolization or surgery)



In 82 % of the cases 16 G or 18 G needle was used (Table 3, Paper IV). Over time there was an overall shift from 14 G to 16 G size (Figure XII and Figure 2, Paper IV). The rate of major complications was similar across all needle types (Table 3, Paper IV), whereas a significant higher rate of gross hematuria was found in the 18 G group compared to the 16 G group.

The percentage of biopsies containing representative tissue was 94.3 % and was not related to the size of the needle. The lowest number of glomeruli was achieved when the 18 G needle was used; the mean number of glomeruli was 10.7, range 0-76, $p<0.001$. The mean number of glomeruli in all the groups together was 12.0 (range 0-86). In 3.0 % of the biopsies no glomeruli were found. In the majority of the patients only one (47.5 %) or two (37.9 %) passes were performed per biopsy procedure.

Figure XII: Kidney biopsy needle sizes (blue: 14 Gauge, green 16 Gauge, Yellow: 18 Gauge, Purple: Unknown)



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The risk factors for major complications were analyzed using logistic regression (Table 4, Paper IV). In children the relative risk was 0.4, and in the patients aged ≥ 60 years the relative risk was 2.3 when compared to adults 18-59 years old. When adjusting for eGFR there was no correlation with age. In the patients with GFR 30-59 ml/min/1.73 m^2 the relative risk was 5.0, and in the patients with GFR < 30 ml/min/1.73 m^2 the relative risk was 16.8 compared to the patients with GFR ≥ 60 ml/min/1.73 m^2. Adjusting for age the relative risks were fairly unchanged; 4.9 and 15.5 respectively. Higher blood pressures were associated with increased risk; a systolic blood pressure of 140-159 mm Hg or ≥ 160 mm Hg showed relative risks of 1.5 and 2.3 respectively. Adjusting for both eGFR and age removed the significance of higher risk of higher blood pressure (odds ratio 1.1 and 1.3 respectively). In unadjusted analyses, acute renal failure had a relative risk of 2.29, in the adjusted analyses the odds ratio was only 1.1 (non significant). When less than 650 biopsies were performed in a centre during the whole study period, the relative risk was 1.9 compared to the bigger centres (≥ 650 biopsies). In adjusted analyses the relative risks were 1.6. The level of proteinuria, diagnosis of rapidly progressive glomerulonephritis, needle size, number of passes and the type of medical specialty of the physician did not inluence the rate of major complications.

5. Discussion

5.1. Methodological considerations.

5.1.1. Functional renal measurements

5.1.1.1. Glomerular filtration rate

As in most previous Fabry disease studies the renal function in Study II was evaluated by creatinine based formula estimation of the GFR. In addition the renal function was measured with plasma clearance of iohexol for comparison of estimated GFR (eGFR) and measured "true" GFR (mGFR). To our knowledge Paper II was the first paper that presented a paired eGFR-mGFR evaluation in children with Fabry disease. This was further explored in a greater patient cohorth involving three centres in Study I. Three different mGFR methods were used that all are generally validated against the gold standard inulin-clearance and adopted as feasible modern gold standard methods (87, 89, 164-173). In addition, all centres have validated their preferred method locally and defined the accuracy (analytical variation) of the method in the respective laboratories. However, the use of different methodology of GFR-measurements is a limitation in Study I and ideally the same mGFR-methods should be used in all centres. To achieve our goal, the use of the different mGFR-methods was the only way to demonstrate the difference between mGFR and eGFR in a relatively large cohort of children with Fabry disease. Fabry disease is a rare disease and therefore in many studies cooperation between centres in several countries is necessary to obtain a meaningful sample size. We believe our comparative data are valid because of similar validated accuracy of the three methods. Secondly, the median values for bias for the different equations were similar in all three cohorts in spite of the different methods used. We believe that the finding of similar normal GFR-levels and stable pattern over time strengthens our results.

The single-point Iohexol method used in the Fabry patients followed in Bergen is shown to correlate well with multiple point methods (89, 164-166), but there is a higher risk of outliers in the single-point method. Preliminary results in a Norwegian multiple-point iohexol study in children show that the single-point method is adequate in children above 10 years with various chronic kidney diseases (174).

The “blind range” of serum creatinine is an important issue in the validation of creatinine based GFR methods, although attenuated this pitfall is not abolished by the use of creatinine-based GFR formulas. The renal function may be reduced as much as about 50 % before the serum creatinine value is above the upper reference value (175). One of our main objectives in this study was to highlight the fact that creatinine-based eGFR-equations, which have been the routine methods for follow-up of patients with kidney disease, have important limitations and should be used with care. The early identification of subtle decline of GFR is the most important point, since this may be a critical limit for the initiation of enzyme replacement therapy .

The complex Schwartz formula includes cystatin C and blood urea nitrogen (BUN) in addition to creatinine, height and age (99). Many centres, including the three centres in Study I, do not have analyses of cystatin C in the routine, and in Fabry studies, i.e. published clinical trials and international Fabry Registries (Fabry Outcome Survey and Fabry Registry) cystatin C has until now not been validated. This is a relevant issue, since many Fabry patients have low body mass index that may influence the accuracy of current GFR-equations. Although the accuracy of the complex Schwartz formula is superior to the abbreviated formula, there still is a relatively great imprecision; 87.7 % within 30 % of mGFR, and only 45.6 % within 10 % (P10) (the values for the abbreviated Schwartz formula were 79.4 % and 37.0 % respectively). As plus-minus 30 % means a high absolute value in the normal ranges of GFR, the value within 10 % is important in this context; a change in GFR of 10-20 ml/min/1.73 m^2 would be highly relevant in the follow up of a child with Fabry disease. In our view the relatively low 10 % precision (P10) of both formulas is a matter of concern, and implies that the risk of missing an early decline in true GFR is relatively high.

Again, this fact strengthens our routinely use of a more precise GFR method (iohexolclearance). Our definition and evaluation of bias, precision and accuracy are in line with others (99, 176, 177).

There are studies that show that cystatin C-based formulas (94, 95) or formulas that combine cystatin C and creatinine (99, 178, 179) are superior to creatinine-based formulas alone. In studies of individuals with normal GFR without diabetes and/or cardiovascular disease cystatin C has not proved superior to creatinine (180), and the diagnostic accuracy of different cystatin C-equations varies with GFR-level (181). Comparison with cystatin C and cystatin C-derived equations is generally highly relevant when all available and refined GFR methods are compared, especially in children with low normal or marginally decreased GFR, which is a main focus in the follow-up of renal disease in Fabry patients. However, the feasibility of the cystatin C derived methods is under debate, and importantly, no reference method or uniform calibration material exists for cystatin C. In addition, the method is more expensive and certainly not a routine method in many hospitals (182, 183). This is in contrast to the routinely used creatinine-based equations that now are based on standardized analyses (IDMS-calibrated) which render comparisons with gold-standard methods more conclusive. In particular, the utility of modern cystatin C methods deserves specific clarification in Fabry populations. Unfortunatly, we did not have cystatin C measurements in our study.

Few patients had three or more GFR-measurements in Study I and the follow-up period was relatively short which limits the value of comparison between repeated measured and estimated GFR measurements. Three or more paired mGFR-eGFR measurements were available for 13 patients. These patients all had stable renal function and we think that the minor variations seen in repeated measurements are due to analytical and biological variation. The concept of critical difference was used to evaluate the difference observed between repeated measurements, and defines the minimal difference needed between two results to conclude that there is a true difference not due to the within-subject biological variation and the analytical

variation (161). This is a valuable tool in the assessment of clinically important changes.

5.1.1.2. Albuminuria

Three morning void urine samples (three consecutive days) were collected from the patients and the median value was used to obtain a standardized procedure and avoid the problems of intra-individual variation in urine albumine excretion. The use of the mean value would introduce the risk of results indicating microalbuminuria based on one single outlier. The cut-off value of our clinical laboratory was > 2.5 mg/mmol creatinine both for men and women (111). Due to the higher muscle mass in males some centres recommend a cut-off value of > 3.4 mg/mmol creatinine in females (112). On the other hand, only one female patient in Study II and Study III had microalbuminuria (ACR 6 mg/mmol). Gubler et al. reported 3 pediatric cases (8, 11 and12 years old) with 0 mg/min proteinuria (2). Four of their adult patients (above 20 yr) had proteinuria in excess of 0.6 mg/min (720 mg/24 h)(2). Probably microalbuminuria was not measured, and they do not describe the protein method used. Generally, protein assays are less sensitive than albumin methods.

Three patients (patient 5, 7 and 11) received treatment with angiotensinII-receptor-antagonist after the baseline biopsy. Despite this additional renoprotective treatment the ACR-values increased in one (patient 7), whereas the microalbuminuria normalized in one (patient 5) and remained stable in one (patient 11). Consequently, the use of angiotensinII-receptor-antagonist did not influence microalbuminuria in an unequivocal direction in these few cases. We do not believe that these cases have critical influence on our results.

5.1.2. Kidney biopsies

5.1.2.1. Kidney biopsies in Fabry disease

A limited number of Fabry disease patients were studied (in total 13 unique patients) in Study II-III. The rather small number of patients in our studies were a consequence of the rarity of Fabry disease and the fact that both studies were single-centre studies based on the consecutive inclusion of patients referred to the specialist team at Haukeland University Hospital. The Fabry Group at Haukeland University Hospital shares with Oslo University Hospital the responsibility for diagnosis and treatment of patients with Fabry disease in Norway (46). The diagnosis and follow-up of patients is performed in line with our local Fabry-protocol that includes a multispecialist team. The recommended examinations include initial mutation analysis and specific enzyme and glycosphingolipid analyses, routine laboratory tests, renal function tests, cardiac examinations including echocardiography and cardiac and cerebral MRI, audiogram, eye examination and neurologic examinations. Kidney biopsies may sometimes be of decisive diagnostic importance in patients with unknown kidney disease, and is recommended in the local Fabry-protocol to assess baseline kidney damage in patients where ERT is initiated. Because of the scarcity of scientific data in patients on ERT with early nephropathy and low total disease burden, we wanted to include all available patients in a systematic review of morphologic and clinical data to obtain new knowledge of indication and effect of ERT. All patients were enrolled on a consecutive basis, and choice of enzyme treatment (Paper III) was balanced to gain experience with both available medications (agalsidase alfa and agalsidase beta) in their respective licensed doses.

The majority of clinical trials in children with Fabry disease treated with ERT have mainly focused on safety aspects as well as quality of life and renal biopsies have not been done in any previous studies (184-187). Only a few pediatric Fabry cases with renal biopsies have been reported in the literature, and they were all without ERT (2). Generally, there has been a reluctance to perform renal biopsies in Fabry disease children in many countries, whereas in Norway the threshold for renal biopsy has

been lower based on clinical experience and safety data from the Norwegian Kidney Biopsy Registry (135, 143). The ERT is financed through the public health care system in Norway, and the treatment is evaluated through yearly applications to HELFO (www.helfo.no) from the responsible doctors on behalf of the different patients.

All pediatric Fabry disease patients on ERT in Norway are followed at Haukeland University Hospital, and the patients were recruited to the Study II and III on a consecutive basis. The inclusion also of young adult males with mild renal disease in Study III allowed statistical comparisons between various treatment regimens in a patient cohort of still relatively young patients with rather low total disease burden and without evidence of irreversible organ damage. Such a patient cohort with rather "early disease" is markedly different from the many other long-term follow-up studies comprising many patients with more advanced disease (49, 188-191). There is a lack of studies that evaluate disease progression and ERT effectiveness in young patients, and no clear guidelines have defined a proper timing when ERT should be initiated. Ideally, a randomized double blind clinical trial should have been performed to compare doses and drugs in Fabry disease. However, such randomized double blind clinical trials are difficult to perform for many reasons; Fabry disease is a multisystemic disorder with a mixture of phenotypes, the drugs have been on the marked for more than ten years, and the treatment is subject to local experience. No such trials have yet been demanded from the health authorites.

Our re-biopsy study was an observational study taking into account the fact that both preparations have been available in Norway since 2001 (32, 192), and many of the patients were involved together with the doctors in the choice of treatment drug. The different dosing regimens are subject to different infusion procedures which also have practical implications for the patients; standard dose agalsidase alfa is normally given over 40 minutes whereas standard dose agalsidase beta initially is given over 4-5 hours reduced over months to a minimum of 1.5 hours (46).

After more than ten years of ERT there still is uncertainty as to the equipotency of the two available enzyme preparations (agalsidase alfa and agalsidase beta) in their licensed doses, although several experimental studies and clinical observations suggest that they may be similar mg per mg (53-56, 58, 62, 193). Both Blom et al. and Lee et al. have shown functionally indistinguishability between the two preparations (51, 194). Sakuraba found a higher enzyme activity by agalsidase beta in cultured human Fabry fibroblasts and kidney, heart and spleen when the same doses were given of agasidase alfa and agalsidase beta (52). Our hypothesis was that morphologic examinations of various kidney cells before and after ERT for a sufficiently long time period might have the capacity to discover dose-dependent differences in the clearing of GL3-deposits. The potential biomarker role of kidney biopsies in Fabry disease is even more important since this method has a potential for discovery and grading of reversible damage and treatment effects (GL3-inclusions) in a phase of clinical silence (normoalbuminuria and normal serum creatinine). Moreover, if such important treatment decisions should be delayed until manifest proteinuria or progressive kidney dysfunction (GFR < 60 ml/min/1.73 m^2) occur, many patients will be in a phase where treatment is ineffective or suboptimal with uncertain long-term ERT benefits (49).

The scoring of the renal biopsies in clinical trials in adults supported by either Genzyme or Shire/TKT 5S has been performed by three different pathologists (44, 45, 48-50, 195). For our single centre observational studies, not supported by the industry, one experienced independent renal pathologist was responsible for the consecutive evaluation of the renal biopsies and this pathologist was blinded to which kind of medical treatment the patients received. In Study III a second renal pathologist blinded for the identity, treatment and order of the biopsies to secure unbiased scoring, re-scored the GL3-inclusions in the podocytes using the scoring system of the International Study Group of Fabry Nephropathy (ISGFN) from 2010 (160). Both the scoring of the GL3-inclusions in the podocytes evaluated in McDowell-embedded tissue as semithin toluidine-blue sections as well as the scoring of the podocyte-vacuoles in PAS-sections from paraffin-embedded tissue was

included in the examinations of the biopsies. The paper of the ISFGN did not combine the scoring of the GL3-inclusions in the toluidine stained sections and the scoring of the vacuolization in the PAS-sections (5). Due to the fact that the vacuolization in the PAS-sections represent the space where the GL3-lipid was located before the fixative was applied, the additional information from the PAS-sections is a valuable supplement to the information found in the toluidine-stained semi-thin sections. We therefore postulate that the use of a "composite score" introduce a higher degree of representativity since the scoring then is based on a substantially higher number of unique glomeruli. Based on this, we chose to expand the scoring methods reported by ISGFN and included a "composite score" to take advantage of as many scorable glomeruli as possible. This is an important issue since sampling error always is a confounder in the characterization of renal morphology, especially in the assessment of non-homogenous changes.

A separate table (Table 5, Paper III) shows the scoring of the GL3-inclusions in the toluidine-stained semithin sections (0-4) and the scoring of the vacuolization in the PAS-sections (0-3). It is a reassuring finding in our study that both scoring methods showed similar significant dose-dependent effects in the treatment induced reduction of deposits. In this table, as well as in Table 6, Paper III, we also chose to include the composite score (0-7), and we suggest that the combination of the two scores (GL3-inclusions and vacuolizations) is a more valid marker of renal disease progression and a potential sensitive biomarker for assessment of adequacy of ERT in terms of renal morphologic damage. The mean number of scorable glomeruli in PAS-sections at baseline was 18.8 ± 7.8 and after five years 15.3 ± 6.4, and in the semithin blue stained sections 9.3 ± 8.2 and 6.0 ± 3.9, respectively.

The semiquantitative method used to score the biopsies has been a standard in evaluation of Fabry disease in line with many other kidney diseases (2, 5, 48, 49, 196). The purpose of the ISFGN-paper was to develop a standardized scoring system of baseline kidney biopsies with potential use also for longitudinal assessment of prognosis and responses to therapy. The podocyte scores (both vacuoles and

inclusions) were among the most robustly scored elements with reasonably well agreement between nephropathologists (5). Our Paper III is the first study where this scoring method has been evaluated for follow-up, and confirmatory studies are needed. However, the ISFGN-paper is the only validated evaluation of a standardized scoring system of Fabry disease specific renal pathology.

A more quantitative analysis of the number of inclusion bodies and assessment of differences in size and structure at least at the level of semi-thin plastic sections, in the various glomerular cells, in selected segments of the tubules, endothelial cells in peritubular capillaries, and the cells of the vascular wall would have been of interest, but this is not an available routine method yet. A stereologic morphometric methodology has recently been tested in Fabry patients not treated with ERT (64), and for finer staging and more detailed evaluation of dose-response effectsof ERT, morphometric methods will probably give valuable additional information. On the other hand, this issue will also be subject to considerable research and need methodologic evaluations in larger patient groups. Electronmicrographic examinations are normally based on only one glomerulus, and the issue of representativity is an argument against giving electron micrograph based on a single glomerulus high weighting in this context.

Because of the limited number of patients we chose to compare one “low dose” group and one “high dose” group, the latter also included three patients with “intermediate dose” (0.4 mg/kg/eow). Given the assumption that a dose-dependent effect exists and that the drugs are similar on a mg-to-mg basis, this is in our opinion the only reasonable way of dividing these patients for initial comparison. We believe the renal response of treatment is hard to evaluate in a sufficiently accurate manner based on eGFR and urine tests alone due to the insensitivity of these methods (e.g. creatinine blind range) (103, 197) and the fact that Fabry disease per se is a disease that varies very much even in male patients with the same mutation. Morphologic characteristics also have the potential to predict future development of albuminuria or loss of renal function and morphological changes are probably the earliest sign of renal damage

that could be identified in these patients. In a recent review of renal complications in Fabry disease in children the importance of kidney biopsies in the evaluation of ERT is highlighted (198)

The small number of patients limited the statistical analyses. The statistical methods were used in a strict regulated way with stepwise evaluation with normality tests, test of equality of variances with use of corresponding values and methods. Correlation statistics between a semiquantitative and quantitative variable has limitations as the p-values often not will be significant. In our case where the p-values are highly significant, we believe that the statistics are adequate.

5.1.2.2. Norwegian Kidney Biopsy Registry

The Norwegian Kidney Biopsy Registry was founded in 1988 and is currently probably the biggest active kidney biopsy registry in the world with more than 11000 biopsies. The registry is basically a quality registry designed for epidemiological research, and our study is the first study of biopsy safety and procedure related complications in Norway. A major strength of our safety study (Paper IV) is that it is based on high numbers of both pediatric and adult cases over a long period of time. The previous publications with comparable numbers are based on review of different mainly small single centre studies (139, 140). An overview over studies during the last 20 years that were not included in the review-articles from Parrish and Korbet are shown in Table III in the introduction of this thesis. Many authors have reported non symptomatic hematoma and gross hematuria together with serious complications such as the need of blood transfusions, catheter based embolization and surgery when discussing major versus minor complications (139, 140, 155). The inclusion of different kinds of hospitals in our study; comprising university hospitals with many biopsies and small local hospitals performing very few biopsies, gives a nationwide perspective with relative homogenous clinical biopsy practice different from the majority of previous litererature based on data from larger single centres.

The validity and sensitivity of the reporting system in the Norwegian Kidney Biopsy Registry may be characterized as high. Only patients that consented to inclusion were included. All nephrologists and renal pathologists are asked to admit all available cases, and the inclusion rate in general is high. Due to Norwegian privacy legislation and no other national way to register the native kidney biopsies in Norway, it is not possible to find out exactly how many patients did not sign informed consent or were not reported to the Norwegian Kidney Biopsy Registry by the responsible doctor. Concerning the sensitivity of the report of complication, cases were excluded when the report form was incomplete. Based on the consistency of reporting quality data among Norwegian nephrologists we believe it is reasonable to anticipate that if a major complication would have been present, the responsible doctor would likely have reported such information. The individuals in the study were already 'pre-screened' (general contraindications to kidney biopsies are well known among nephrologists), giving a study of safety in patients for whom the risk of biopsy was found lower than the potential benefit. There are no absolute exclusion criteria in terms of age or eGFR.

We believe that registered clinical and histopathological data from the Norwegian Kidney Biopsy Registry are representative of the entire population as nearly all biopsies performed in Norway are included in the Norwegian Kidney Biopsy Registry, and the health system in Norway is public and available for free for all inhabitants. There is no information in the reports about race, ethnicity and socioeconomic status. For the time period 1988-2010 the mean yearly proportion of Caucasians was 97 % in the Norwegian population (199). There has been an increase of biopsies in general in elderly people in line with general improved health and care for elderly. Generally, indications for biopsies are guided by potential benefit. 5.9 % of the biopsies in the study period were rebiopsies due to 5.6 % of the patients having more than one biopsy performed during the study period. As this percentage is low, we believe nclusion of the same individuals more than once has not an important influence on the data.

Currently there is a debate in the healthcare system if ultrasound guided percutaneous renal biopsy should be conducted in a day care setting for low risk patients. This organisational change is based on the fact that great majority of complications occur within the first 12 hours post biopsy(149). We have not analysed the time span from procedure to a complication or day care activity in our study as this information was not available in the Norwegian Kidney Biopsy Registry in the time period studied. Potential reporting bias and the lack of detailed information about other potential complications not pre-specified in the registry report form are limitations in our study.

In the analyses of risk factors for having major complications, we used logistic regression analyses. These analyses can be adjusted for possible confoundes and give a measure of relative risk associated with the given risk factors. The importance of adjusted analyses was clearly demonstrated as only two variables were significant after adjustments, low eGFR and small centre size. This means that the apparent risk in subjects with high blood pressure or older age (significant in the unadjusted analyses) could be accounted for by low eGFR. This does of course not mean that blood pressure is irrelevant, but when adequately taken into account at the time of the procedure, the blood pressure seems to be less important than might have been expected, and does not seem to be a significant risk factor. As the major outcome in the study was relatively rare, it is important to keep the number of adjustment factors as low as possible and we therefore chose to only adjust for categories of age and GFR

5.2. Discussion of the findings

5.2.1. Renal evaluation in young Fabry disease patients

5.2.1.2. Glomerular fitration rate in pediatric Fabry disease

The main finding of Study I is that the widely used original Schwartz formula (91, 92) overestimated measured GFR substantially by 50.6 ml/min/1.73 m^2 with a very low accuracy of only 4 % of the values within ± 10 % of the value achieved with measured GFR. The same tendency was found in the the comparison of eGFR and mGFR measurements in the preceeding Study II. The original Schwartz formula is the GFR-equation that for many years has been used in the international Fabry registries, Fabry Outcome Survey and Fabry Registry, registries that have important world wide impact on prognosis estimates and treatment decisions. The use of this formula has made authors report high occurrence of hyperfiltration in Fabry disease both in data from the registries as well as from clinical studies (33, 200). The new abbreviated Schwartz formula published in 2009 (99) showed the best performances of the tested equations in our study with a mean GFR overestimation of 5.3 ml/min/1.73m^2 and 79 % of the eGFR calculated within ± 20 % of measured GFR and 49 % within ± 10 %. The Counahan-Barratt formula (90) was only slightly inferior to the new abbreviated Schwartz formula, due to a minor difference between the quotients in the two formulas. Our findings of the very low accuracy of the original Schwartz-formula in children with Fabry disease have contributed to a change to the more accurate new abbreviated Schwartz formula in the international Fabry registries as well as in clinical trials in pediatric Fabry disease (34, 198, 201, 202).

Less than half of the estimated GFR calculations by the new abbreviated Schwartz equation were within ± 10 % of the measured GFR in our cohort with normal mGFR. It is well known that serum creatinine and all creatinine based GFR equations are hampered by inaccuracy in the “creatinine-blind window”. This fact and our results show that the use of measured GFR should be part of the routine assessment of the

kidney function in Fabry children and young adults, since this method is more accurate and reduces the risk of missing the diagnosis of early decline of GFR. This is especially clinically important when it comes to decisions of initiation of ERT; several studies have shown that the effect on nephropathy is attenuated when ERT is delayed until the patients reaches CKD stage III (GFR < 60 ml/min/1.73 m^2) (34).

The slightly higher mGFR values found in the Dutch group (not statistically different from the Norwegian cohort) could be due to a true difference in GFR in this relatively small group (11 patients) of slightly older children (compared to the UK group) and mostly female (9 of 11) patients (with corresponding slightly lower creatinine values; Table 1, Paper I). In fact, Vedder et al. (61) demonstrated in the Dutch Fabry cohort that glomerular hyperfiltration was more frequent in the female group compared to male patients; as many as 78 % of female patients had hyperfiltration defined in that study as GFR > 125 ml/min. The same Iothalamate-method was used in the Dutch cohort in our study and the quality of the method is documented by Apperloo et al. (167). Secondly, a systematic bias of Iothalamate clearance is conceivable since Iothalamate is subject to minor tubular secretion (203). More important, our finding of similar median values for bias for the different equations in the three patients groups in spite of the different methods used argues against a systematic bias in our study.

Patient 3 had a great discrepancy between the first mGFR and eGFR, whereas the second and the third pair of measurements corresponded pretty well. Two reasons for these discrepancies are possible: An analytical random error probably explains the difference seen at the first pair of measurements. This patient had serious Fabry related akroparestesias, and after introduction of ERT quality of life improved substantially. As a consequence, a potential increase of muscle mass due to more physical activity may explain better correspondence between GFR methods (higher and more correct eGFR) at the second and third GFR timepoint. It has been shown by others that body composition (lean patients) may lead to overestimation by eGFR (177). This case also illustrates the importance of using exact GFR methods in the

evaluation of GFR in Fabry disease patients, especially male patients tend to have lower BMI.

Importantly, a major conclusion in our studies is that probably all GFR-methods (and especially the estimated methods), as well as the identification of increasing albumin excretion, are “late methods” compared with renal biopsy in the diagnosis of early CKD-progression in Fabry disease. Therefore, we think that the use of the best available GFR method i.e. mGFR at defined intervals is mandatory in addition to a kidney biopsy in the routine monitoring of early Fabry nephropathy when ERT is considered.

5.2.1.2. Renal biopsies and effect of enzyme replacement therapy

Study II was the first kidney biopsy study including male Fabry disease children with minimal albuminuria (2, 34), and showed substantial GL3-accumulation in multiple celltypes as well as podocyte effacement in all patients. The finding of prominent glomerular and vascular morphologic changes in children with Fabry disease long before decrease in GFR and development of albuminuria/proteinuria has added impotant information to the litterature. Further studies in young Fabry disease patients have shown significant correlation between GL3-accumulation and patient age and suggest that the cellular damage progresses with increasing age (64). Our patient cohort is young and consists of patients with low total disease burden, essentially before organ dysfunction is manifest, in a phase where valid biomarkers of disease progression currently are unavailable. As a consequence, this cohort represents patients with early disease where enzyme replacement therapy (ERT) may have a potential for preventing progressive kidney disease.

Study III is the first systematic re-biopsy study after long-term ERT in children. It is also the first report of almost complete clearance of podocyte deposits in a young boy after 5 years ERT with agalsidase beta 1.0 mg/kg/ every other week (Figure 3, Paper III). Previous studies have demonstrated the capacity of agalsidase beta to reduce GL3-inclusions in podocytes (44, 48, 49). Our study is the first time re-biopsies after

long term ERT with agalsidase alfa have been studied in a cohort. Our demonstration of a cumulative dose effect of the available drugs, irrespective of drug preparation is new, and indicates that kidney re-biopsies after 5 years of ERT may be relevant to evaluate disease progression and treatment effect as well as allowing for dose adjustments if failure of cellular clearance or progressive disease is found. Deterioration of GFR was shown in the only available head-to-head comparison of agalsidase beta 0.2 mg/kg/eow and agalsidase alfa 0.2 mg/kg/eow over 24 months in 21 patients with mostly CKD stage I (55) . No kidney biopsies were performed in that study. Further studies are needed to clarify the issue; of dose-dependent morphologic beneficial effects, and also whether disease duration (patient age) or burden of total disease influence the efficacy of drug- induced cellular clearance of GL3-deposits.

To our knowledge, Paper III is the first demonstration that partial GL3-clearance of podocytes is achieveable with standard or double dose agalsidase alfa (0.2-0.4 mg/kg/eow), as shown in patient 4 (slight clearance) and 5 (substantial clearance) (Table 5, Paper III). It is also the first demonstration of total clearance of GL3 in mesangial cells. In the randomized controlled trial of agalsidase alfa published in 2001, 14 patients received agalsidase alfa for six months and twelve of these had a renal biopsy. In this trial there was no significant change in total GL3-score in the kidneys, but a significant reduction in mesangial widening and vascular endothelium GL3-deposits was shown (45). Renal biopsies have not been included in long-term trials with agalsidase alfa (193, 204). Schiffmann et al. published a case report after treatment with agalsidase alfa for 2.5 years in a 47 years old man who died, and post mortem examinations showed lack of GL3 in the vascular endothelium, wheras all other kidney cells showed extensive GL3-accumulation (205). In our study we found a highly significant correlation between GL3-clearance in the podocytes and cumulative alfa-galactosidase dose (Figure 2, Paper III), which may indicate a continuous clearance over time. Our findings are in line with previous preclinical studies (206) and early phase clinical studies (43) showing a dose-response effect that indicates that higher doses are needed to enter the podocytes. In a recent case report GL3-deposits remained unchanged in podocytes in Fabry donor kidneys for up to 12

years after transplantation despite normal levels of native alfa-Galactosidase in two kidney recipients without Fabry disease (207). ERT in patients is different from the situation the natural non-Fabry-case and ERT results in high peeks when the infusion is given as infusion every two weeks. ERT has a short plasma half-life, but longer tissue half-life (42). An important observation in our study was that at least in some of the patients treatment with agalsidase alfa in standard or dobbel dose (0.2 mg/kg/eow or 0.4 mg/kg/eow) was sufficient to achieve some clearance in the podocytes. AN important observation in our cohort was that no patients showed increasing morphologic damage over the 5 year ERT period, and GFR was stable in all patients.

Moreover, our study supports and expands the dose-dependent effects suggested by Lubanda et al. who demonstrated morphologic improvement across many renal cell types including podocytes after treatment with agalsidase beta 1.0 mg/kg/eow for 6 months, and a composite of renal scores was maintained after dose reduction to 0.3 mg/kg/eow in a subset of proteinuric patients with CKD stage 1 or 2 for a period of one year, while progressive cellular damage was observed in some patients after dose reduction (50). A more robust decline in urinary GL3 has also been observed when higher enzyme dose (agalsidase beta 1.0 mg/kg) was given to patients with neutralizing antibodies (56). Recently, an increase of plasma lyso-Gb3 in 17 clinically stable Fabry disease patients was observed one year after a switch from agalsidase beta 1.0 mg/kg/eow to agalsidase alfa 0.2 mg/kg/eow or upon dose reduction of agalsidase beta, further indicating a dose-response relationship (58, 62).

In our study (Paper III) all patients that had substantial clearance of the GL3 in the podocytes (three patients treated 5 years with agalsidase beta 1.0 mg/kg/eow and one patient treated with agalasidase alfa 0.4 mg/kg/eow for four years followed by agalsidase beta 1.0 mg/kg/eow for one year) also showed less podocyte effacement. Among the eight patients with mainly unchanged podocyte-GL3 deposits (agalsidase 0.2 or 0.4 mg/kg/eow) two patients had less podocyte effacement. The partial clearance of podocyte GL3 in three of four patients receiving agalsidase beta 1.0

mg/kg/eow for the whole or part of the study period is similar to what was shown in long-term agalsidase beta treatment by Germain et al. (49). The fourth high-dose (1.0 mg/kg/eow) patient being the youngest in the study showed almost complete clearance of GL3 in the podocytes at the age of 12 years after 5 years of agalsidase beta 1.0 mg/kg/eow (Figure 3, Paper III). There is no other case in the literature demonstrating high levels of GL3-deposits in the podocytes before start of ERT and almost normal looking podocytes after long-term ERT. Whether this conspicuous beneficial finding is due to early treatment of a very young patient or specific genetic factors or combination of factors, remains to be shown.

In the Phase III trial in agalsidase beta treated patients (1.0 mg/kg/eow) re-biopsies from the kidneys showed that GL3 deposits were cleared after five months (19 patients) in 100 % of the glomerular capillary endothelial cells and 100 % of the mesangial cells (48). The placebo-group that switched to active treatment after five months also showed a 100 % clearance after 6 months agalsidase beta in the glomerular capillary endothelial cells and 90 % of the mesangial cells. The podocytes showed a reduction (partial clearance) in 18 % after 11 months of treatment and in 50 % of the tubular epithelial cells. Another study from Japan with renal biopsies after a 5 months trial in 13 male Fabry patients on agalsidase beta confirmed the findings from the Phase III-trial (44, 195) showing complete clearance of the endothelial cells.

In our renal biopsy studies (Paper II and III) we used the scoring method published by the International Study Group of Fabry Nephropathy (ISGFN) (5). This is the first time this model for scoring of potential prognostic histologic markers has been tested in a longitudinal study (Paper III). In line with the scoring system of the ISGFN, we did not evaluate partial clearance of GL3 in the distal tubuli. On the other hand, complete clearance of the distal tubular endothelial cells inclusion was found in two of our patients and both were treated with agalsidase beta 1.0 mg/kg/eow for five years (patient 1 and 3).

As a consequence, it seems that all treatment regimens have the capacity to clear GL3 deposits in endothelial and mesangial cells, whereas only higher enzyme doses (0.4-1.0 mg/kg/eow) result in substantial reduction of Gl3-accumulation in the podocytes. The effect on podocytes, a crucial cell in the glomerular filtration barrier, may be a more relevant cellular biomarker regarding progression of Fabry nephropathy than endothelial or mesangial cells. This concept is further strengthened in our study by the very intriguing observation of a clear structural - functional relationship; we found concomitant reduction of ACR and podocyte GL3-clearance in the patients treated with the highest enzyme dose (Figure 4, Paper III). The clear dose-dependent effect on clearance of GL3-deposits from podocytes may indicate that the licensed doses of the two available drugs (agalsidase alfa 0.2 mg/kg/eow and agalsidase beta 1.0 mg/kg/eow) are not clinically equipotent, at least not when it comes to renal effects, and supports previous experimental (51, 52) and clinical (55) observations of a similar mg-to-mg potency. Our findings have to be confirmed by others in a larger patient cohort before any firm conclusions can be drawn, and probably by more sophisticated morphometric methods describing podocyte size and fractional volume of deposits in line with the studies reported by Najafian et al. (64). On the other hand, if clear morphologic deterioration is found after 5 years, we think enzyme dose increase is appropriate in most early case patients. Individualizing of dosing regimens with the possibility of either increase or decrease of dose may be a new and relevant treatment strategy if podocyte damage proves to be a relevant biomarker of progressive disease, and we suggest that a routine repeat kidney biopsy after about 5 years should be implemented in the surveillance program for young Fabry disease patients with likely treatable nephropathy.

5.2.2. Safety aspects of percutaneous kidney biopsies

In Studies II-III there were no cases with severe complication after renal biopsy. Due to a general fear and resistance to perform kidney biopsies especially in children in many centres, no previous biopsy data have been published in Fabry children on ERT (208). To explore the risk of complications after kidney biopsies in a large patient cohort we decided to perform a study based on data from 9288 biopsies registered in the Norwegian Kidney Biopsy Registry during over more than twenty years. The main finding in Paper IV is that a kidney biopsy can be regarded as a safe procedure in all ages when modern techniques and guidelines are used. We found that the low rate of blood transfusion (0.85 %) was even lower in children (0.14 %) than in adults (0.85 %) (p=0.03) and surgical intervention including catheterbased embolization was on similar low levels in both children (0.14 %) and adults (0.19 %)(Table 2, Paper IV). A major complication was seen in only two children. Moreover, we found lower complication rates han reported in the two main reviews in the literature: In Parrish's report from 1991 of more than 10 000 kidney biopsies performed since the 1950s a rate of interventional surgery of 0.29 % was found (139). The mortality rate in Parrish's study was 0.12 %, whereas in our study no deaths were reported. Excluding the numbers from Parrish's study Korbet reported in 2002 a rate of blood transfusions of 1.24 %, rate of surgery of 0.23 %, and a mortality rate of 0.03 % in about 7500 biopsies (140). In the additional studies we reviewed; a total of about 9500 biopsies performed the last 20 years, a higher rate of blood transfusions (1.22 %), rate of intervention (0.32 %) and mortality rate (0.02 %) was found (Table VI, Introduction).

In our study gross hematuria appeared in 1.7 % of the children and 1.95 % of the adults and should not be considered a serious complication unless bleeding is of a magnitude causing a significant decline in haemoglobin concentration. The great majority of the patients (97.9 %) had neither macroscopic hematuria, nor blood transfusion or surgery/embolization in line with other studies (139, 140, 148, 150).

Asymptomatic minor perirenal hematoma is not a reliable predictor of a serious adverse outcome and should be regarded an epiphenomenon due to the high incidence (60-90 %) seen in several prospective studies that include examination with ultrasonography or computertomography (140-142, 158). Hematomas are reported to be symptomatic in only 1-2 % (146, 151, 158). The higher number of hematomas in children in our study is probably due to the lack of definition of the complication "hematoma" in the Norwegian Kidney Biopsy Registry and a difference in the interpretation of hematoma as complication among the pediatricians compared with the nephrologists being responsible for the adult patients.

The higher relative risk for major complicatons in patients with ower eGFR (Table 4, Paper IV) may be related to the general increased bleeding tendency seen in kidney failure patients (209) and has been demonstrated by others (144, 149, 210, 211). The increased bleeding tendency is probably also the reason for the doubled risk in acute renal failure patients in the unadjusted analysis. To minimize the biopsy risk, clinical routines to avoid inappropriate use of anticoagulant medication one week prior to as well as after the biopsy procedure is important especially when eGFR is reduced.

There was a lower relative risk of major complications in the more experienced and active centres e.g. university hospitals performing more than 30 biopsies per year supporting the view that modern kidney biopsy procedures are safe in experienced hands (140). The overall low complication rate also in the smaller centres indicates that the routines are adequate also in our smaller centres. Greater variability among different centres has recently been demonstrated in other countries (155, 212).

Morphologic biomarkers assessed by kidney biopsies are important in the diagnosis and follow-up of kidney disease in general and is an important prognostic and therapeutic guide in many common as well as rare diseases (5, 131-133). There is an increased focus on the need of high quality clinical trials in children from the Food and Drug Administration and European Medical Association. These important changes have implications for the need of scientifically well-founded decicions in the treatment of children, including for instance the indication for renal biopsies in

children when appropriate (69). Skepticism towards performing a renal biopsy in clinical care and clinical trials is often due to the fear of complications especially in children, and complications are defined in many papers in a way that includes events of minor clinical relevance. Most of these studies are single centre studies in the early phase of modern kidney biopsy techniques. Safety data from our large nation-wide study do not support an attitude of reluctance to do kidney biopsies to provide essential morphologic markers of disease progression or therapeutic adequacy, neither in adults nor in children.

6. Conclusions

Estimated GFR should be used with caution and measured GFR should be preferred in children with Fabry disease. When estimated GFR is used the new abbreviated Schwartz formula should be preferred.

Renal biopsy gives valuable information about early renal disease before clinical signs are visible, and re-biopsies after five years may characterize beneficial effect of ERT in patients with normal and stable kidney function. Similar effects on reduction of glycosphingolipids (GL3) were found in endothelial and mesangial cells after five years of agalsidase alfa 0.2 mg/kg/eow and agalsidase beta 1.0 mg/kg/eow. The clearance of GL3-deposits in the podocytes showed dose-dependent effect and correlated with cumulative doses of agalsidase. Our findings indicate that agalsidase alfa and agalsidase beta are not equivalent in their respective licenced doses. A functional-structural relationship was demonstrate by correlation between GL3-elimination in the podocytes and change in microalbuminuria.

Renal biopsy is a safe procedure in children and adults when modern guidelines are followed. Serious complications were seen in less than 1 % and lower GFR and smaller centre-size are risk factors for major complications.

7. Future perspectives

Estimated GFR has important limitations and should be replaced by measured GFR in the follow up of young Fabry patients. Iohexol as a non-radioactive substance is a good alternative for evaluation of measured GFR and has become a commonly used standard tool in many clinical trials. Further studies are needed and are currently underway to evaluate single-/few-point methodology as well as finger-prick methods in children.

Renal biopsies are important in the evaluation of treatment of kidney diseases, especially in diseases where changes in GFR, albuminuria or other clinical biomarkers are inconclusive or do not seem sufficiently sensitive, which is the fact in the orphan disease Fabry disease. Based on the present data, kidney biopsies should be regarded safe when modern guidelines are followed, and the threshold for performing the procedure in the evaluation of treatment effect should be lower in many centres. Our findings in kidney biopsies from Fabry disease patients need to be confirmed in larger patient cohorts. Secondly, a major issue would be further methodologic developments in the characterization of the glycosphingolipid deposits in the podocytes and other kidney cell types, and more robust data on structural – functional relationships. Thirdly, since no firm evidence up to now has shown that progressive kidney disease can be avoided in the long run, we also think that longer follow-up of patients with repeat kidney biopsies e.g. after 10 years would provide important information regarding the sustainability of the beneficial 5 years data in patients with early nepropathy. In this context, it would also be of paramount interest to test the effect of dose-adjustments in patients that either remain stable (dose reduction?) or show signs of disease progression (dose increase?), and whether the morphologic responses in young or older patients behave similar. When it comes to ERT and drug doses, it is unlikely that a complex metabolic disease like Fabry disease, with highly variable phenotypes, will remain a “one-size-fits-all” disease which has been the prevailing treatment philosophy up to now. Fourthly, there is a

great need to compare the morphologic cellular changes in the kidney with new and older non-invasive biochemical markers. Fifthly, the issue of equipotency of the licensed drugs has to be further explored. If our findings of a mg-to-mg similarity regarding clinical efficacy in early nephropathy hold true, and if further studies confirm that a lower dose will be sufficient in many patients, these observations will have substantial beneficial societal consequences for the cost of treatment of Fabry disease.

The newly introduced need of pediatric investigation plans for all new drugs that also can be used in children, illustrates the great importance of good study design with adequate methods including renal biopsies as well as gold standard measured GFR when needed.

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