Hereditary Renal Disease in the Norwegian Population, with a Focus on Fabry Disease

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Contents

Scientific environment

This work was carried out within the Renal Research Group, Department of Clinical Medicine, Faculty of Medicine, University of Bergen and Department of Medicine, Haukeland University Hospital, Bergen.

Collaboration partners were the Department of Pathology, Department of Pediatrics, Department of Medical Genetics, and Molecular Medicine and Laboratory of Clinical Biochemistry, Haukeland University Hospital, Bergen.

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Abstract

Background: Clinical experience and studies suggest that end stage renal disease (ESRD) without known Mendelian origins may aggregate in families, and increased risk of death has been reported in relatives of patients with ESRD. In the X-linked Fabry disease, deficient alpha-galactosidase activity causes progressive accumulation of globotriaosylceramide in renal cell types and increased risk of chronic kidney disease.

Aims: To investigate the excess risk of ESRD and death associated with having a first-degree relative with ESRD, and to investigate the effects of enzyme replacement therapy with agalsidase-α or –β in patients with classical Fabry disease.

Methods: Papers I and II were retrospective cohort studies. Datasets were obtained through linkage of the Norwegian Population Registry, The Norwegian Nephrology Registry, and The Cause of Death Registry. Relative risk of ESRD and hazard ratios for death were calculated using Cox regression analyses, adjusted for sex, number of first-degree relatives and birth year. Papers III and IV were observational clinical studies, including patients with classical Fabry disease treated with long-term enzyme replacement therapy, who had undergone serial kidney biopsies. The kidney biopsies were evaluated using the scoring system of the International Study Group of Fabry Nephropathy.

Results: Paper I included 5 119 134 individuals, 8203 developed ESRD during follow-up, 27 046 had a first-degree relative with ESRD. Paper II included 5 130 600 individuals. 828 022 individuals died during follow-up, of whom 4105 had a first-degree relative with ESRD. Having a first-degree relative with ESRD was associated with a relative risk of developing non-hereditary ESRD of 3.7 (95% CI 3.1-4.4). Relative risks of ESRD due to glomerular disease or interstitial diseases were 5.2 (95% CI4.1-6.6) and 4.7 (95% CI 3.1-7.3) respectively. Adjusted hazard ratio (aHR) for all-cause death was 1.13 (95% CI 1.09-1.16) in those with a first-degree relative with ESRD. aHR for death due to cardiovascular death was 1.15 (95% CI 1.10-1.21) and aHR for death due to non-hereditary diseases of the kidneys and ureters was 2.29 (95% CI 1.81-2.91). In Paper III reduction and re-accumulation of podocyte Gb3 inclusions

was seen in three young Fabry patients after 5 years of agalsidase-β 1.0 mg/kg/every other week and subsequent dose reduction respectively. Dose dependent reduction of podocyte Gb3 inclusions was observed, r=0.693, p=0.001, in the cohort (n=20) included in Paper IV. Podocyte Gb3 reduction was observed in the lower fixed-dose group (p=0.004) as well as the higher dose group (p=0.002), the reduction was significantly greater in those who received agalsidase-β 1.0 mg/kg/every other week leading up to the final biopsy (p=0.01). More patients in the higher dose group cleared the arterial/arteriolar intima of Gb3 inclusions, no statistical change was seen in medial Gb3 burden in either group.

Conclusions: Having a first-degree relative with ESRD was associated with a significantly increased relative risk of ESRD, and increased the hazard ratio for death. Taken together this argues for polygenic contributions to risk of ESRD and death in first-degree relatives of patients with ESRD. Agalsidase was found to reduce podocyte Gb3 burden in classical Fabry patients treated for a median of 9.5 years in the lower fixed-dose group and the higher dose group. Dose dependent effects were seen. Limited effects on arteries and arterioles raises concerns regarding the long-term effects on the vasculature.

List of Abbreviations

ACR	Albumin creatinine ratio
AD	Autosomal dominant
AR	Autosomal recessive
CI	Confidence Interval
CKD	Chronic kidney disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration Equation
DNA	Deoxyribonucleic acid
eGFR	Estimated Glomerular Filtration Rate
EOW	Every Other Week
ERT	Enzyme replacement therapy
ESRD	End stage renal disease
Gb3	Globotriaosylceramide
GFR	Glomerular filtration rate
GLA	The human alpha-galactosidase A gene
GWAS	Genome wide association study
HapMap	Haplotype Map
HbA1c	Glycated hemoglobin
HR	Hazard ratio
ICD	International Classification of Diseases
ISFGN	International Study Group of Fabry Nephropathy
KDIGO	Kidney Disease Improving Global Outcomes
LysoGb3	Globotriaosylsphingosine
mGFR	Measured glomerular filtration rate
mRNA	Messenger ribonucleic acid

miRNA	Micro ribonucleic acid
NF-κB	Nuclear factor κB
NHGRI	National Human Genome Research Institute
NIH	National Institute of Health
NNR	Norwegian Nephrology Registry
NPR	Norwegian Population Registry
PAS	Periodic acid-Schiff
PCR	Protein creatinine ratio
RAS	Renin angiotensin system
RASAL1	RAS protein activator like 1
RRT	Renal replacement therapy
SNP	Single Nucleotide Polymorphism
TGF-β	Transforming growth factor beta

List of publications

Paper I

Familial clustering of ESRD in the Norwegian population.

Skrunes, R., Svarstad, E., Reisæter, A. V.,Vikse, B. E.

Clin J Am Soc Nephrol. 2014;9(10):1692-700.

Paper II

End Stage Renal Disease Predicts Increased Risk of Death in First Degree Relatives in the Norwegian Population.

Skrunes, R., Svarstad, E., Reisæter, A. V., Marti, H. P.,Vikse, B. E.

PLoS One. 2016;11(11):e0165026.

Paper III

Reaccumulation of globotriaosylceramide in podocytes after agalsidase dose reduction in young Fabry patients.

Skrunes, R., Svarstad, E., Kampevold Larsen, K., Leh, S.,Tøndel, C.

Nephrol Dial Transplant. 2017 May 1;32(5):807-813. doi: 10.1093/ndt/gfw094

Paper IV

Long-term dose dependent agalsidase effects on kidney histology in Fabry Disease.

Skrunes, R., Tøndel, C., Leh, S., Kampevold Larsen K., Houge, G., Davidsen, E.S., Hollak, C., Kuilenburg, A.B.P., Vaz, F.M., Svarstad, E.

CJASN CJN.01820217; published ahead of print June 16, 2017, doi:10.2215/CJN.01820217

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1. Introduction

«The question of heredity as an etiological factor in nephritis has been studied and reported upon from time to time. Fortunately the condition is rare, and the literature shows only half a dozen families affected in this way" *AC Alport* (1).

The concepts of heredity and heritability are often in every-day use, however few of us consider the underlying genetic pathways when we attribute the presence of traits such as eye color, height or mannerisms in ourselves or others to a parent or close relative. In general, a human being carries 23 paired chromosomes in all cells, except the gametes. In every chromosome pair one chromosome is derived from the maternal line, whereas the other is derived from the paternal line. Together they make up the blueprint for a human being. Each chromosome contains roughly 400-3000 genes, which in turn are made up of millions of base-pairs (2). As one copy of each chromosome is inherited from each parent, two copies (alleles) of every gene are present. The expression of this vast genome is tightly controlled, as expression of all of the genes all of the time would likely result in chaos, and not the carefully orchestrated symphony of the human body.

The concept of heritability is not new, and though Cecil Alport reported in 1927 that only a dozen or so families were afflicted with a hereditary nephritis, heritability of disease itself had long been established. The Babylonian Talmud states that some families have "loose blood", and that boys born to women of such families need not be circumcised due to risk of exsanguination (3). The mode of transmission of hereditary traits, or indeed how these traits were carried in affected individuals remained a mystery.

Usually only one copy, one allele, of an active gene is expressed. Alleles that are always expressed are termed dominant, whereas alleles that are only expressed when they are not paired with a dominant allele are termed recessive. The terms dominant and recessive inheritance were coined by Gregor Mendel, as he described how different traits were transmitted down the generations of seedlings derived from selectively pollinated plants (4). The seedlings in Mendel's laboratory bloomed, not

only into differently colored pea-plants in a predictable pattern, but in time also bloomed into what we now refer to as Mendelian inheritance. A significant technological gap was bridged in the mid-17th century with the invention of the proper microscope, allowing scientists to examine tissues in far greater detail than had previously been possible. Deoxyribonucleic acid (DNA) was isolated for the very first time in 1869, and though Miescher recognized it as an important phosphorus rich factor in cell nuclei, the function of what he termed "nuclein" remained unknown (5), and it is not until the discoveries of Avery (6), Chargaff (7), Watson and Crick (8) in the mid- 20th century that DNA was born.

As knowledge of the human genome grows, it becomes clear that many known disease entities have genetic contributions, or are indeed Mendelian disorders. Families where kidney disease seems to aggregate can now be investigated with intent to pinpointing a culprit gene. Resolving the etiology of aggregated kidney disease is important to families and to treating physicians. When the culprit gene is identified, medical advice can be adapted, and counselling with respect to future generations can be given. Recently several new genes causing steroid resistant nephrotic syndrome in adults have been identified (9). If such a gene is identified in a pedigree, steroid treatment need not be attempted, and the patient can be spared potential side-effects. However, kidney disease may aggregate in some families without suspected Mendelian disorders. Epidemiological studies have shown a significant increase in kidney disease in first-degree relatives of individuals with end stage renal disease (ESRD) (10-12). The observed increased risk of chronic kidney disease in relatives of patients with ESRD is likely not explained by monogenic disorders. Rather genetic susceptibility to kidney disease, predisposing select individuals to kidney disease, is likely part of the explanation. The inherited vulnerability may be activated as a response to the environment, or as a response to transcription of neighboring genes. Susceptibility genes may vary from population to population, as environmental pressure unrelated to kidney disease may cause selection of alleles within populations. A notable example is the APOL1 gene, where alleles conferring increased resistance to the parasite causing African sleeping sickness have been selected in African

populations. Unfortunately the same alleles confer increased risk of developing non-diabetic chronic kidney disease, with a 17-fold increase in risk of focal and segmental glomerulosclerosis in homozygous carriers (13).

At present, there is often no disease specific treatment for genetic kidney disease, but identifying the underlying genetic cause may spare patients unnecessary investigations, or futile, and possibly cumbersome treatment attempts.

1.1.1 Mendelian inheritance

In Mendelian inheritance, a single gene determines a defined trait. Each parent contributes a copy of half of their original DNA to the offspring, 22 autosomes and 1 sex chromosome. Importantly, the maternal and paternal DNA is inherited independently of each other. Some genes, when present, will always be expressed as a phenotype. This is dominance, which affects the phenotype derived from the genotype. In a setting of complete dominance, a single gene is fully responsible for the expressed phenotype. Co-dominance occurs when both parental alleles are expressed as a phenotype in the offspring. A recessive gene is not expressed as a phenotype when paired with a dominant gene, but is expressed as a phenotype in the case of a homozygote recessive genotype. The patterns of dominant and recessive alleles are not always straight forward, as allelic interactions may influence dominance. Allele 1 may be dominant when paired with allele 2, codominant when paired with allele 3 or recessive when paired with allele 4.

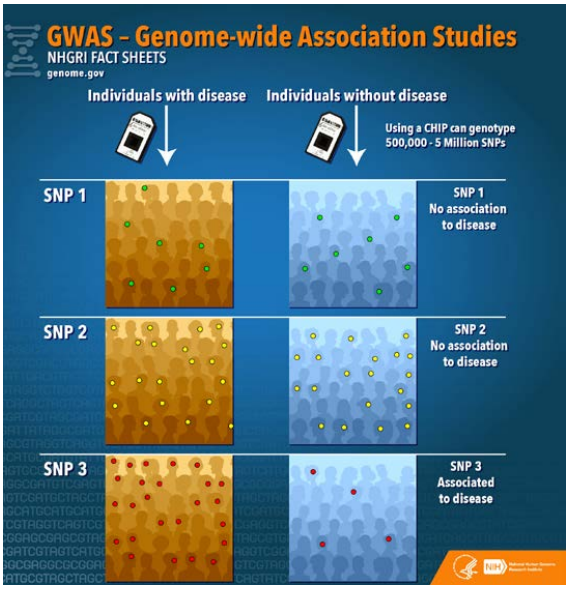
Multiple and slightly variable alleles may exist within a population, though only two alleles can co-exist in a single individual. This is termed polymorphism, indicating that some variation within the nucleotide sequence of certain genes is normal. In genes where several alleles are found, multiple genotype/phenotype combinations are possible. An example of such inheritance is the ABO blood types found in man. Three alleles are found for this trait, two of which are co-dominant. Consequently, four phenotypes are possible; A, B, AB, and O, the recessive phenotype.

1.1.2 Polygenic inheritance

Phenotypes that depend on the accumulation of several different genes are due to polygenic inheritance, and eye color is an example of this. The color of the human iris depends on genes in different loci, regulating production, transport and storage of melanin. Significant polymorphism in several of these genes is described, accounting for the variety in eye color we see in everyday life (14).

Polygenic disorders require accumulation of several risk alleles at multiple loci acting together to produce a phenotype, and the phenotype/genotype correlation is often not a very tight (15). Disease manifestation typically occurs during adulthood, in contrast to the early onset seen in single gene recessive disorders. The number of risk alleles necessary to produce a phenotype may run in the hundreds. Though several factors, including environment, are needed to produce a phenotype, polygenic disorders are much more common than single gene Mendelian disorders. Polygenic risk is best investigated with genome wide association studies (GWAS), where the genome is interrogated for polymorphisms in single nucleotides which may be associated with a trait or disorder. The phenotype is thus established first, and the genome is subsequently investigated for single nucleotide polymorphisms (SNPs) present in individuals with the phenotype in question, and not present in controls (Figure 1). This technology may be applied to rare disorders, and to more common disorders such as cardiovascular disease, diabetes or cancer. SNPs with a prevalence of at least 5% in the population are often viewed as common (16).

Figure 1. Investigation of single nucleotide polymorphisms using genome wide association studies. Reproduced with permission from NHGRI/NIH.



Cardiovascular disease is a common cause of morbidity and mortality world-wide, and is the most common cause of death in Norway, though risk of cardiovascular death has decreased over the past decade (17). Several rare causes of cardiovascular disease are known (18), however polygenic and environmental contributions to cardiovascular disease are more common. A family history of premature cardiovascular disease is a risk factor for cardiovascular disease, which is reflected in recommendations in both American and European guidelines (19, 20). A large meta-analysis of 14 genome-wide association studies on individuals of European descent reported 23 loci associated with increased risk of coronary artery disease, 13 of which were new (21). The risk increase associated with the new loci identified ranged from 6-17 % per allele, only three of which showed a significant association with traditional risk factors such as hypercholesterolemia or hypertension (21). Seventeen of the 23 confirmed risk alleles were not associated with traditional cardiovascular risk factors, indicating that SNP-associated cardiovascular risk is mediated through yet unknown pathways.

Blood pressure is heritable (22), and several loci influencing systolic and diastolic blood pressure have been identified (23-25). Hypertension is a cause of morbidity, including chronic kidney disease, and mortality. In a study identifying 29 different loci implicated in blood pressure control, two loci were thought to connect to blood pressure through genes implicated in renal physiology or disease in individuals of European descent (25). A genetic risk score was used to assess the association of the identified SNPs with hypertension, hypertension related complications, chronic kidney disease or measures of kidney function. Several associations to hypertension and its complications were seen, however the genetic risk score was not significantly associated with chronic kidney disease (25). The authors hypothesize that an increase in blood pressure may in part be a consequence and not the cause of sub-clinical kidney disease.

The world prevalence of diabetes mellitus in adults was estimated to 6.4% in 2010, and is expected to increase to 7.7 % in 2030, affecting over 400 million adults (26). The majority of the more than 400 million expected patients with diabetes will suffer type 2 diabetes. Risk of type 2 diabetes is mediated through traditional risk factors such as obesity, dietary factors, smoking and physical inactivity, but also through poly- and epigenetic risks. More than 60 susceptibility loci for type 2 diabetes have been identified through genome wide association studies (27). The effect size of individual risk alleles is unknown, several loci have also been implicated in metabolic traits such as body mass index (28, 29), triglycerides (30) and insulin resistance (31). However several of the SNPs associated with type 2 diabetes appear to influence beta cell function and insulin secretion rather than insulin resistance (32). Diabetic kidney disease is currently the most common cause of ESRD in the world (33), and CKD is a common complication of diabetes mellitus. Familial aggregation of diabetic kidney disease has been described (34, 35), though all patients with diabetes do not develop diabetic kidney disease. There is evidence for genetic susceptibility to diabetic kidney disease in selected individuals, conferring risk independent of long-term exposure to high levels of blood glucose levels (36). Individuals with type 1 or 2 diabetes who have a sibling or parent with diabetic nephropathy are at increased risk of developing

diabetic kidney disease themselves (35, 37). Elucidating the exact genetic mechanisms behind diabetic nephropathy has however been difficult. The genetics of the inflammatory response in diabetic nephropathy have been investigated (38), increase in cytokines such as interleukin-1β (39), interleukin-6 originating from hepatic or adipose tissues (40, 41), and tissue necrosis factor-α (42) have been shown in patients with type 2 diabetes. Activation of cell signaling, transcription factors, and cytokines may result in abnormal translation or transcription of genes, which in turn may trigger the development of diabetic nephropathy (43).

Polymorphism in loci associated with kidney function and decline has recently been investigated to further elucidate the mechanisms behind the variability in risk of CKD associated with e.g. hypertension or diabetes mellitus. Common allelic variants have been identified in several genes known to code for nephrogenesis, podocyte function, angiogenesis and metabolic functions of the kidney in a population of mainly European descent (44). Some of the identified polymorphisms seem to be associated with creatinine production or secretion, rather than eGFR or CKD (44), highlighting the importance of careful evaluation of identified SNPs with respect to causality. The overall effect of the identified polymorphisms on CKD and eGFR was modest, though the prevalence of CKD seemed to increase with increasing genetic risk score. This may indicate clinical significance when several risk alleles are accumulated within one individual (44). Polymorphisms identified within one ethnic group may not be readily transposed to populations of different descent. The genomes of the participants in The Chronic Renal Insufficiency Cohort Study (CRIC) were recently interrogated for alleles conferring increased risk of CKD or CKD progression (45). The authors investigated candidate gene regions in Americans of different ethnicities, and whether the genetic associations were consistent across ethnic groups. 50% of the candidate gene regions investigated yielded SNPs associated with decline in eGFR in both African-Americans and Americans of European decent, different SNPs were identified in the two ethnic groups. Two distinct SNPs in a single gene expressed in the glomeruli was found to be associated with progression of CKD in non-diabetic

individuals, one SNP in individuals of African-American descent and one SNP in Americans of European descent (45).

1.1.3 X-linked inheritance

In man, gender is determined by the X- and Y-chromosomes, collectively termed the sex chromosomes. The male genotype is XY, whereas the female genotype is XX. Females inherit one X-chromosome from the paternal line, and one from the maternal line. Only one X-chromosome remains active in the cells, as one X-chromosome is inactivated at random (46). The inactivation pattern may sometimes be skewed, so that either the maternally or paternally derived X-chromosome is more frequently inactivated. When a mutation occurs in a gene located on the X-chromosome, male carriers must express the phenotype coded for by the mutant gene, as they have no other healthy copy of the gene. In females with balanced X-inactivation, roughly 50% of the cells will express the normal X-chromosome, with the remaining cells expressing the X-chromosome carrying the mutant gene. Thus, females may be asymptomatic carriers of X-linked disorders. The most prominent example of an X-linked disorder is probably hemophilia, sometimes also known as “the royal disease”. Hemophilia entered the British royal family through Queen Victoria of England, and spread to the royal houses of Russia, Prussia and Spain through two of her asymptomatic daughters carrying the defective gene (47).

It has previously been postulated that X-linked disorders can be either recessive or dominant, with recessive inheritance affecting males almost exclusively, whereas X-linked dominant inheritance may affect males and females, with an excess of affected females in the pedigree (48). The concept of dominant and recessive X-linked inheritance may not always hold true. In females with skewed X-inactivation, predominantly inactivating the healthy X-chromosomes, a more severe phenotype may occur. Fabry disease is an example of an X-linked disorder where the classical phenotype is usually expressed in males who carry a defective GLA gene. A phenotypically wide spectrum can be observed in females, ranging from no disease to the classical phenotype. Random X-inactivation is likely not the only explanation for

the varied phenotypic spectrum seen in Fabry females, additional factors such as penetrance and epigenetic inheritance may also impact on disease severity.

1.1.4 Epigenetics

Only 1% of the human genome (the exome) is protein encoding. To transcribe DNA, the double helix must be opened and the two strands of DNA separated from each other. A promoter region within the DNA signals the starting point of the transcription of a gene, a terminator sequence (stop codon) is located at the end of the coding DNA sequence. RNA polymerase assembles a new complimentary RNA molecule at the template strand, each nucleic acid is paired with its appropriate counterpart in the newly formed RNA molecule. The transcription unit is separated from the transcription site, and serves as a template for protein synthesis.

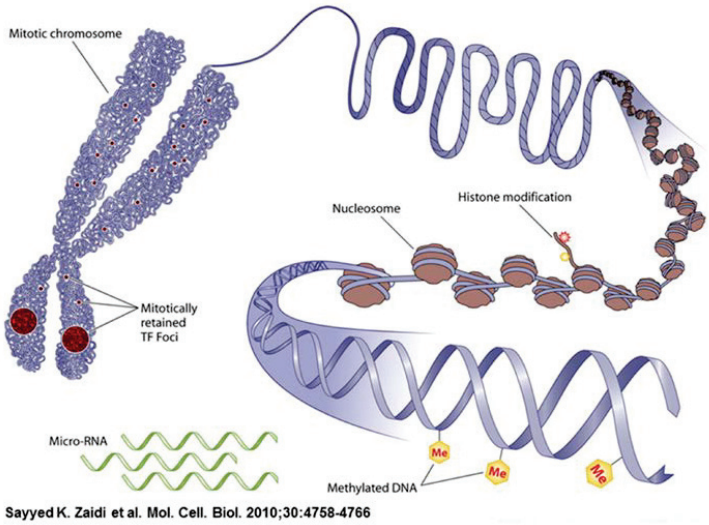
The protein coding exome may be impacted by other heritable genetic factors besides alterations in the primary nucleotide sequence (49). Epigenetic changes do not involve changes in the underlying nucleotide sequence, the effects are mediated through up or down regulation of gene expression (49), regulation of DNA sequences which are capable of changing their positions within the genome (transposable elements), and adjustment of gene dosage as seen in e.g. random X-inactivation (49). The effects are mediated through DNA methylation/demethylation and histone modification. Methylation of DNA and histone acetylation and deacetylation are dynamic processes, with regulatory effects on gene expression (50) (Figure 2). Methylation of GpG (cytocine guanosine dinucleotides) islands within promoter regions of genes can silence gene transcription (51). Epigenetically mediated hypermethylation of DNA may thus silence genes, whereas hypomethylation may cause gene activation. Histones are the primary protein components of chromatin, and are also subject to epigenetic modifications. Active and un-methylated regions of DNA are rich in acetylated histones, rendering the DNA segment easily accessible to transcription factors. Conversely methylated DNA is rich in deacylated histones, which confers a more compact and unfavorable configuration with respect to transcription (50). Epigenetic changes can be mediated through e.g. environmental

factors, physical activity or diet (52), toxins such as alcohol and nicotine can also mediate epigenetic change.

Down regulation of protein coding sequences can be effectuated through double stranded RNA and microRNA (miRNA). RNA interference may decrease the levels of messenger RNA, whereas short, non-coding miRNA impairs the transcription of RNA into protein (53). As such non-coding miRNA is important in vital posttranscriptional processes affecting cell proliferation, differentiation and apoptosis.

Fetal phenotype may be the result of genomic parental imprinting. Parental epigenetic changes are usually not transmitted to the offspring, however epigenetic change can affect the oocyte or spermatozoa. Through acquired DNA hypermethylation of one allele of a gene, monoallelic expression of an allele originating from either the maternal or paternal line can occur, with phenotypic consequence (54).

Figure 2. Mechanisms of heritable epigenetics. Reprinted with permission (55).



Renal fibrosis is a complex, dynamic process often initiated by inflammation (56) and fibrosis, and loss of functional renal tissue is important in the development and progression of chronic kidney disease. Fibroblasts are important in tissue repair, but are also principal players in development of scarring and fibrosis. Preliminary evidence suggests that some instigators of fibrosis may exert their deleterious effects through epigenetic change. In the fibrotic state, there is evidence of perpetual activation of fibroblasts. Hypermethylation of the RAS protein activator like-1 (RASAL1) promoter, a Ras oncoprotein inhibitor, has been shown to cause irreversible activation of RASAL1, with subsequent perpetual activation of renal fibroblasts and fibrosis by silencing Ras-GTPase activity in fibroblasts in mice (57). Hypermethylation of RASAL1 is mediated through DNA methyltransferase 1, and renal fibrosis was reduced in mice which had a 70 % reduction in expression of DNA methyltransferase 1 compared to the wild type (57).

Chronic kidney disease is associated with a high risk of cardiovascular disease, progression of cardiovascular disease, and cardiovascular death. Epigenetic change may also play a role in establishing and accelerating atherosclerotic disease through up regulation of atherosclerosis-susceptibility genes and/or down regulation of genes with protective effects on atherosclerosis (58). DNA hypermethylation has been associated with inflammation and risk of all-cause death and cardiovascular death in patients with CKD 3-5 as compared to healthy controls (59). Elevated levels of homocysteine and its precursor S-adenosylhomocysteine have been reported in CKD and ESRD (60). The homocysteine precursor may lead to DNA hypomethylation through competitive inhibition of S-adenosyl methionine dependent methyltransferase when faced with hyperhomocysteinemia, such as can be seen in uremia (50, 60). However, the evidence for homocysteine as an instigator of epigenetic change leading to cardiovascular disease in uremia remains unresolved. Hypomethylation has been reported in males with high homocysteine levels at hemodialysis start compared to healthy controls (61). A subsequent study investigated whether global DNA methylation was associated with renal function in patients with CKD stage 2-4. No association was reported, moreover folate therapy did not alter the methylation status (62). The study designs and CKD

stages investigated by Ingrosso and Nanayakkara were different, which may have contributed to the differing results. A meta-analysis reporting on nearly 11 000 individuals with all stages of CKD found that lowering homocysteine levels with folate therapy did not reduce cardiovascular events in the CKD population (63).

Diabetes nephropathy is a frequent cause of ESRD, and vascular complications continue to occur in diabetic patients even after adequate glycemic control has been achieved. “Metabolic memory” in relation to glycemic control was first described in 1987 (64). Accumulation of advanced glycation end products have been proposed as a mechanism for metabolic memory, and epigenetic changes related to exposure to hyperglycemia is likely part of this memory. There is increasing evidence for epigenetic change in the development of diabetic kidney disease, stemming from animal models and human studies (65). Increased risk of diabetic kidney disease may stem from the accumulation of several polymorphisms acting in concert to increase risk, and epigenetic modification of proinflammatory and profibrotic pathways. NF-κB is an important transcription factor in relation to proinflammatory genes, and hyperacetylation of promoter regions within histones due to hyperglycemia can result in a proinflammatory state (65). Hyperglycemic exposure has been shown to alter NF-κB mediated expression of inflammatory genes, and post-translational histone modification have been found to modulate NF-κB mediated gene expression in vascular cells and monocytes (66). Hyperglycemia can also increase the action of transforming growth factor β (TGF-β) (67). The sum epigenetic influence on profibrotic and proinflammatory pathways can thus lead to acceleration of CKD in patients with diabetes mellitus. Glucose levels in utero may also impact on epigenetic risk of future disease, gestational diabetes mellitus has been shown to epigenetically affect genes predominantly involved in metabolic disease pathways (including diabetes mellitus) in the offspring (68).

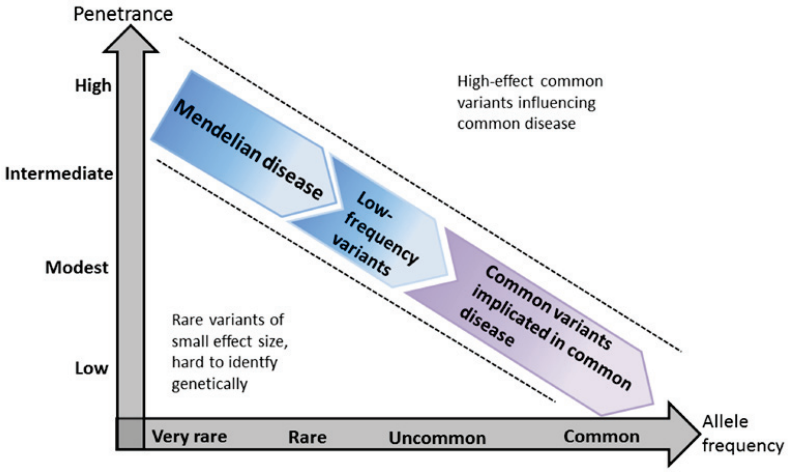
1.1.5 Causality and risk alleles

Correlating a phenotype to genotype can sometimes be difficult. Single gene autosomal recessive disorders usually have full penetrance, in that all carriers of a

specific genotype express a clearly defined phenotype. In autosomal dominant disorders, the phenotype may be less tightly defined, and the complete phenotype may not be expressed by all carriers of the genotype. Autosomal dominant polycystic kidney disease exemplifies this, where all carriers develop cystic kidneys bilaterally, but all do not progress to end stage renal disease. Polygenic disorders are more common than single gene disorders, and are often the result of accumulation of several risk alleles. Typically penetrance is low, with great phenotypic variability (69) (Figure 3). Usually increase in risk of kidney disease is only seen when accumulation of a large number of risk alleles occurs within an individual. A notable exception is APOL1, as harboring a APOL1 risk allele confers significantly increased risk of focal and segmental glomerulosclerosis (70-72).

The clinical course of chronic kidney disease is highly variable, and may depend on factors such as underlying disease, smoking, lipid profile or compliance with medical advice. Hypertension and diabetes mellitus are two leading causes of chronic kidney disease throughout the world, with a significant proportion of sufferers progressing to end stage renal disease (73). All patients with hypertension and/or diabetes mellitus do not progress to ESRD. Environmental factors or the presence of additional risk alleles may compound the deleterious effects of hypertension and hyperglycemia on renal tissues. Several genome-wide association studies have identified significant genetic contributions to eGFR, CKD, and ESRD in non-diabetic individuals (44, 45), which support the findings of previous large cohort studies (10, 11, 35). Single nucleotide polymorphisms may vary between ethnic groups, and it is therefore important to validate potential SNPs associated with GFR decline or time to ESRD within relevant populations.

Figure 3. Penetrance versus frequency of genetic contribution to disease. Modified from McCarthy et al. (74)



1.1.6 Hereditary kidney disease

During the 20th century kidney disease was reported to cluster within certain families, and with the advent of DNA analysis the genetic basis for disease entities such as adult polycystic kidney disease, Alport syndrome, Fabry disease, and other monogenic kidney diseases were discovered. Single gene defects often have distinct phenotypic expressions, though penetrance may vary in autosomal dominant conditions. Recessive conditions, such as nephronophthisis, are more likely to have full penetrance, and sufferers of recessive genetic disorders display the complete phenotype at an early age (15). Autosomal dominant polycystic kidney disease is the most common monogenic renal disorder (75), which may be caused by a mutation in the PKD1 gene (76) or PKD2 gene (77). The prevalence has been reported to vary somewhat according to ethnicity, with a prevalence of 100 per 100 000 reported in Denmark (78), 11.7 per 100 000 in Japan (79) and 32.5 per 100 000 in South-Western Germany (80). Although this disease entity has a clearly defined genetic basis, the significant variation in disease severity observed within families, and between

different families, point to the possibility of other genetic and environmental modifying factors (75). These factors are largely unknown.

There are probably several monogenic kidney diseases where the culprit gene or genes are unknown. Several genes causing hereditary focal and segmental glomerulosclerosis have identified after next generation sequencing was developed (9), many of which are involved in the podocyte cytoskeleton, and may have a more varied penetrance and phenotype.

Polygenic kidney disease is caused by an accumulation of several risk alleles, and may be triggered by a "second hit" mechanism of possibly environmental or epigenetic origin. IgA nephropathy is an example of polygenic disease, where GWAS studies have identified discrete risk loci of interest (81).

1.1.7 Investigating genetic kidney disease

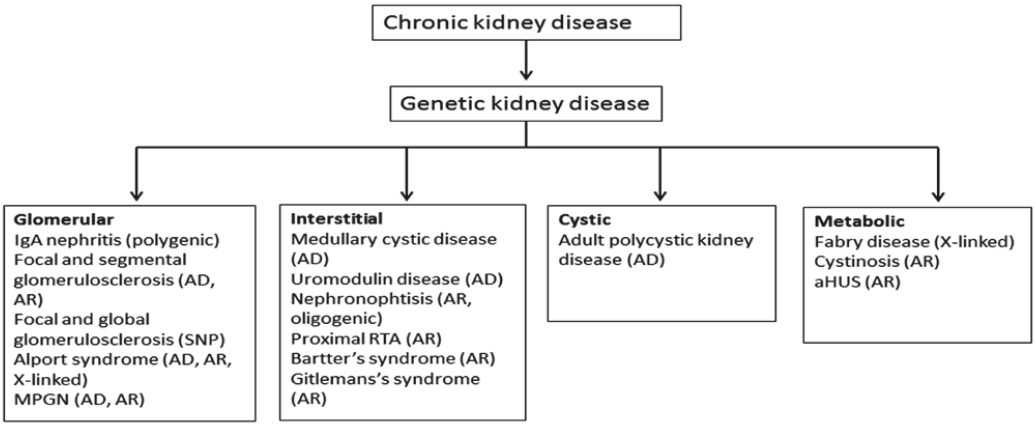
In clinical practice, genetic kidney disease may be recognized or suspected through a constellation of specific symptoms, kidney biopsy findings, radiology findings and/or the referral of several individuals within the same family. When constructing a pedigree, an impression of the mode of heritability may be gained, and further genetic counselling and investigations can be planned. When a monogenic disease with a well described culprit gene is suspected, such as autosomal dominant polycystic kidney disease, Alport syndrome or nephronophthisis, targeted investigation is possible (Figure 4). However, when faced with a strong suspicion of hereditary renal disease without a known culprit gene, the investigatory net must be cast wider.

Next generation technology, with massively parallel sequencing, efficiently interrogates the whole exome at relatively low cost. As this technology targets all protein coding DNA, the dataset generated is vast and comprehensive, and may yield secondary findings of genetic variation associated with increased risk of e.g. common complex diseases such as cardiovascular disease, diabetes or cancer, which may generate ethical dilemmas when reporting the data. Using whole genome exome sequencing (82), a success rate for finding the culprit gene of around 25% is

commonly reported (83), some authors claim that whole exome sequencing may identify the culprit gene in as much as 50% of clinically well-defined Mendelian disease (84). Indeed, the advent of whole exome sequencing has led to the identification of several new genes causing Mendelian disease, and more are likely to be discovered. This may not only facilitate clinical diagnosis, but may in time also facilitate personalized medicine.

Investigating single nucleotide polymorphism within candidate genes may focus the search for culprit genes. Using microarray technology, deletions, duplications and regions of homozygosity may be identified within the genome. When such regions are detected, they can focus next generation sequencing, based on the assumption that a SNP allele is inherited along with a disease-causing allele due to close physical proximity (85).

Figure 4. Overview of selected genetic causes of chronic kidney disease and mode of inheritance.



AD: autosomal dominant. AR: autosomal recessive. SNP: Single nucleotide polymorphism. MPGN: membranoproliferative glomerulonephritis. RTA: renal tubular acidosis. aHUS: atypical hemolytic uremic syndrome.

1.1.8 Epidemiology of chronic kidney disease

Chronic kidney disease is defined as abnormal kidney function or structure for three or more months (86) (Table 1, Figure 5). The incidence of chronic kidney disease varies across populations, which may in part be due to variations in genetic susceptibility, demographic composition or ascertainment. The Global Burden of Disease study ranked death due to chronic kidney disease as the 18th most common cause of death globally in 2010. Kidney disease related death may in fact have been more prevalent, as death due to diabetes related kidney disease was found to be significantly underestimated (87). Only 20 years earlier death due to chronic kidney disease globally was ranked as number 27 (87). The majority of patients receiving renal replacement therapy live in affluent countries with an ageing population (88), and costs related to CKD, co-morbid conditions, and renal replacement therapy are considerable (89). Incidence of ESRD varies within countries where access to health care should be universally available. Compared to the USA, the incidence of ESRD in Europe is much lower. The incidence of ESRD also varies markedly within the United States, according to ethnicity. The prevalence of ESRD among African Americans is 3.7 times that of Americans of European decent, and the prevalence of CKD stages 1-5 was 14.8 % in the general population in 2011-2014, with CKD 3 being the most prevalent CKD stage (90). A comparison of prevalent CKD in the Norwegian population versus that of the United States was published in 2006 (91). During 1995-1997 the total CKD prevalence in Norway was 10.2%, which was not dissimilar to the prevalence reported in the United States during the same time-period. However, a 2.5-fold increase in relative risk of progression from CKD 3 or 4 to ESRD in the general American population compared to the Norwegian population was observed, and a 2-fold increase in relative risk in Americans of European decent compared to the Norwegian population, despite age and GFR at initiation of dialysis being comparable was also noted (91).

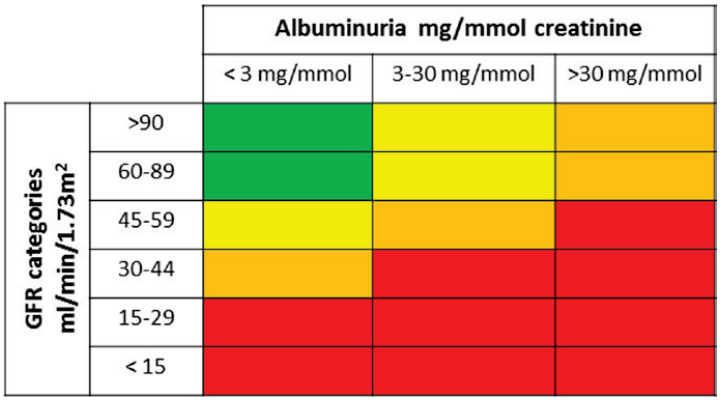
Calculating glomerular filtration rate by measuring external filtration tracers such as Iothalamate, Chromium EDTA or Inulin is rarely done in general clinical practice, single sampling plasma Iohexol GFR is available in many centers and is

usually well tolerated (92, 93). Glomerular filtration rate can be estimated based on a single time-point creatinine measurement, using different GFR equations. Creatinine is dependent on muscle mass, dietary intake, tubular secretion and extra-renal removal, and differences between populations can be expected. The CKD-EPI equation (94) is recommended by KDIGO (86). GFR equations adapted to the pediatric population must be used in children as muscle mass increases during growth (95).

Table 1. Overview of stages of CKD. Modified from the KDIGO guidelines (86).

GFR ml/min/1.73m^2	CKD stage	
≥90	1	if there is further evidence of kidney damage such as histology, radiological findings or albuminuria
60-89	2	if there is further evidence of kidney damage such as histology, radiological findings or albuminuria
45-59	3a	
30-44	3b	
15-29	4	
<15	5	

Figure 5. Heat map of risk of progressive kidney disease according to eGFR and level of albuminuria. Modified from the KDIGO guidelines (86).



Green: Low risk in non-CKD individuals. Yellow: Moderate risk. Orange: High risk. Red: Very high risk.

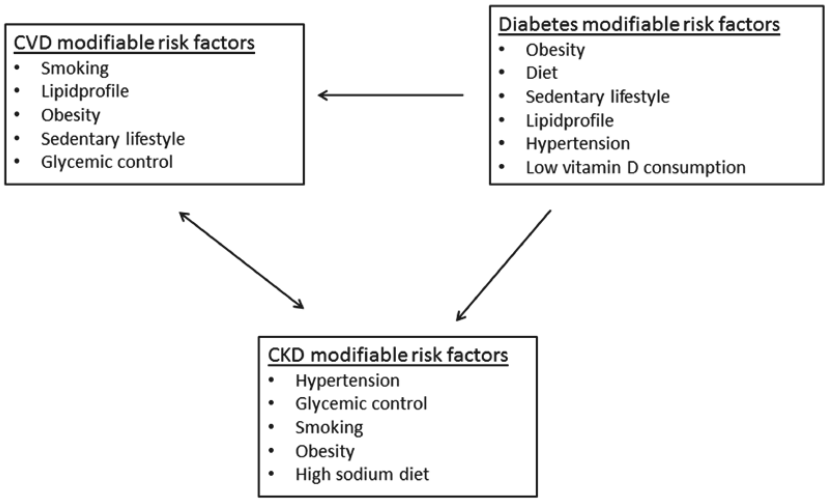
Sustained decrease in renal function is associated with an increase in risk of cardiovascular disease, cardiovascular- and all-cause death (96, 97). Diabetes mellitus and hypertension contribute to the majority of chronic kidney disease, whereas adult polycystic kidney disease is the most common cause of monogenic renal disease (75, 98). The cost effectiveness of screening for CKD in the general population is unclear. However, when CKD is discovered, some measures should be adopted in attempt to slow progressive loss of GFR. Adequate and individually tailored antihypertensive treatment should be offered, with blockers of the angiotensin-renin system as the drug of choice in individuals with proteinuria (86). Good glycemic control is imperative, and lipid lowering therapy should be considered.

1.1.9 Environmental and modifiable risk factors

Gene translation and transcription can be influenced by environmental factors, some risk factors are well known and exert their actions in obvious and readily understandable ways, while the mechanisms underlying other risk factors remain elusive. The interplay between environment and the human organism may result in excess risk in the individual as well as in the population. Cigarette smoking confers excess risk of common complex diseases such as cardiovascular disease, cancer and diabetes mellitus, risk also increases in those who are exposed to passive cigarette smoking. Smoking habits may be transferred from parent to offspring, and prevalence of cigarette smoking has been found to be higher among adolescents with parents who smoke (99-101). Like cigarette smoking, obesity and dietary habits may be inherited, not through genetics, but through habit (102, 103). Modifiable lifestyle risk factors such as smoking habits, obesity and physical inactivity may be imprinted on children, adding to risk of common complex diseases in adulthood. Dietary habits which may be rooted in cultural context may increase or decrease risk of common diseases. The Mediterranean diet has been shown to decrease risk of cardiovascular disease (104), whereas obesity has been linked to risk of cancer (105). In chronic kidney disease, several modifiable risk factors are known, many of which overlap with other common diseases (Figure 6). Poorly treated hypertension, impaired glycemic control, dyslipidemia, obesity and smoking have all been linked to development of CKD or

progression of CKD. Many of the same environmental and/or modifiable risk factors are important in the development of cardiovascular disease and diabetes mellitus type 2. CKD, cardiovascular disease and diabetes mellitus adversely impact risk of morbidity and mortality, with risk of all cause increasing with decreasing eGFR (97). Risk of cardiovascular death has been shown to increase with decreasing eGFR and proteinuria (96, 97).

Figure 6. Modifiable risk factors of cardiovascular disease (CVD), diabetes mellitus and chronic kidney disease (CKD)

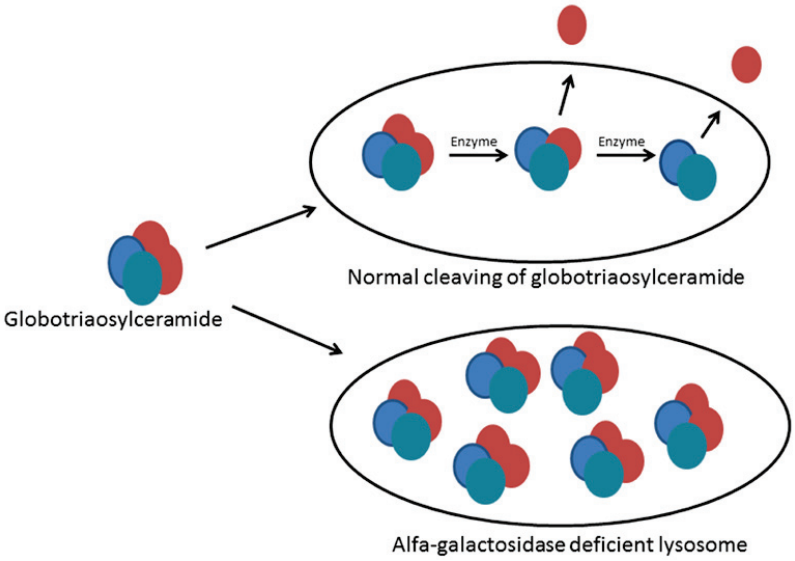


1.2.1 Fabry disease

Fabry disease is a monogenic X-linked disorder, where mutations in the GLA gene result in a severe defect in the enzyme alpha-galactosidase. The lysosymal enzyme alpha-galactosidase hydrolyses the terminal alpha-galactosyl moieties from the sphingolipid globotriaosylceramide (Gb3). The lysosome is a cellular organelle which was first described in 1966 (106). The primary function of the lysosome is phagocytosis, through enzymatic digestion and recycling of complex structures within the cell. When alpha-galactosidase activity is sufficiently reduced, lysosomal accumulation of Gb3 occurs (Figure 7). Inappropriate storage of Gb3 within lysosomes

takes place in various cell types in the body. In time, multi-organ dysfunction and failure ensue.

Figure 7. Gb3 accumulation in the lysosome due to alfa-galactosidase deficiency.



The phenotypical spectrum associated with mutations in the GLA gene is wide, and Fabry disease can affect males and females. The most severe phenotype is usually seen in males with very low residual enzyme activity. Females will in most cases have some residual enzyme activity, as they nearly always also carry an unaffected X-chromosome. Skewed X-inactivation, with predominant inactivation of the healthy X-chromosome, may produce a clinical picture similar that of the hemizygote male (107). Homozygosity of a GLA mutation has been reported in females (108), however this is rare.

One of the cardinal signs of classical Fabry disease is angiokeratomas of the skin. The characteristic, but not pathognomonic, skin lesions were first described by two dermatologists, independently of each other, in 1898. Both Johannes Fabry (109) in

Bonn and William Anderson (110) in London noted the characteristic skin lesions in several related individuals.

As globotriaosylceramide accumulates in multiple cell types, the phenotype associated with classical Fabry disease is one of multi-organ disease. Patients may develop thin fiber neuropathy during childhood, manifesting as burning pain in the hands and feet, exacerbated by exercise or febrile episodes (Table 2). Fabry related end-organ damage occurs in males and females, onset of Fabry related complications is usually seen at a younger age in males compared to females (111). Kidney disease typically develops during the third decade (112), followed by cardiac and cerebrovascular disease in the fourth decade (113, 114). The heavy disease burden seen in classical patients reduces life expectancy by approximately 20 years in males (111), and 10 years in females (111).

1.2.2 Incidence

The reported incidence of Fabry disease is variable. World-wide incidence has been estimated to 1:40 000, to 1:117 000 (115). The incidence appears to be similar across ethnic groups, though regional differences may occur due to founder effects. The frequency is somewhat higher in at-risk populations, such as dialysis patients, where a prevalence of 0.33% and 0.10% for men and women respectively has been reported (116). Newborn screening has revealed a higher than expected incidence of GLA variants. The variants detected through newborn screening or screening in the general population often confer late onset, are often missense mutations, and are often associated with cardiac disease. In an Austrian cohort, the incidence of a GLA mutation was found to be 1 in 3859 births (117), an incidence of 1 in 3100 births has been reported in an Italian cohort (118), and a frequency of 1 in 1250 births was reported in Taiwan (119).

1.2.3 Phenotypes in Fabry disease

The GLA gene is small, with a large scope for nucleotide variations. Mutations are associated with significantly reduced alfa-galactosidase activity, particularly in males.

The phenotypic spectrum associated with Fabry disease is in part related to residual enzyme activity, with a more severe phenotype in males with very low enzyme activity (120, 121). Traditionally two distinct phenotypes have been described in the literature, the classical and non-classical phenotypes. The classical phenotype is more common in males, and typically includes early onset of symptoms, thin fiber neuropathy with acroparesthesia, cornea verticillata, hypohidrosis, and clustered angiokeratomas in a pattern related to Fabry disease (Tables 2 and 3). Some, or most, of the features associated with the classical phenotype may be absent in the non-classical phenotype. The non-classical phenotype may have greater residual enzyme activity, with later onset of symptoms. It is often associated with cardiac disease in the form of hypertrophic cardiomyopathy and disturbances in the conductive circuits of the heart. Distinguishing between the different phenotypes may be difficult, particularly in women. The biomarker globotriaosylsphingosine (lysoGb3) may aid the diagnostic process, as markedly elevated levels of lysoGb3 are always found in classically affected males (122, 123). A recent study found that all classically affected males and females had significantly higher lysoGb3 values than controls. LysoGb3 levels did not increase with age, suggesting that levels are related to phenotype rather than age (122). The same study also found that though most classically affected males had elevated plasma Gb3 levels, less than 5% of classically affected females showed an elevation in this biomarker. Non-classical Fabry disease usually has a later onset, and may lack some or all of the clinical hallmarks of the classical phenotype (124-126). Late onset disease, often dominated by cardiac disease, is now a well-recognized phenotype. In females without skewed X-inactivation, the healthy X-chromosome will be expressed by roughly 50% of all cells in the body, consequently some residual enzyme activity is found. This may account for the wide spectrum of symptoms seen in women.

Table 2. Organ specific manifestations of Fabry disease. Modified from Germain (127).

Organ system	Disease manifestation
Central and peripheral nervous system	Acroparesthesia Heat intolerance Autonomic dysfunction Tinnitus Neurogenic hearing loss White matter lesions on cerebral MRI Stroke
Skin	Hypo- or anhidrosis Clustered angiokeratomas (umbilicus, buttocks, genitals or in the hands or feet)
Eyes	Cornea verticillata Lenticular opacities Vasculopathy in the retina and/or cornea
Kidneys	Progressive albuminuria/proteinuria Progressive loss of glomerular filtration rate Accumulation of Gb3 in renal cells Increased podocyturia
Heart	ECG abnormalities Impaired heart rate variability Arrhythmias Left ventricular hypertrophy, particularly increase septal and posterior wall thickness Late gadolinium enhancement on cardiac MRI
Gastrointestinal tract	Postprandial bloating and pain Diarrhea Nausea (vomiting)

Table 3. Diagnostic criteria for classical Fabry disease. Modified from Smid et al. (128)

	Males	Females
Classical Fabry disease	Variant in the GLA gene **and** <5% of the normal mean leukocyte agalsidase activity combined with a minimum of 1 of the following: • acroparesthesia • cornea verticillata • angiokeratoma • significantly increased plasma lysoGb3 or Gb3 **or** A family member with classical Fabry disease according to the above criteria	Variant in the GLA gene **and** a minimum of one of the following criteria • acroparesthesia • cornea verticillata • angiokeratoma • significantly increased plasma lysoGb3 **or** A family member with classical Fabry disease according to the above criteria
Uncertain diagnosis	Do not fit the classical criteria Further organ specific investigations are necessary. Gold standard: Histologic investigation for characteristic lysosomal storage, e.g. lamellated inclusions	Do not fit the classical criteria Further organ specific investigations are necessary. Gold standard: Histologic investigation for characteristic lysosomal storage, e.g. lamellated inclusions

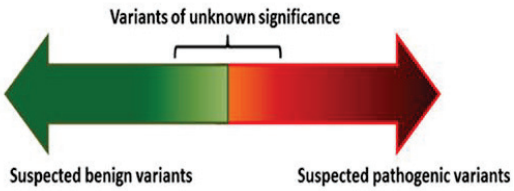
1.2.4 The GLA gene and Fabry disease

The GLA gene is located on the long arm of the X-chromosome, and the structure was described in the 1980's by Bishop et al. (129). To date over 400 variations in the GLA gene can be found in the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/?term=301500[MIM]), and the list is likely not exhaustive. All variations are not disease causing, some are polymorphisms and variants of unknown significance (Figure 8). Screening programs tend to identify more individuals with genetic variants of unknown significance than individuals with classical Fabry disease (130). Discriminating between non-classical Fabry disease and a genetic variant of uncertain significance may be difficult, and diagnostic algorithms have been proposed (131, 132).

Fabry disease may be caused by nonsense, missense, frameshift or splice site mutations, with nonsense and missense as the most frequently occurring mutations. Each amino acid in a protein is coded for by a triplet of nucleotides, a codon. In nonsense mutations a point mutation results in a premature stop codon, leaving the alpha-galactosidase enzyme truncated or incomplete, with little or no function. Missense mutations are usually less severe. A single nucleotide is altered, causing a different amino acid to be substituted for the original. This may significantly impair alpha-galactosidase activity, and very severe missense mutations are associated with very little or no residual alpha-galactosidase activity. In frameshift mutations, several nucleotides are either inserted or deleted, causing a change in how the gene is read and translated. Depending on the mutation, the aberrant end-product may be several amino acids longer or shorter, and function is likely to be diminished. In splice site mutations nucleotides are either inserted or deleted at the boundary of an exon and an intron. Precursor messenger RNA (mRNA) transcribes the coding sequence (exon) and the non-coding sequence (intron). Only the exon is required to produce the end-product, and conversion into mature mRNA is achieved through the removal of the non-coding introns. If nucleotides are inserted or deleted at the intron/exon boundary, the maturation of precursor mRNA into mature mRNA is disrupted, resulting in a mutant end-product.

Figure 8. Classification of genetic variations. Modified from Richards et al. (133)

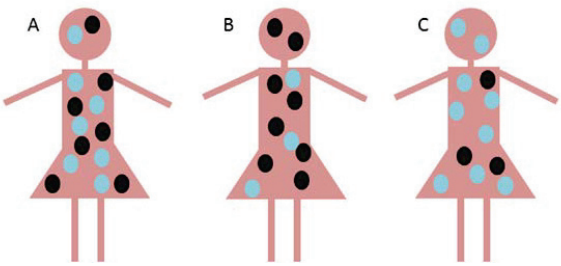
Risk classification	Clincial significance
5	Definitely pathogenic
4	Likely pathogenic
3	Uncertain
2	Likely benign
1	Definitely benign



1.2.5 Lyonization

The female genotype is XX, and one X chromosome is inactivated at random in every female cell. This process is known as random X-inactivation or lyonization, and was first proposed in 1962 by Mary Lyon (46). Skewed X-inactivation may occur, whereby either the maternal or the paternal X-chromosome is inactivated in the majority of cells (Figure 9). Lyonization may become important when dealing with an X-linked disorder such as Fabry disease. When a defective GLA gene is inherited by a female, half of her cells should express the healthy X-chromosome, carrying a normal GLA gene coding for the normal alpha-galactosidase. Should skewed X-inactivation take place, favoring the X-chromosome carrying the mutated GLA gene, a more severe phenotype is likely to occur. Conversely, should the woman be skewed towards the healthy X-chromosome, a more favorable disease course may be expected.

Figure 9. Lyonization of the X-chromosome in females, balanced and unbalanced X-chromosome inactivation.



1.2.6 Enzyme replacement therapy

The lysosome was serendipitously discovered by the Belgian cytologist and biochemist Christian de Duve. He suggested the concept of enzyme replacement therapy for lysosomal storage diseases, and saw ERT become a reality for several such diseases before his death in 2013. Alpha-galactosidase was identified by Brady et al in 1967, and the first treatment attempts in Fabry disease, using normal plasma infusions, were published in 1970 (134). Enzyme replacement therapy with ceramidetrihexose (alpha-galactosidase) purified from human placental cells was subsequently attempted in two Fabry patients in 1973 (135). Recombinant galactosidase was made available to the European market in 2001, and two products were marketed, intravenous agalsidase-α 0.2 mg/kg every other week (human cell-line) and agalsidase-β 1.0 mg/kg every other week (Chinese Hamster Ovary cell-line) (136, 137). Based on available evidence, the European Medicines Agency determined that agalsidase-α and –β should be licensed in different doses, as equipotent treatment. Only agalsidase-β 1.0 mg/kg every other week reached the North-American market, as only one product can be licensed in the treatment of orphan diseases by the Food and Drug Administration.

Although different doses of agalsidase-α and –β are given in vivo, in vitro studies suggest that the two products have similar properties milligram per milligram (138, 139). In a randomized controlled trial of agalsidase-α 0.2 mg/kg/eow versus agalsidase-β 0.2 mg/kg/eow, no difference in left ventricular mass or adverse clinical events could be ascertained between the treatment groups after 12 and 24 months of ERT (140). Unfortunately most studies have been underpowered, and the question of whether the two licensed doses are indeed equipotent remains largely unanswered nearly 15 years after ERT first became widely available. The vast phenotypical spectrum also hampers the design of robust comparative studies. Selection bias may hamper studies based on data from the two Fabry patient registries (Fabry registry and Fabry Outcome Survey) run by the two respective manufacturers of agalsidase, as not all agalsidase treated patients are included, and the incentive may be stronger to include patients with more advanced Fabry disease.

Few studies have included kidney biopsies, and follow-up time has been relatively short. In studies including kidney biopsies, capillary endothelial cells are cleared of globotriaosylceramide irrespective of agalsidase dose (136, 137, 141-143). Reaching the podocyte, a terminally differentiated cell with a longer lifespan than that of endothelial cells, has proven more difficult. Agalsidase-β 1.0 mg/kg/eow has been shown to reduce podocyte Gb3 load in adult patients treated for 54 months (144) and in young patients treated for 5 years (143). Tøndel et al. were also able to show a dose dependent reduction of podocyte Gb3 load (143). Enzyme replacement therapy has been found to improve quality of life for sufferers of Fabry disease (145-147), and though there is evidence for beneficial effects on kidney disease, the evidence is not equally strong for effects on other organ manifestations of this multisystem disease (148). Patients continue to have clinical events in the face of "state of the art" treatment (149, 150). The mechanism behind Fabry vasculopathy have yet to be fully elucidated, and the treatment currently available is inefficient at preventing cardiac fibrosis and stroke. Indeed, cardiac death is more common than progression to end stage renal disease in Fabry disease (151, 152).

1.2.7 Chaperone therapy

The feasibility of pharmacological chaperone therapy is based on the presence of an amenable missense mutation in the GLA gene, resulting in a structurally aberrant and partially defective protein. The mutant protein is detained at the endocytoplasmic reticulum, preventing its transport to the lysosome where it exerts its primary action. The pharmacological chaperone binds to the active sites of the mutant alpha-galactosidase, shifting the aberrantly folded protein towards the normal configuration. The enzyme is thus able to escape the endocytoplasmic reticulum and enter the lysosome, where the chaperone dissociates from the enzyme (153-155). Close to 90 amenable missense mutations have been identified to date. The list is likely to increase as more mutations are evaluated for amenability. In cases where a misfolded, partially active enzyme is produced, the patient should be able to benefit from endogenously produced enzyme, if the enzyme can be aided into the lysosome (154, 156).

Currently one oral chaperone is available on the market for Fabry disease. In a randomized controlled placebo controlled cross over study, no difference between groups in reduction of Gb3 inclusions per renal interstitial capillary could be found (157). However, all patients were not treatment naïve, and interstitial capillary Gb3 load in treated patients was likely already low due to prior ERT. Plasma lysoGb3 was significantly lower after 6 months of chaperone therapy as compared to 6 months of placebo (157), indicating a biological effect of the chaperone therapy which may have been difficult to ascertain histologically, due to the study design and methods. An open label study of patients with amenable GLA mutations randomized patients already treated with ERT to switch to an oral chaperone, or to continue with previously prescribed ERT (agalsidase-α 0.2 mg/kg/eow or agalsidase-β 1.0 mg/kg/eow) (158). After 18 months of treatment, no statistical difference in change of eGFR or mGFR was found between the two treatment groups. Plasma lysoGb3 levels were stable after switch from ERT to the oral chaperone, and the oral chaperone was associated with a decrease in left ventricular mass (158). The half-life of the two currently available agalsidase products is relatively short, and co-administration of ERT and a chaperone may remedy this (159).

1.2.8 Neutralizing antibodies towards agalsidase

The hemizygous male has very little endogenous alpha-galactosidase (121), and infusion of recombinant agalsidase-α or –β may elicit a humoral immune response as the immune system is exposed to high levels of enzyme of foreign origin (160-162). Agalsidase-α is derived from a humane cell-line (137), which in theory might cause less triggering of the immune system. Agalsidase-β is produced in Chinese hamster ovary cells (136), and infusions of 1.0 mg/kg/eow expose patients to a relatively high load of non-humane protein. Antibody formation was reported initially in both products, with a greater proportion of patients treated with agalsidase-β developing antibodies (137, 163). A Dutch study of 18 patients treated with either agalsidase-α 0.2 mg/kg/eow, agalsidase-β 0.2 mg/kg/eow or agalsidase-β 1.0 mg/kg eow showed development of IgG antibodies of 11 patients, only two female patients remained antibody negative. Complete cross reactivity was observed, in that patients who had

antibody titers toward one product were found to have the same titer toward the other product. Marked in vitro inhibition of enzyme activity was seen, irrespective of which product the patient had received (160). A similarly designed study detected agalsidase antibodies after 6 months of ERT in 4 out of 10 males treated with agalsidase-α 0.2 mg/kg/eow, 6 out of 10 males treated with agalsidase-β 0.2 mg/kg/eow and 8 out of 10 males treated with agalsidase-β 1.0 mg/kg/eow (164). Lubanda et al. reported IgG antibodies towards agalsidase-β in 18 of 21 patients, with three patients reverting to seronegative status over time, and two patients maintained low levels of antibodies (142). Statistical power was not sufficient to adequately test the impact of antibody formation on Gb3 levels in plasma or tissue sections. Development of agalsidase antibodies may adversely impact ERT efficacy. Antibody positive males have been shown to have significantly higher levels of lysoGb3 as compared to those without antibody formation, and urine Gb3 levels remained unchanged from baseline (165).

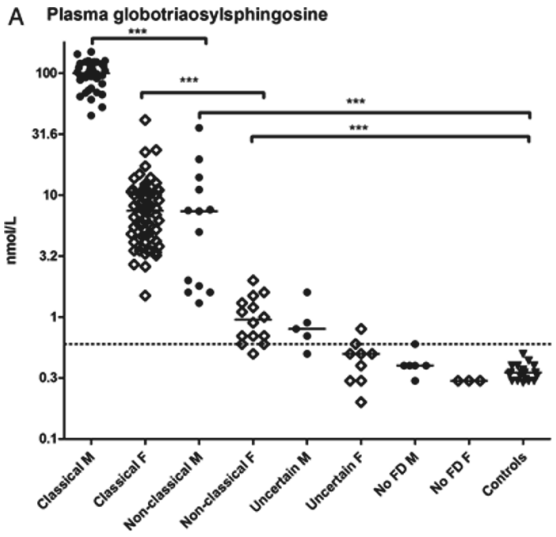
There are however some caveats when comparing studies which report on antibody formation. Different criteria may have been used to assess antibody formation, and differences in cut-off points, sample dilution and the timing of testing may be present, which in turn may affect the reported results.

1.2.9 Biomarkers in plasma and urine

A biomarker is an objective indication of a particular medical state, which can be accurately measured in a reproducible manner (166). The National Institutes of Health Biomarkers Definitions state that it is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (167). In Fabry disease, the sphingolipid globotriaosylceramide has been the main disease specific biomarker in plasma and urine (168). Gb3 is the substrate for alpha-galactosidase, and faced with a severe enzyme deficiency, the substrate will accumulate in plasma and lysosomes. Women with Fabry disease are heterozygous for the GLA mutation, and most retain some enzyme activity (169). Serum Gb3 may therefore be within the normal range. This is also true of some missense mutations which result in some residual enzyme

activity. Urinary Gb3 likely originates from lysosomes in tubular cells, shedding of podocytes laden with Gb3 could also be a source of urinary Gb3. The effects of ERT on Gb3 in plasma and urine do not seem to correlate with clinical disease, and reduction in the biomarkers may be observed as clinical disease progresses (140, 170, 171). Globotriaosylsphingosine (lysoGb3) is another Fabry specific biomarker. LysoGb3 is significantly elevated in all classically affected hemizygotes, but GLA variations associated with mild or atypical Fabry disease can have lysoGb3 levels which are only marginally elevated, or even within the normal range (172). LysoGb3 is important in the diagnostic work-up of Fabry disease, and is reliable in identifying classical disease in males and females (122) (Figure 10), however the study was unable to discern groups of patients with non-classical, uncertain status or no Fabry disease from each other. The authors conclude that normal lysoGb3 levels in males is strongly indicative of absence of Fabry disease, whereas normal levels in a female is not necessarily compatible with absence of Fabry disease (122). The main value of lysoGb3 appears to be in the diagnostic work-up of suspected Fabry disease, and while lysoGb3 may be reduced by ERT (173), levels are not normalized (174). LysoGb3 has been shown to increase smooth muscle cell proliferation (175), and has been associated with an increase in the profibrotic cytokine TGF-β1 (176), which may play a part in the development of Fabry associated vasculopathy. LysoGb3 has also been linked to an upregulation of Notch1 in podocytes in vivo, which may cause a NF-κB dependent proinflammatory response in the podocyte (177).

Figure 10. Plasma lysoGb3 grouped by phenotype and gender. Reprinted with permission from Smid et al. (122).



Upper normal limit is defined as 2 SD above the mean of healthy controls, and is delineated by the dotted horizontal line. ***= $p<0.001$.

Loss of podocytes in the urine may be seen in both Fabry patients and healthy controls, with higher levels seen in Fabry patients (178), and may precede albuminuria. Although podocyturia is not specific to Fabry disease, it has been found to correspond to severity of Fabry nephropathy in males (178, 179). Increasing density of podocyte Gb3 load has been linked to podocyte damage (180, 181). Loss of podocytes in glomerular disease may be multifactorial, but factors such as biomechanical stretch or change in proteins anchoring the podocyte to the basement membrane likely play a role (182, 183). Accumulation of Gb3 within the podocytes has been associated with an increase in podocyte volume, with concomitant volume reduction as Gb3 is cleared from the podocytes by ERT (181). The increasing volume of the podocyte in Fabry nephropathy, with accompanying foot process widening, may induce biomechanical stretch, dislodging the podocyte from the glomerular basement membrane.

Increase in serum creatinine is not specific to Fabry nephropathy, nor is podocyturia. A significant proportion of normal kidney function must be lost before any change in serum creatinine is seen, and creatinine is thus not a very good biomarker in the early stages of kidney disease (184). Estimated glomerular filtration rate based on a single creatinine measurement may also miss subtle changes in kidney function during the early phases of chronic kidney disease. This is particularly true of Fabry nephropathy (185-187), where low muscle mass may contribute to an overestimation of true GFR. To remedy this, measured GFR should be performed at regular intervals, in tandem with clinical assessment and estimated GFR. Fabry nephropathy is a progressive disease, without a generic time-point for ERT start. This is largely due to the vast clinical heterogeneity seen in patients with Fabry disease, and meticulous clinical examination and investigations are necessary to tailor initiation of ERT to the individual patient.

Glomerular albuminuria and proteinuria may occur in chronic kidney disease irrespective of etiology, and is caused by changes in the glomerular filtration barrier, composed of podocytes, the basement membrane, fenestrated endothelium and the endothelial cell surface layer (188). Both proteinuria and albuminuria can be evaluated in spot urine samples, and is reported per unit of urinary creatinine (86). Increasing levels of albumin or protein excretion in the urine is associated with further loss of GFR (189) and increased cardiovascular and all-cause mortality (96) in chronic kidney disease. In patients with Fabry disease, low grade albuminuria may precede loss of GFR, and may serve as an indication for starting ERT.

1.2.10 Indications for Fabry specific therapy

Fabry disease is an inborn disease, and accumulation of Gb3 can be seen in the fetal side of the placenta (190). Although the enzymatic defect is present at birth, Fabry related symptoms take years to develop. In patients with the more severe phenotype, clinical symptoms such as acroparesthesia and dyshidrosis may develop during childhood and mainly precede clinical evidence of organ damage (albuminuria, loss of GFR, left ventricular hypertrophy) by many years. Individuals who have a less severe

phenotype may have only mild acroparesthesia with later onset, and Fabry disease may be diagnosed as a result of family screening, or targeted investigations for specific organ damage during adulthood. Neuropathic pain may be managed with oral therapy, such as paracetamol, carbamazepine, gabapentin or pregabalin (191). There is some evidence that ERT may reduce neuropathic pain in adults (137, 192), however this effect may be less pronounced in clinical practice (150). The optimum time to start ERT has been much debated, and no randomized controlled trials have investigated this question. The threshold for starting ERT may vary from country to country, and the literature offers only opinion based guidelines. There is consensus that treatment should start before irreversible organ damage is manifest (191), however the phenotypical spectrum is wide, and timing of ERT must likely be individualized to suit the classical phenotype, the late phenotype and Fabry diseased in women. A consensus statement for when to initiate ERT in Fabry patients was recently published. The treatment guidelines were stratified by organ specific symptoms, gender and phenotype (193). Fabry manifestations in the renal, cardiac, central nervous system, peripheral nervous system and GI domains were identified as indications for starting ERT, and ERT was recommended when potentially reversible organ damage became apparent. Concerning the renal domain, initiation of ERT was recommended when microalbuminuria, proteinuria or early signs of renal insufficiency (eGFR 45-90) became apparent, irrespective of phenotype or gender (193). Importantly, the consensus group also discussed whether initiation of ERT may be too late, and when ERT might be in vain. Severe end-organ damage with a short life expectancy was recommended as indications for not starting ERT, whereas non-compliance with infusions or follow-up visits, severe and life-threatening infusion reactions or severe end-organ damage or cognitive decline were some of the identified stop criteria (193). No consensus could be reached for treatment criteria for patients below the age of 16 years, and a recent KDIGO controversies conference stated that starting ERT should be balanced against the burden of biweekly infusions (191). There is evidence for beneficial ERT effects on different organ systems, however evidentiary quality is often hampered by sample size, heterogeneous cohorts or reporting bias. Evidence suggests

that ERT may improve Fabry related left ventricular hypertrophy, if there is no fibrosis present when ERT is started (194, 195). When cardiac fibrosis is present, Fabry cardiomyopathy may progress in the face of ERT. There is no evidence to support reversal of arrhythmias or ECG changes on ERT (191). Long-term ERT may slow progression of or stabilize Fabry nephropathy in adults (144), and has also been shown to improve renal histology in young Fabry patients (143), with dose dependent reduction of podocyte Gb3.

1.2.11 Disease progress on ERT

Fabry disease is caused by an enzymatic defect, and one might expect that enzyme substitution in adequate doses would remedy all manifestations of the enzyme deficiency, and prevent further disease progression. ERT has been found to ameliorate signs of kidney disease and to reduce podocyte Gb3 load (143, 181), and agalsidase-α and –β have been reported to have beneficial effects on Fabry cardiomyopathy, if treatment is started before fibrosis develops (195, 196). Agalsidase-β has been reported to have beneficial effects on left ventricular mass (197), and a randomized placebo controlled trial reported regression of left ventricular hypertrophy in 15 male patients after only 6 months of agalsidase-α treatment (194). However, no additional decrease in left ventricular mass was observed in the 10 patients who continued in an open label study after an additional two years of treatment (194). An open label study of agalsidase-β 1.0 mg/kg/eow reported on 26 patients (6 females) who started ERT at a mean age of 41.4 ± 8.9 years (198). Only 9 patients had mGFR > 90ml/min/1.73m^2, the rest were in varying stages of CKD, including dialysis. Twelve endpoints were seen in 9 patients after a mean treatment time of 23 ±8 months, strikingly all end points were seen in patients with mGFR less than 60 ml/min/1.73m^2. ERT did not halt CKD progression in individuals with established CKD, nor was ERT found to alter left posterior wall thickness (198). A comparison between a cohort of Fabry patients treated with either agalsidase-α 0.2mg/kg/eow or agalsidase-β 1.0 mg/kg/eow (13 patients received agalsidase-β 0.2 mg/kg/eow in a clinical trial for parts of the study period) and a “natural history” cohort was performed at the Academic Medical Center (148). Fifteen patients developed a major Fabry complication on ERT, compared to 19

in the “natural history” cohort. No difference in time to first complication was observed between the two groups, nor was time to a second complication different. The risk of developing a complication did however decrease with increasing treatment time. Some differences were noted, renal function and left ventricular mass were found to remain stable in the female ERT treated participants, and increase in left ventricular mass mainly occurred in males with CKD (148).

Cerebrovascular disease is a feature of Fabry disease. A prospective study reported a frequency of Fabry disease of 1.2 % in young stroke patients (199), and data from the Fabry registry showed a stroke prevalence of 6.9 % and 4.3 % in untreated males and females respectively (114). Thus far there is little evidence to suggest that ERT prevents cerebrovascular complications in Fabry disease, on the contrary most evidence suggest that cerebrovascular complications continue to develop in the face of ERT (148, 150, 200). Atrial fibrillation and ventricular arrhythmias are associated with Fabry disease, becoming more frequent with age (201). ERT likely has little impact on the risk of developing malignant arrhythmias (202). The presence of atrial fibrillation may further increase the risk of stroke in patients with Fabry disease.

The discouraging frequency of complications reported in ERT treated patients caused an editorial asking whether there is a role for ERT in Fabry disease (203). As sustainable agalsidase replacement therapy became available, the initial hopes were for ERT treated patients to have marked improvement in Fabry related organ damage, and certainly to avoid further complications. After nearly 16 years of clinical use, it has become apparent that though ERT may not improve or halt disease manifestations in all, it may impede disease progress in select patients and organ systems.

1.2.12 Kidney biopsy

Kidney disease is often classified according to histology, e.g. IgA nephritis, focal and segmental glomerulosclerosis, minimal change disease, different classes of Lupus nephritis. A kidney biopsy can also identify cases where a hereditary nephropathy should be suspected. Foam cells should alert the nephrologist to the possibility of

Alport syndrome, whereas lamellated inclusions in the podocyte may herald Fabry disease.

Percutaneous kidney biopsies were first performed in the 1950's, and as ultrasonography was developed and became more widely available, real-time ultrasound guided kidney biopsy became the method of choice (204). The frequency with which native kidneys are biopsied varies across the world (205), the possibility of procedure related complications, and differing local guidelines, may influence the number of biopsies performed in different parts of the world. In an Australian report the biopsy frequency was 25.4 per 100 000 at a single center (206), whereas the observed biopsy rate for native kidneys was 4.8 per 100 000 in Spain (207). The annual rate of native kidney biopsies in Norway has been roughly 11 per 100 000 inhabitants since the late 1980's (208).

The value of renal histology remains undisputable (205), and has been found to alter the diagnosis and clinical management in a significant number of patients. In a single center experience the clinical management was changed in 42% of all cases, due to the results from a kidney biopsy (209).

The decision on whether to biopsy or not is usually founded on local guidelines and practice. The lack of an international consensus on when to perform a kidney biopsy may also in part explain the geographical differences in biopsy frequency. The ERA-EDTA Immunonephrology Working Group published a review of the current literature in 2015, which concluded that nephrotic syndrome in adults, coexisting hematuria and proteinuria in adults, steroid resistant nephrotic syndrome in children, acute kidney injury when pre- and post-renal causes have been excluded, and renal disease due to systemic disease were firm indications for performing a biopsy (205).

The level of risk associated with a kidney biopsy is generally low, when contraindications such as bleeding diatheses and uncontrolled hypertension have been eliminated. A single center publication of 1055 kidney biopsies performed from 1983 to 2012 reported minor complications in 8.1 % of biopsies, of which 4.5 % were macroscopic hematuria. Major complications were reported in 6.6 % of biopsies, of

which 5.3 % required blood transfusions (210). In a Norwegian study including 9288 kidney biopsies from 1988-2010, macroscopic hematuria was reported in 1.9 % of the biopsies, and transfusions were performed after 0.9% of biopsies. Surgical intervention or renal catheterization was performed in 0.2% of the biopsies (211). Thus, kidney biopsies can be considered low risk, when using modern technology and respecting contraindications.

2 Aims of the thesis

The aims of the thesis were to investigate whether having a first-degree relative with ESRD adversely impacted the risk of developing ESRD due to non-Mendelian disease, and to investigate whether first-degree relatives of ESRD patients were at increased risk of premature death, and causes of death. Furthermore, clinical symptoms, renal morphology and GFR in the X-linked disorder Fabry disease were investigated to evaluate the effects of long-term enzyme replacement therapy in a known hereditary disorder causing CKD and ESRD, for which there is disease specific treatment.

3 Materials and Methods

3.1 Registries

3.1.1 The Norwegian Population Registry (NPR)

The Norwegian population Registry was established in 1960, and records administrative data for all citizens residing in the realm, organized by an eleven-digit personal identification number. Registration in the population registry is mandatory, and not subject to individual consent. Maternal and paternal information is virtually complete for all individuals born in Norway after 1952. First degree relatives, defined as a parent, offspring or a full sibling, were identified using data from this registry in Paper I and Paper II (212, 213).

3.1.2 The Norwegian Nephrology Registry (NNR)

The Norwegian Nephrology Registry has since 1980 registered all individuals in Norway with renal replacement therapy (RRT), defined as either dialysis or kidney transplantation. Data are reported to the registry by the treating physician upon initiation of RRT, and annually thereafter until either discontinuation of treatment or death. Informed consent is obtained from all patients before data are reported to the registry. Data were available through medio 2009 for Papers I and II.

3.1.3 The National Cause of Death Registry

Reporting the cause of death is mandatory on the Death Certificate, which is completed by a physician. Causes of death are registered by the National Cause of Death Registry, using the International Classification of Diseases system. Data were available through 2008 for Paper II.

3.1.4 Linking the data

After obtaining consent from the Regional Ethics Committee (REK 2009/2051), data from the registries were linked using the Norwegian personal identification number. The linked dataset was de-identified, and subsequently anonymized, and used in Papers I and II.

3.2 Evaluation of Fabry disease

3.2.1 Clinical evaluation of Fabry patients

About two thirds of the known patients with Fabry disease in Norway have clinical follow-up visits at Haukeland University Hospital, most of whom contribute to the clinical follow-up study REK 2010/02483. Patients who receive ERT are followed annually, with clinical examination, Iohexol GFR and urine albumin to creatinine ratios. Additional organ specific investigations may be added to the yearly visit on individual indication. A comprehensive visit is scheduled every three to five years, with a standardized multi-disciplinary follow-up regimen, often including a kidney biopsy. All patients included in papers III and IV, twelve males and eight females, had

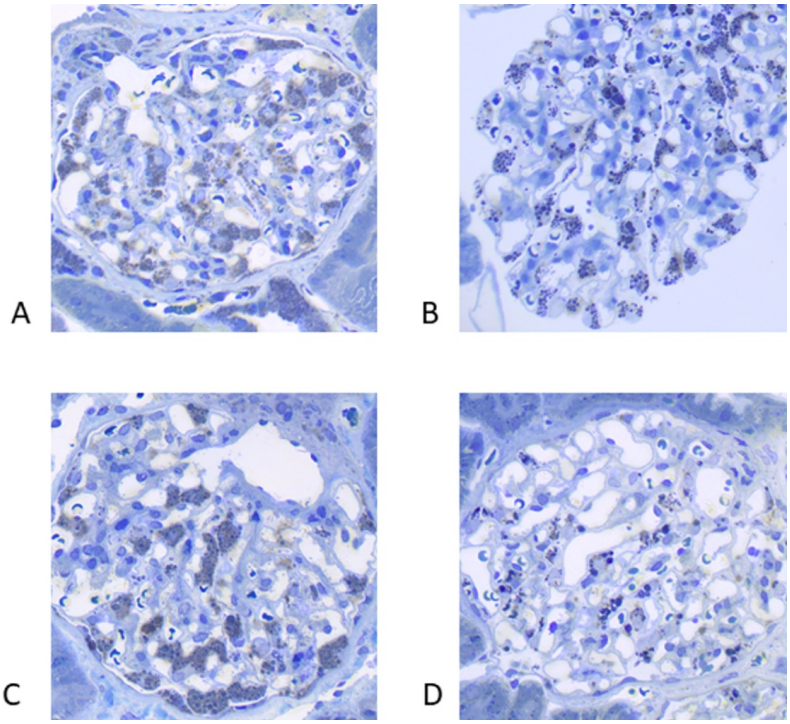
baseline and follow-up kidney biopsies along with comprehensive clinical evaluations during long-term treatment with ERT. Informed consent was signed in all cases, and the studies were performed according to the Declaration of Helsinki.

3.2.2 Renal evaluation

Glomerular filtration rate was measured by single point Iohexol analyses at four hours (214). Urine albumin and protein excretion were measured in three consecutive morning voids. A median albumin to creatinine ratio above 2.5 mg/mmol creatine was defined albuminuria, proteinuria was defined as a median protein/creatinine ratio of >20.0 mg/mmol creatinine.

All individuals included in Papers III and IV underwent serial kidney biopsies to evaluate disease progress and effects of ERT. The formalin fixed tissue was stained with periodic acid Schiff (PAS), whereas tissue fixed in McDowell's medium was stained with toluidine blue (Figure 11). Electron microscopy was performed on plastic embedded tissue. The kidney biopsies were evaluated by experienced nephropathologists, who were blinded with respect to treatment. The kidney biopsies were scored according to the scoring system of the International Study Group of Fabry Nephropathy (215), where maximum scores of 3.0 and 4.0 were possible in PAS and toluidine stained tissue respectively. These scores were combined to improve representativity, resulting in a maximum composite score of 7.0.

Figure 11. Serial kidney biopsies, sections stained with toluidine blue. Male patient treated with agalsidase-α 0.2 mg/kg/eow.



Male patient who started agalsidase-α 0.2 mg/kg/eow at 11 years of age. **A**: Baseline. **B**: 3 years of ERT. **C**: 5 years of ERT. **D**: 10 years of ERT. Marked reduction of podocyte Gb3 is seen after 10 years of treatment.

3.3 Summary of Methods

3.3.1 Methods Papers I and II

Papers I and II included all individuals born in Norway who had a minimum of one registered first-degree relative in the Norwegian Population Registry. Individuals without registered first-degree relatives were excluded from the cohort. First-degree relatives were defined as a parent, a full sibling or offspring. The exposure variable in

both Paper I and II was defined as having a first-degree relative with ESRD. The outcome variable for Paper I was onset of ESRD in the included individuals, defined as either initiation of dialysis or kidney transplantation. Individuals with ESRD due to a known hereditary cause were excluded from the main analyses. Those who did not develop ESRD were followed until the end of 2009 or death. As no outcomes were registered prior to 1980, the data were left truncated. Parental information was registered in the Norwegian Population Registry from the 1970s, and information on first-degree relatives were virtually complete for individuals born after 1952. For this reason, the main analyses were repeated for the cohort born between 1953 and 2009. The outcome variables in Paper II was death, and causes of death. Data on death and causes of death was available from January 1969, consequently the data were left truncated. Causes of death were reported using the old ERA-EDTA classification (216), and were classified according to the International Classification of Diseases (ICD) versions 8 through 10. ICD 8 was used from 1969-85, ICD 9 from 1986-1995, and ICD 10 was implemented in 1996. Only small changes were made in the coding system when moving from ICD version 8 to 9, however a significant revision was made when transitioning to ICD 10. To compare causes of death over periods spanning different ICD versions, a shortlist translating "old" causes of death to the newer ICD version was prepared by Eurostat. A Norwegian version of this shortlist was used to group causes of death to be able to estimate hazard ratios for disease specific causes of death in the dataset.

To more robustly evaluate the risk of premature death associated with having a first-degree relative with ESRD, individuals who died during the first year of life were excluded from the cohort in Paper II. A cohort design was used in both papers, and relative risk was estimated by Cox regression statistics. The analyses were adjusted for sex, number of identified first-degree relatives and birth year. Data were reported with 95% confidence intervals, and p values of < 0.05 were considered statistically significant.

3.3.2 Methods Papers III and IV

Twenty patients with classical Fabry disease, treated with long-term agalsidase were included in Paper IV. Three of the patients were included in Paper III, which included all treatment naïve patients started on agalsidase-β 1.0 mg/kg every other week who were switched to agalsidase-α 0.2 mg/kg/every other week due to the global shortage of agalsidase-β (n=3, males=2, female=1). Baseline and long-term follow-up clinical data, measured GFR and kidney biopsies corresponding to changes in agalsidase dose were reported for the three patients included in this case series. Due to the small number of included individuals, no statistical analyses were possible.

Paper IV, and observational clinical study, included twenty patients with classical Fabry disease. The 12 males and 8 females had received ERT for a median time of 9.4 (range 5-13) years, all had undergone serial kidney biopsies along with comprehensive clinical assessments at Haukeland University Hospital during the treatment period. Upon starting ERT, all patients were intended for fixed dose of the prescribed product. Agalsidase dose or product was changed either due development of Fabry complications, inadequate symptom relief or lack of agalsidase-β.

The cohort was divided into two groups based on the received agalsidase dose. All patients underwent baseline kidney biopsies, two patients had their first biopsy after two years of ERT, whereas one patient was biopsied after three years of ERT. The kidney biopsies were scored according to the scoring system of the International Study Group of Fabry Nephropathy (215), and change in podocyte globotriaosylceramide inclusions and vacuolizations was analyzed. Globotriaosylsphingosine (lysoGb3) was analyzed at the Academic Medical Center, the Netherlands, and change in lysoGb3, mGFR, plasma sphingolipids and urine albumin to creatinine ratio performed in parallel with renal biopsies were analyzed for statistical significance. Linear regression was used to ascertain agalsidase dose dependent clearing of podocyte globotriaosylceramide accumulation. Mixed model regression analysis was used to adjust for individual variables. Student's t-test was used to compare normally

distributed variables, and Mann-Whitney U test was used for non-normally distributed variables. A p-value < 0.05 was considered statistically significant.

4 Summary of main results

4.1 Results Papers I and II

Paper I included 5,119,134 individuals, 27,046 had a first-degree relative with ESRD. Of the 8203 individuals who developed ESRD during follow-up, 313 individuals had a first-degree relative with ESRD. Mean birth year for individuals with ESRD was 1940 ±18, as compared to 1963 ±28 in the group without ESRD. Individuals with ESRD were more likely to be male, and to have fewer recorded first-degree relatives, mean number of first-degree relatives was 3.4 ± 2 as compared to 4.0 ± 2.1 in the group without first-degree relatives. The relative risk of all-cause ESRD in individuals with a first-degree relative with ESRD was 7.2 (95% CI 6.5-8.1) when compared to those who did not have a first-degree relative with ESRD. This risk decreased to 3.7 (95% CI 3.1-4.4) when individuals with known hereditary nephropathies were excluded from the analysis. The risk estimates remained virtually unchanged after adjustments for birth year, number of first-degree relatives and sex. The highest risk estimates were seen in individuals whose first-degree relatives had glomerular or interstitial disease, with relative risks of 5.2 (95% CI 4.1-6.6) and 4.7 (95% CI 3.1-7.3) respectively. As the dataset was nearly complete for individuals born 1953 or later, the analyses were repeated for this subset, which consisted of nearly 60% of the entire cohort, but only 22% of those who developed ESRD. Relative risk of ESRD due to any cause was 10.2 (95% CI 8.7-12) in this cohort, which decreased to 5.3 (95% CI 4.1-6.8) after individuals with known hereditary disease were excluded from the analysis.

Paper II included 5,130,600 individuals, 27,508 had one or more relatives with ESRD. During follow-up 828,022 individuals died, and of those 4150 had a first-degree relative with ESRD. Individuals who died during the first year of life or had no recorded first-degree relatives were excluded from the analyses. Deceased individuals

were more likely to be male, and had fewer recorded first-degree relatives. The unadjusted hazard ratio for all cause death in individuals who had a first-degree relative with ESRD was 1.07 (95% CI 1.04-1.11), compared to individuals without a first-degree relative with ESRD. When individuals with known hereditary renal disease were excluded from the analyses, the hazard ratio decreased to 1.06 (95% CI 1.02-1.09). In analyses adjusted for birth year, sex and number of first-degree relatives the hazard ratio for all-cause death was 1.13 (95% CI 1.09-1.16) in individuals who had a first-degree relative with ESRD as compared to individuals who did not have first-degree relative with ESRD. After excluding individuals with known hereditary renal disease from the adjusted analyses, hazard ratio for all cause death decreased to 1.12 (95% CI 1.08-1.16).

The most common causes of death during follow-up were cardiovascular disease, cancer and pulmonary disease. However, death due to cardiovascular disease, diabetes mellitus and diseases of the kidneys and ureters were associated with the highest hazard ratios for death. Individuals with a first-degree relative with ESRD had an adjusted hazard ratio for cardiovascular death of 1.15 (95 % CI 1.10-1.21), of which cerebrovascular disease was associated with the highest aHR 1.34 (95% CI 1.22-1.47). Death due to hypertensive disease was not statistically significant, aHR of 1.30 (95% CI 0.97-1.76) when individuals with a first-degree relative with ESRD were compared to those without. Death due to non-hereditary, non-congenital diseases of the kidneys and ureters was associated with an aHR of 2.29 (95% CI 1.81-2.91). Death due to glomerular diseases was associated with the highest aHR, 5.69 (95% CI 3.88-8.34), followed by death due to renal failure of uncertain etiology (1.80 (95% CI 1.26-2.56)). Individuals who had first-degree relatives with ESRD also had increased aHR for death due to diabetes mellitus, 1.68 (95% CI 1.35-2.10), compared to individuals without ESRD in first-degree relative. Conversely, diabetic nephropathy in a first-degree relative was associated with an aHR of death from any cause of 1.21 (95% CI 1.13-1.29). ESRD due to hypertensive nephropathy in a first-degree relative also increased the risk of all cause death (aHR 1.24 (95% CI 1.14-1.34)), although ESRD in a first-degree relative was not associated with an increase in aHR for death due to

hypertensive disease. The highest increase in absolute excess mortality risk was seen in the oldest subset of the cohort. Individuals born from 1920 to 1939 had an absolute risk increase of 148 per 100,000, whereas an absolute risk increase of 218 per 100,000 was found in the cohort born 1900 to 1919.

4.2 Results Papers III and IV

Paper III included two males and one female, aged 7, 11 and 18 years old respectively at the start of ERT. All had severe acroparesthesia, gastrointestinal symptoms and normal mGFR prior to starting ERT. Kidney biopsies performed at baseline revealed full ISGFN scores inn all three patients. After initiation of ERT, symptom-severity decreased, and kidney biopsies performed after five years of agalsidase-β 1.0 mg/kg/eow showed a reduction in podocyte Gb3 scores from 7.0 in all patients at baseline to 0.4, 2.9 and 4.9 in Patients 1, 2 and 3 respectively. Mesangial and endothelial cells were completely cleared of Gb3. Due to the global shortage of agalsidase-β, the patients were switched from agalsidase-β 1.0 mg/kg/eow to agalsidase-α 0.2 mg/kg/eow. An increase in pain and gastrointestinal symptoms were reported by all three patients after the switch. One patient suffered a Fabry related drop in mGFR, whereas one patient developed de novo glomerulonephritis during follow-up. Kidney biopsies performed three years after dose reduction showed re-accumulation of podocyte Gb3 in all three patients, and they were switched to agalsidase-β 1.0 mg/kg/eow as soon as the product again became globally available. A kidney biopsy was performed in Patient 1 two years after being switched back to agalsidase-β 1.0 mg/kg/eow, and renewed decrease of podocyte Gb3 was observed. The decrease in mGFR observed in Patient 3 after dose-reduction was reversed after reinstating agalsidase-β 1.0 mg/kg/eow.

Paper IV included twenty patients with classical Fabry disease treated with long-term enzyme replacement therapy. Median age at start of ERT was 21 (range 7-62) years. The higher dose group was a median of 10 years younger at start of ERT compared to the lower fixed-dose group, this did however not reach statistical significance. Median number of years on ERT was 9.4 (range 4.8-13.8) years. There

were no differences between the lower fixed-dose group and the higher dose group at baseline with respect to mGFR, albuminuria or plasma Gb3. Eleven patients had evidence of Fabry related complications at the baseline kidney biopsy. Both treatment groups had a significant reduction in composite podocyte Gb3 score from baseline to final kidney biopsy, mean reduction of podocyte Gb3 burden in the lower fixed-dose group was -1.39 (95% CI -2.19 to -0.59) p=0.004, whereas mean reduction of podocyte Gb3 burden in the higher dose group was -3.16 (95% CI -4.87 to -1.45) p=0.002. In a linear regression model, reduction of podocyte Gb3 load was found to correlate with cumulative ERT dose, r=0.693, p=0.001, and there was a significant difference in clearing of podocyte Gb3 when the lower fixed-dose group was compared to individuals who received agalsidase-β 1.0 mg/kg every other week for a minimum of 2 years leading up to the kidney biopsy. Those who received the higher dose leading up to the final biopsy had significantly better clearing of podocyte Gb3, p=0.01. Gb3 inclusions in the intima of arterioles and/or arteries were more frequent in the lower fixed-dose group at the end of follow-up, p=0.02. Similarly, medial inclusions were more frequent in the lower fixed-dose group at the end of follow up as compared to the higher dose group, p=0.17. However, change in medial Gb3 inclusions did not reach statistical significance when the groups were compared, p=0.046. Plasma lysoGb3 did not normalize in any patients, however residual lysoGb3 levels at the time of the last kidney biopsy was significantly lower in the higher dose group as compared to the lower fixed-dose group (p=0.04). Furthermore, residual lysoGb3 levels were found to correlate with cumulative ERT dose in males, r=0.711, p=0.01, but not in females. Serum was analyzed for inhibitory antibodies to agalsidase, only one patient (no 13) developed a significantly elevated antibody titer (≥ 5) after a total of 10 years of ERT.

5 Discussion

5.1 Methodological discussion

5.1.1 Study designs and cohorts

A retrospective nationwide cohort study design was used for Papers I and II. The datasets were obtained through linkage of the Norwegian Population Registry, the Norwegian Nephrology Registry, and the Cause of Death Registry, as described in the Methods section. Data reporting to the Norwegian Population registry and the Cause of Death Registry is mandatory. Registering of data in the Norwegian Nephrology Registry is subject to informed consent, after which data is reported to the registry by the treating physician. Data on ESRD is virtually complete in the Norwegian Nephrology Registry. In using nationwide registries with virtually complete coverage, the issue of "lost to follow-up" is significantly reduced. All citizens who were alive when the Norwegian Population Registry was established in 1960 were registered, parental information was incomplete for the cohort born prior to 1953, as this information was collected from the 1970s. This may have affected the results in Paper I, as individuals born prior to 1953 were older and more likely to develop ESRD, whereas those born in 1953 or later were more likely to have complete information on first-degree relatives. Incomplete information on first-degree relatives in a cohort with a higher proportion of individuals with ESRD may have resulted in underestimation of the relative risk of developing ESRD associated with having a first-degree relative with ESRD. For this reason, risk estimates were calculated separately for the cohort born after 1952 in Paper I. The assumption of underestimation of relative risk due to incomplete ascertainment of first-degree relatives was supported, as higher risk estimates were seen in the cohort with complete information on first-degree relatives. Several factors may contribute to an underestimation of the relative risk of ESRD associated with having a first-degree relative with ESRD; the prevalence of ESRD increases with increasing age, and it is unfortunate that the dataset was incomplete with respect to identifiable first-degree relatives for the oldest subset of the cohort. Progression of CKD to ESRD occurs over time, and ESRD is relatively rare in the

Norwegian population. The low absolute risk of ESRD meant that some of the risk estimates for specific causes of ESRD may have been imprecise, due to the low number of outcomes. Additionally, though causes of ESRD were reported to the Norwegian Nephrology Registry by the treating physician, all diagnoses were not verified by a kidney biopsy. Some cases may have been mislabeled as non-hereditary, when in fact a hereditary cause may have been the cause of ESRD. The dataset included all known cases of ESRD treated with long-term renal replacement therapy from 1980 to 2009. Several advances have been made in technology and molecular genetics in the recent years, and new monogenic causes of e.g. focal and segmental glomerulosclerosis have been identified since the dataset was generated. It is likely that some of the cases of ESRD would have received a diagnosis of genetic kidney disease had the cause of chronic kidney disease been investigated with the diagnostic tools of today.

The dataset included all individuals born in Norway up to 2009, which meant that some individuals were very young when included, and thus had a very short follow-up time. CKD progresses over time, and is more common in the older age groups, similarly older individuals are more likely to die. To overcome this hurdle, individuals should ideally be included over a defined inclusion period, and followed for several years, if not decades. Had studies I and II been designed in this way, standardized mortality rates would have been available, and a more precise estimate of potential years of life lost in at risk individuals. A prospective cohort design would however take a very long time to bear fruits, as progression of CKD to ESRD can occur over a long period of time.

The Cause of Death Registry registers causes of death through the mandatory death certificate which must be completed by a physician at the time of death. The immediate cause of death must be stated, and any illness leading up to death or contributing to cause of death may also be stated. Cause of death need not be verified by an autopsy, and the reported causes of death in the dataset used for Paper II may thus have been inaccurate in some cases.

Cox regression statistics were used in Papers I and II. The datasets used included individuals born from 1900 onwards, but outcomes were only registered after 1980 for Paper I and 1969 for Paper II. To compensate for this, the analyses were left truncated so that an individual would not be included until an event was registered (217). In using the method of Cox proportional hazards, equal effect of the exposure variable throughout time is assumed. This assumption implies similar risk of ESRD or death attributable to having a first-degree relative with ESRD in a 50-year old individual in e.g. 1995 and 2005. In our opinion, this is a reasonable assumption, not likely to have affected the results significantly. Cox regression analysis is a robust method where the effect size of the exposure variable can be investigated in different groups over a defined time-period. Additionally, hazard ratios can be adjusted for continuous and categorical variables. Analyses were adjusted for birth year and number of first-degree relatives in both Papers I and II to reduce confounding variables. The relative risk of ESRD did not alter significantly after adjustments for said variables were performed in Paper I. Similar adjustments were performed in the multivariate analyses in Paper II, without significant alteration in relative risks of death and causes of death. ESRD and death is more likely to occur in older individuals, and older individuals are more likely to have a first-degree relative with ESRD. Residual confounding may however still be present, as the datasets did not contain information on traditional risk factors for kidney disease and death such as smoking, lipid profile or hypertension.

Papers III and IV are observational clinical studies. The studies included classical Fabry patients (128), who received clinical follow-up at Haukeland University Hospital, were treated with long-term ERT, and underwent baseline and follow-up kidney biopsies. The observational design is less rigorous than a clinical trial, and all eligible Fabry patients were included in the studies, irrespective of age and gender. The large age span and inclusion of females may have further contributed to the heterogeneity often seen in Fabry cohorts. All included patients exhibited a classical phenotype, and were thus expected to follow a more similar disease course. Furthermore, the median age at initiation of ERT in the Paper IV cohort is significantly younger than most cohorts derived from Fabry registries (218, 219), and

it is therefore plausible that our cohort has a lower and more homogenous risk profile. Seven different GLA mutations were found in the included Fabry patients, four missense mutations and three non-sense mutations. Theoretically genotype could influence the response to ERT, some mutations might respond more promptly to therapy than others. We were however not able to adjust for this in the statistical analyses, due to lack of power. It would be of interest to attempt to include 10 or more genotypes in future Fabry cohorts, as this would likely give adequate statistical power to evaluate the effect of genotype on the observed effects of Fabry specific therapy.

The patients were arbitrarily prescribed either agalsidase-α or agalsidase-β. Change in agalsidase product or dosing was made when needed, based on clinical or histologic findings in individual patients, or due to the shortage of agalsidase-β during 2009-2012. During the treatment period described in Papers III and IV, dose changes were made in 8 out of 20 patients. Two patients were treated with agalsidase-β 0.2 mg/kg every other week, they started ERT upon enrolment in a comparative trial of agalsidase-α and –β 0.2 mg/kg/eow (140). The reduced dose of agalsidase-β was continued as they were clinically well. The fact that dose and/or product was not constant during the whole follow-up period also contributed to heterogeneity in the cohort, and reflects the individualized approach to the management of Fabry patients at Haukeland University Hospital, combined with a period of agalsidase-β shortage. Papers III and IV are hampered by the small sample size, which is inevitable when dealing with an orphan disease, and few authors have reported serial kidney biopsies in cohorts treated with long-term ERT. One published clinical trial on long-term ERT in Fabry disease included kidney biopsies, where kidney biopsies in 8 out of 58 included patients were reported (144). In an ideal setting, patients would have been included in a randomized controlled trial, and randomly assigned one or the other enzyme product, which would have been given without interruption, or dose change, to a larger cohort. In a setting of a randomized controlled trial, performing kidney biopsies after the same number of months or years of therapy could also have contributed to a more homogenous dataset. Ensuring an adequate sample size is difficult when investigating rare diseases, which must limit the statistical scope of such studies. The varying dosing

of agalsidase, and the fact that some patients have received both products at different time-periods during follow-up, may have influenced the statistical analyses.

The sample sizes in Papers III and IV were considerably smaller than those of Papers I and II. Consequently, a different statistical approach was necessary. As the sample size of the case series in Paper III was only three, no statistical analyses were possible. For Paper IV each variable was evaluated with respect to the normal distribution curve. Student's t-test was used for normally distributed variables, whereas non-parametric tests were used for the non-normally distributed variables. In mixed model regression, the analysis of correlation between reduction of podocyte Gb3 burden and cumulative agalsidase dose received was adjusted for age at start of enzyme replacement therapy. When using adjusted analyses, it is important to calibrate the number of adjustments in the analyses to sample size. Adjustment for more than one variable at a time in a cohort of this size would have resulted in loss of statistical power. In theory, different GLA mutations may have varying responses to agalsidase, and to agalsidase dose, a confounding effect which might have been detected had it been possible to adjust for GLA mutations in the mixed model regression analysis. The number of GLA mutations in the cohort was too small to be adequately powered for such analyses.

5.1.2 Renal histopathology data

The patients included in Papers III and/or IV underwent baseline and follow-up kidney biopsies to evaluate disease burden in the kidney, effects of ERT, signs of progressive Fabry nephropathy, and the presence of non-Fabry pathology. Fabry disease is a rare disorder, and few studies have thus far investigated the long-term effects of ERT on renal tissues. Kidney biopsy findings have been reported by several authors, who have, to some extent, used different methods to evaluated different cell types (137, 141, 143, 180, 192, 215, 220, 221). In Papers III and IV, we elected to evaluate the podocyte and arterial/arteriolar Gb3 burden in the kidney biopsies of the included patients using the ISGFN scoring system (215). The podocyte is a terminally differentiated cell, and accumulation of Gb3 is over time expected to impact on cell

function. Using light microscopy, the ISGFN scoring system evaluates all non-sclerosed glomeruli in sections stained with periodic acid-Schiff and toluidine blue respectively. The ISGFN system ensures a comprehensive evaluation of the available material, which may be of particular importance in females, as different glomeruli may have different scores due to random X-inactivation. The scoring system is semi-quantitative, and depends on an experienced nephropathologist. The stereological morphometric method of Najafian et al. (180) uses electron microscopy to evaluates 1-5 glomeruli in plastic embedded tissue, irrespective of the total number of glomeruli contained within the biopsy specimen. Stereological morphometric evaluation of the glomeruli is an unbiased method, but evaluates a smaller proportion of the available glomeruli, and requires electron microscopy facilities. Electron microscopy is not available in all centers, and where present may not be available in routine clinical practice. Currently, stereological morphometry is only used for research (181). The ISGFN scoring system uses light microscopy, which is available in all departments of pathology, and accessible to the clinician in general practice. The semi-quantitative nature of the method does however leave some room for subjective interpretation of the podocyte Gb3 burden.

The seminal paper of Gubler et al. (220) semi-quantitatively described Gb3 inclusions and vacuolizations in various renal cell types in patients with Fabry disease, using light- and electron microscopy. It laid the foundations for the early clinical trials of ERT (137, 192), which focused on the capillary endothelium. In turn, this later led to the development of a light microscopy based quantitative scoring system, focusing on the endothelium of the peritubular capillaries (221). It has previously been shown that reduction of capillary endothelium Gb3 load occurs after less than 12 months of agalsidase-β 1.0 mg/kg/eow (141, 192), and that after 5 years of ERT the reduction occurs irrespective of agalsidase product or dose (143). Choosing a scoring system that evaluates terminally differentiated cells in the kidney, where dose dependent effects have previously been shown (143) may be a more robust measure of potential differences in the therapy currently available for the treatment of Fabry disease. The podocyte is vital in maintaining the normal filtration barrier at the basement

membrane, and podocyte Gb3 burden and effects of Fabry specific therapy on the podocyte may yield more clinically relevant information when assessing Fabry nephropathy.

5.1.3 Iohexol GFR

A discrepancy between estimated and measured GFR has been reported in Fabry patients (185-187), with eGFR formulas overestimating GFR when compared to measured GFR. This may in part be due to decreased muscle mass in patients who experience acroparesthesia on physical exercise. In Papers III and IV a single point Iohexol GFR was used to evaluate renal function (214). A drawback of measured GFR is the need for a peripheral line and injection of a tracer, followed by perfectly timed blood samples. While eGFR may be adequate when following individual GFR trajectories in every day clinical practice, in the context of aiming for optimal timing of very costly treatment for a disease without firm guidelines on when to initiate treatment, clinical follow-up should be meticulous, and quantification of kidney function as accurate as possible.

5.1.4 Albuminuria and proteinuria

In Papers III and IV urinary excretion of albumin was measured in spot urine samples. Albuminuria and proteinuria occur when there is damage to the glomerular filtration barrier (222), and often precedes changes in serum creatinine and eGFR. Proteinuria has been shown to be an independent risk factor for progression to ESRD (223). In Papers III and IV, samples from three consecutive early morning voids were collected. Urinary albumin excretion was expressed as milligram per mmol of urine creatinine. Values in excess of 2.5 mg/mmol creatinine were considered abnormal (224), and median values were used in analyses. Urinary albumin excretion can vary significantly within a single individual (225, 226). To minimize this bias in statistical analyses, the method of median value of three consecutive early morning voids was adopted. Albuminuria may precede overt proteinuria, and measuring proteinuria alone may miss the very early stages of kidney disease. In addition to heralding kidney disease progress, albuminuria, and change in albuminuria levels, has been shown to

increase risk of all-cause death, and cardiovascular death (96, 227). Urine protein to creatinine ratios were also measured in early morning void spot samples, however few of the included patients had proteinuria above the upper reference range of 20 mg/mmol creatinine. The initial studies of agalsidase-α and agalsidase-β did not report on proteinuria or albuminuria (136, 137, 192), though kidney biopsy results were reported. Baseline proteinuria data were collected from the phase I/II study of agalsidase-β (136). The data were reported by Germain et al., who found that baseline proteinuria >1g/24 h was associated with a higher likelihood of a subsequent renal event in patients treated with long-term ERT (144). Studies on ERT and chaperone therapy have focused on proteinuria, and not albuminuria (140, 142, 157, 228). Fabry nephropathy develops over years. By only measuring protein creatinine ratio, the very early phases of Fabry nephropathy where therapeutic intervention might be more effective could be missed. Measuring albuminuria when evaluating CKD is recommended by KDIGO (86), and should also be included in the evaluation of Fabry nephropathy.

5.2 Discussion of the main results

5.2.1 Risk of ESRD in Norwegians and their first-degree relatives

In Paper I, 12.1% of the included individuals developed ESRD due to a known hereditary nephropathy, of whom 55.3% had a first-degree relative with ESRD. The 36-fold increase in relative risk of ESRD due to a hereditary nephropathy was expected, as the most common hereditary cause of ESRD in the Norwegian population was autosomal dominant polycystic kidney disease, a Mendelian disorder. To quantify the excess risk of ESRD not attributed to known hereditary causes, all individuals and first-degree relatives with known hereditary ESRD were excluded from further analyses. The relative risk of developing ESRD of any cause was still significantly increased, at 3.7 (95% CI 3.1-4.4). A Kaplan Meier plot showed that the increase in relative risk was already evident by the age of 20-30 years. Our findings support previously published reports of familial aggregation of perceived non-hereditary ESRD. The strength of the association may vary between populations. The 3.7-fold

increase in relative risk of ESRD in Norwegians who had at least one first-degree relative with ESRD is comparable to risk estimates from a Caucasian cohort in Canada (11). A case control study in a Caucasian cohort from the US reported that having a first or second-degree relative with ESRD was associated with an odds ratio of 2.7 for developing ESRD. In an American study of dialysis patients, nearly 23% of 25 833 incident dialysis patients reported that they had either a first- or second-degree relative with ESRD not due to a known hereditary or urologic disorder (10). This is a significantly greater proportion than what was found in the Norwegian population, where 1.8 % of those with ESRD due to a non-hereditary cause had a first-degree relative with ESRD. American dialysis patients of African American descent were more likely to report a family history of ESRD, and those who had glomerular disease, hypertensive nephropathy or diabetic nephropathy were the most likely to report a positive family history of ESRD (10). The specific causes of ESRD in the family members were however not reported. In the Norwegian population, having a first-degree relative with ESRD increased the relative risks of glomerular and interstitial disease the most, at 5.2 and 4.7 respectively. The relative risks of hypertensive or diabetic nephropathy were also significantly increased, but to a lesser degree compared to glomerular or interstitial disease. The difference in risk estimates between the different studies may in part depend on differing study designs. O'Dea et al. and Freedman et al. utilized a case control design, whereas nationwide registries were used to ascertain ESRD in the Norwegian population. Differences in coding practices may also have contributed to the differences observed in disease specific risk of ESRD. Risk of ESRD is known to vary between populations (229). Even though the prevalence of CKD is similar in the US and Norway, progression to ESRD is more common in the US population compared to the Norwegian population (91). The incidence of ESRD in the United States was 348 per million in 2010 (230), compared to 102 per million in the Norwegian population (231). The difference is even greater when incidence of ESRD in the Norwegian population is compared to that of African Americans (230). The background risk of ESRD appears to be lower in the Norwegian population compared to that of Americans, the underlying reasons for this difference

are not fully known. Universal health coverage at minimal personal cost is available to all Norwegian citizens, whereas Americans must access primary and/or specialized health care at personal expense. An American study reported that 6.4% of individuals Caucasian descent, aged 45 years or older, had a positive family history for ESRD (232). Additionally, the authors reported that women were more likely to report a family history of ESRD (232). In contrast, only 0.5 % of the included Norwegians, who did not develop ESRD themselves, were found to have a first-degree relative with ESRD (Paper I, table 1). No significant difference in risk estimates could be found between males and females in the Norwegian population.

Reported risk estimates for disease specific causes of ESRD vary between populations (11, 233). The highest risk estimates in Paper I, were observed for ESRD due to glomerular or interstitial disease. O'Dea et al. reported that ESRD due to hypertensive nephropathy was associated with the highest risk estimates in a Canadian population (11), whereas Spray et al. found that US patients with ESRD due to diabetes or glomerular disease were more likely to have positive family history for ESRD (233). ESRD is a rare outcome in the Norwegian population, and low numbers in some disease categories may have affected the outcomes. Environmental factors play a role in risk of common complex diseases such as cardiovascular disease (234), and risk of ESRD. First-degree relatives are more likely to share these risk factors, be it the physical environment or nutritional factors impacting traditional risk factors such as cholesterol levels or glycemic control.

In view of the recent advances in molecular genetics, it is likely that some of the non-hereditary cases were mislabeled, and that ESRD did indeed occur as a result of a hereditary disorder. As this is likely also the case for other published cohorts, it does not fully explain the observed differences in risk of disease specific ESRD.

Paper I quantified the excess risk of ESRD associated with having a first-degree relative in Norwegians. The observed association is important, in that extra attention to attenuation of traditional and modifiable risk factors such as lipid profile, glycemic control and BMI can be given. Prevention is more cost effective than treating manifest

disease, and regular monitoring of urine albumin to creatinine ratio, serum creatinine, blood pressure and blood glucose may be advisable in individuals with a family history of ESRD. The prevalence of chronic kidney disease and ESRD is increasing world-wide, largely due to cardiovascular disease and diabetes mellitus. Chronic kidney disease, and ESRD in particular, is associated with increased risk of morbidity and mortality (97, 235, 236). Many patients with CKD are unaware of their renal function, an American health screening program found that only 7.8% of those screened with CKD stage 3 were aware of having CKD, whereas 41% of those with CKD 4 were aware of their condition (237). The financial impact of CKD and renal replacement therapy on healthcare systems is significant (89, 238), and a significant part of this financial burden is carried by the patients and their families in countries without general health coverage. In placing a heavy and unequally distributed burden on society, with evidence of possible, but not yet implemented preventative measures, CKD can be said to be a public health issue (239). However, the possibility of identifying modifiable risk factors, followed by implementation of preventative measures may decrease the societal and personal burdens of CKD.

5.2.2 ESRD in first-degree relatives as a risk factor for premature death

Increased risk of mortality has been reported in patients with CKD and ESRD (96, 97, 240), in Paper II risk of premature death in first-degree relatives of patients with ESRD was explored.

In the Norwegian population, individuals who had a first-degree relative with ESRD of any cause were found to be at increased risk for premature death, adjusted hazard ratio for all-cause death was 1.13 (95% CI 1.09-1.16). The observed risk decreased somewhat when individuals who developed ESRD and first-degree relatives with a hereditary nephropathy were excluded. The most common cause of death was cardiovascular death, which was associated with an aHR of 1.15 (95% CI 1.10-1.21) in those who had a first-degree relative with ESRD. The largest increases in aHRs were observed for death due to non-hereditary diseases of the kidneys and ureters, 2.29 (95% CI 1.81-2.91) and death due to diabetes mellitus; 1.68 (95% CI 1.35-2.10).

The observed increase in risk of cardiovascular disease was comparable to findings in a cohort consisting of Americans of mainly European decent (241). In our cohort, first-degree relatives of ESRD patients were at increased risk of cardiovascular death, and having a first-degree relative with ESRD due to hypertensive disease added to the observed increased risk of all-cause death. There is strong and consistent evidence for heritability of cardiovascular disease, and a positive family history for premature cardiovascular disease has been reported to be a risk factor for cardiovascular disease, independently of other traditional risk factors (242-244). Sudden death below the age of 65 years in a parent and parental myocardial infarction have been linked to increased risk of primary cardiac arrest in offspring in a case control study (245). Conversely, CKD is known to be a risk factor for cardiovascular disease (97, 240), with cardiac death as the leading cause of death in dialysis patients (230, 246). Norwegians with a first-degree relative with ESRD had a significantly increased aHR for death due to diabetes mellitus. Accumulation of diabetes mellitus in families have been observed (247, 248), as have familial clustering of diabetic kidney disease (34). Additionally, diabetes mellitus has been linked to increased risk of all-cause mortality as well as cardiovascular mortality (249). Taken together this argues for shared genetic risk factors in ESRD patients and their first-degree relatives contributing to the observed excess risk of death. Shared genetic or environmental risk factors may also have contributed to the excess risk of cardiovascular death reported in Norwegian living kidney donors (250).

Paper II showed that individuals with a first-degree relative with ESRD had significantly increased aHR of death due to non-hereditary disease of the kidneys and ureters, with an aHR for death due to primary glomerular diseases of 5.69 (95% CI 3.88-8.34). An increase in mortality rates has previously been described in a Korean cohort of patients with biopsy verified primary glomerular disease. Significantly higher standardized mortality rates were found in glomerulonephritis patients when compared to sex and age matched cohorts (251). This excess in mortality was however not appreciable in patients with normal blood pressure, preserved renal function and low levels of proteinuria (251). Data on blood pressure and proteinuria were not

available for the Norwegian cohort, and we were not able to ascertain whether hypertension and/or proteinuria in the included individuals impacted the hazard ratio for death due to glomerular diseases associated with having a first-degree relative with ESRD. Hazard ratios for death in the Norwegian population were also analyzed according to cause of ESRD in first-degree relatives, and ESRD due to primary glomerular disease in a first-degree relative was not associated with an increase in aHR for all-cause death.

The aHR for death due to renal disease was significantly lower in the Norwegian population compared to an American cohort (12). Differences in methodology may account for the difference in observed hazard ratios. In the Norwegian cohort hazard ratios were calculated for the primary cause of death alone, whereas Goldfarb-Rumyantzev et al. (12) included CKD and ESRD as the primary or contributing cause of death in their risk estimates.

Survival curves indicated that having a first-degree relative with ESRD was associated with increased risk of premature death, and absolute increase in mortality rates per 100 000 person years were calculated according to birth year. The highest increase in absolute mortality rates were seen in the oldest individuals, i.e. those born between 1900 and 1939. Death due to cardiovascular disease and renal disease were the main factors influencing mortality rates in this age-range. Individuals born between 1940 and 1959 had the highest aHR for cardiovascular death, 1.41, with higher risk estimates in women. Post World War II, tobacco use in women increased and peaked during the 1970s (252). Excess risk of myocardial infarction, hospitalization and death due to chronic obstructive pulmonary disease have previously been reported in female smokers compared to male smokers (253). Women may be more vulnerable to the deleterious effects of tobacco use, which may explain some of the increased risk of cardiovascular death in women who had a first-degree relative with ESRD.

The increased risk of premature death associated with having a first-degree relative with ESRD observed in Paper II was found using data from nationwide registries. This method allowed complete ascertainment of ESRD and death, but did not allow

adjustments for traditional risk factors such as lipid profile, glycosylated hemoglobin, body weight and smoking. First-degree relatives may have similar lifestyle risk factors, and may share environmental risk factors. However, some of the excess risk observed may be due to shared genetic risk factors, or shared epigenetic change due to exposure to similar environmental triggers.

Chronic kidney disease and ESRD may be the result of polygenic inheritance, epigenetic modifications as a response to environmental pressures or monogenic disorders. There are likely genetic contributions to the risks of developing diabetic or hypertensive nephropathy (10, 43, 65), and monogenic causes of known disorders may also likely be discovered in the future.

5.2.3 Fabry disease in the Norwegian population

In Papers III and IV a known monogenic cause of CKD and ESRD was explored. Few studies have reported effect of long-term agalsidase therapy on renal histology in Fabry disease. Beneficial dose dependent effects of agalsidase on podocyte Gb3 load after 5 years of ERT were described by Tøndel et al in 2013 (143), and the findings of Papers III and IV support this finding in relatively young cohorts treated up to 14 years.

In Papers III and IV we included classical Fabry patients who had undergone serial kidney biopsies, and who had received long-term ERT. Median treatment time in Paper IV was 9.4 years, the treatment groups were statistically similar at baseline. Reduction of ISGFN podocyte score (215) was found to correlate with cumulative agalsidase dose, with a greater reduction in podocyte Gb3 score in those who had received the higher cumulative agalsidase doses, $r=0.693$, $p=0.001$. However, some reduction in podocyte Gb3 scores was also seen in individuals who had received a lower cumulative agalsidase dose. A statistically significant reduction of podocyte Gb3 load was observed from first to final kidney biopsy in both treatment groups. This is in contrast to the five-year material previously published (143), where no discernable statistical impact of agalsidase 0.2 mg/kg/eow was found on the podocyte. Treatment duration likely had an impact on the ability of the lower dose agalsidase to

remove Gb3 from the podocyte. Five years of treatment may not have been enough for the lower dose to reduce podocyte Gb3 sufficiently to warrant a decrement in ISGFN score. Individual differences in treatment response despite identical GLA mutations and agalsidase dose may also have contributed. The cohort in Paper IV is larger than that published by Tøndel et al. (143), as age was not an exclusion criteria in Paper IV. The increase in sample size may have increased the statistical power, so that the effects of agalsidase 0.2 mg/kg/eow reached statistical significance.

Evaluation of podocytes by unbiased stereological morphometry has previously linked increasing podocyte Gb3 burden to podocyte size, podocyte damage and albuminuria (180, 181). The change in podocyte Gb3 load was not found to correlate with albuminuria in Paper IV, nor did albuminuria rates change significantly during follow-up. Though no correlation to albuminuria was found in Paper IV, a decrease in podocyte Gb3 load may improve general podocyte health, which in turn may lessen excess podocyturia linked to progressive Fabry nephropathy (178).

Many Fabry related complications involve the vasculature, and appear to progress in the face of current state of the art therapy. In our cohort, significantly fewer patients in the higher dose group had arterial/arteriolar Gb3 intima and media inclusions as compared to the lower-fixed dose group. The median 10-year difference in age at start of ERT between the groups, favoring the higher dose group, may have impacted this observation. Levels of plasma lysoGb3 prior to ERT have been shown to be significantly elevated in Fabry patients, however baseline levels may vary between classical and non-classical patients, as well as in males versus females (122). Baseline lysoGb3 was only available in 5 of the 20 patients. None of the twenty patients had lysoGb3 levels within the reference range at the first available measurement. Subsequent measurements of lysoGb3 showed that males who had received a higher cumulative agalsidase dose had lower residual lysoGb3 levels at the final time-point. This finding may indicate dose dependent effects on lysoGb3. Currently the implications of persistently elevated lysoGb3 levels are not fully understood. LysoGb3 has been shown to decrease significantly during the first year of ERT (173, 174), and

considerable decrements in lysoGb3 levels were seen in Paper IV in patients where true baseline levels were available. LysoGb3 is related to disease severity (122), and has been reported to be an independent risk factor for white matter lesions in males and left ventricular hypertrophy in females (123). Elevated plasma lysoGb3 levels have also been linked to age and sex independent vascular damage (254). Its usefulness in determining risk of future complications is however undetermined, as is the question of whether lysoGb3 is directly involved in the development of Fabry disease manifestations. Choi et al. reported an influx of $Ca2^{+}$ into peripheral sensory neurons and mechanical allodynia when lysoGb3 was injected into the paws of healthy mice, whereas normal saline injection was not associated with allodynia (255). This may suggest that lysoGb3 plays a direct role in the pathomechanisms of acroparesthesia. Plasma lysoGb3 has been shown to promote secondary mediators of glomerular injury (256) as well as pro-fibrotic and pro-inflammatory cytokines in cultured human podocytes (177), and may have a detrimental effect on long term kidney function in Fabry patients through these mechanisms. Thus evaluation of lysoGb3 is important, and baseline levels may indicate risk of future complications of Fabry disease. As lysoGb3 levels fall within the first year of ERT (173, 174), regular monitoring in a clinically stable patient may not yield further information. Monitoring lysoGb3 when development of complications or non-compliance with ERT is suspected, as well as when Fabry specific treatment is altered, may be a reasonable approach to the use of this biomarker.

The median mGFR remained stable in both treatment groups during the study period, however a decline in mGFR was observed in individual patients. Mean end of follow-up mGFR was less, and statistically different, than the baseline value in the higher dose group. The higher dose group received more agalsidase per kilogram, and the difference in change in mGFR compared to the lower fixed dose group was likely due to selection bias, as patients not on agalsidase-β 1.0 mg/kg/eow who showed signs of decreasing renal function were switched to agalsidase-β 1.0 mg/kg/eow.

The clinical spectrum of Fabry disease is heterogeneous, and individual risk assessment and tailoring of therapy are cornerstones of good clinical management of Fabry patients (191). Patients who share the same GLA mutation may develop different clinical manifestations of Fabry disease, despite being treated with the same ERT product. Likewise, different individual mGFR trajectories may become evident in patients who are otherwise matched with respect to age, gender and ERT product and dose. A baseline kidney biopsy can establish the disease burden attributable to Fabry disease, as well as other pathology such as e.g. nephrosclerosis. The effects of Fabry specific therapy and the possible development of additional renal pathology (vasculopathy) can be evaluated in follow-up biopsies. Including a kidney biopsy in the clinical work-up of unexpected renal events is beneficial, and may unveil significant renal pathology of non-Fabry origin.

For the first time, we were able to show a statistically significant reduction of podocyte Gb3 burden in the lower fixed dose group, treated with agalsidase-α 0.2 mg/kg/eow, when mean podocyte Gb3 scores from first to final biopsy were compared. Though a greater reduction of podocyte Gb3 load was found in patients who received agalsidase-β 1.0 mg/kg/eow leading up to the final kidney biopsy. Paper IV confirmed and further expanded the understanding of dose dependent clearing of podocyte Gb3, using a light microscopy based scoring system (215).

Paper III showed a dose dependent reduction and re-accumulation of podocyte Gb3 burden in three young Fabry patients who were switched from agalsidase-β 1.0 mg/kg/eow to agalsidase-α 0.2 mg/kg/eow due to the global shortage of agalsidase-β. Effects of dose reduction after initial treatment with agalsidase-β 1.0 mg/kg/eow, was reported in an open label trial (142). The peritubular capillary endothelium was cleared of Gb3 in all patients treated with agalsidase-β 1.0 mg/kg/eow, which was maintained in 90% of the patients after switch to agalsidase-β 0.3 mg/kg/eow, indicating re-accumulation in 10% of the patients. Re-accumulation of podocyte Gb3 was also indicated in 2 out of 19 patients after dose reduction in this study. Superiority of agalsidase-α 0.2 mg/kg/eow versus agalsidase-β 0.2 mg/kg/eow was investigated in a

randomized controlled open label trial (140). No statistical differences in eGFR, proteinuria or adverse events between the two groups after 12 and 24 months of treatment were observed, kidney biopsy data were not reported (140). Two of the patients included in the lower fixed-dose group in Paper IV were included in the head to head study of agalsidase-α and -β (140), and received agalsidase-β 0.2 mg/kg/eow. Their clinical and histologic trajectories were on par with the patients treated with agalsidase-α 0.2 mg/kg/eow. Taken together, the findings of Papers III and IV add to previously published data suggesting that agalsidase-α and -β have similar properties milligram per milligram (138, 139, 143).

5.2.4 Applicability of results to external populations

The Norwegian population is mainly Caucasian, with a low prevalence of ESRD compared to other populations, such as Americans of all ethnicities (230). Some of the observed increase in relative risk of non-hereditary ESRD and premature death in first-degree relatives of ESRD patients may be due epigenetic factors, which are influenced by the environment. As the environment differs from population to population, and indeed sometimes within a population, this may reduce the generalizability of the observed relative risk. However, as prevalence of ESRD is low in the Norwegian population (231), and progression of CKD to ESRD has been found to be less in Norwegians as compared to Americans (91), the observed risk in the Norwegian population may in fact underestimate risk in other populations.

In comparing results from the Fabry cohort at Haukeland University Hospital to other Fabry cohorts, there may be dissimilarities which can affect the generalizability of our results. The mean age of the patients included in Papers III and IV are lower than many other long-term ERT publications. In addition, high level albuminuria or proteinuria is not a prominent feature of the Haukeland cohort, and when albuminuria is present, is at a significantly lower level than other published cohorts (150, 228, 257). As proteinuria is a risk factor for further loss of GFR (258), the lack of high grade proteinuria in our cohort may have contributed to a less steep decline in GFR. Few studies have published data on kidney biopsies, particularly kidney biopsy findings

after long-term ERT. Different scoring systems have been used in publications which include data on renal histopathology findings, focusing on e.g. capillary endothelium (137, 157, 192), other semi-quantitative scoring by light microscopy (141-144), or stereological electron microscopy assessment of podocytes (178). The efficiency with which Fabry specific treatment reduces Gb3 load varies from cell type to cell type in the kidney. The capillary endothelium is cleared after a relatively short period of ERT, whereas clearing of podocyte Gb3 is more difficult, and requires longer exposure to ERT (142, 143, 192). A stereological method of scoring podocyte Gb3 inclusions may be more sensitive to change in the limited number of glomeruli scored (180), however the ISGFN (215) scoring system includes all non-sclerosed glomeruli, thus taking a larger portion of the biopsy into account. This method is likely not sensitive to smaller changes in podocyte Gb3 load when the load is extensive, as in some cases a decrease in podocyte Gb3 load may be apparent on light microscopy, while still fulfilling the criteria for a maximum score. However, the ISGFN scoring system appears to be sufficiently sensitive to capture clinically meaningful change in podocyte Gb3 burden.

6 Conclusions and future perspectives

In the Norwegian population individuals who have a first-degree relative with ESRD not due to known hereditary disease have a three-fold increase in relative risk of developing ESRD themselves. The highest increases in risk estimates were observed for relative risk of ESRD due to glomerular diseases or interstitial diseases. Hypertension and diabetes mellitus are the two leading causes of ESRD world-wide, both of which are common complex diseases with genetic contributions. Relative risks of ESRD due to diabetes mellitus or hypertensive disease were increased if a first-degree relative with non-hereditary disease was identified, however the risk estimates were lower than those of glomerular and interstitial ESRD. The increase in observed relative risk in individuals who have a first-degree relative with non-hereditary ESRD may indicate shared genetic risk factors, predisposing to CKD and ESRD.

A small, but statistically significant increase in risk of premature death was found in first-degree relatives of ESRD patients. Cardiovascular disease was the most

common cause of death in the cohort, of which death due to cerebrovascular disease was associated with the highest risk estimates. First-degree relatives of ESRD patients were also more likely to die from renal disease and diabetes mellitus when compared to those without a first-degree relative with ESRD. An increase in absolute mortality rates was also observed in the older birth cohorts, the risk of premature death due to cardiovascular disease appeared to be stronger in women born 1940-1959 compared to men born during the same time-period. The higher relative risk of cardiovascular death in women born during and after the Second World War may in part be explained by the gender conversion of tobacco use in Norway during the 1970s, and the apparent stronger susceptibility to the deleterious effects of tobacco use in women. Shared genetic risk factors which may impact on risk of both ESRD and death may underlie the observed increased risk of premature death in first-degree relatives of ESRD patients. Shared environmental risk factors may also have impacted on the observed risk estimates. Extra vigilance with respect to modifiable risk factors and monitoring of renal function may be advisable for first-degree relatives of ESRD patients.

Dose dependent effects of agalsidase treatment were found in classical Fabry patients, followed by serial kidney biopsies over a median period of 9.4 years. The greatest reduction in podocyte Gb3 inclusions was seen in the group which received agalsidase-β 1.0 mg/kg/eow leading up to the final biopsy, however a smaller but statistically significant reduction in podocyte Gb3 load was also found in patients treated with agalsidase-α 0.2 mg/kg/eow for the duration of the study period. Reduction of podocyte Gb3 load, with subsequent re-accumulation was observed in the three individuals who were switched from agalsidase-β 1.0 mg/kg/eow to agalsidase-α 0.2 mg/kg/eow due to the global shortage of agalsidase-β. Renewed clearing of podocyte Gb3 was observed in the single patient who underwent a kidney biopsy after agalsidase-β was reinstated. Meticulous clinical monitoring and individualized therapy are important in Fabry disease.

The constant advances of molecular genetics open new diagnostic avenues for chronic kidney disease. Familial aggregation of kidney disease without known

Mendelian causes may still be due to predisposing genetic factors. It would be of interest to combine epidemiological studies with genome wide association studies, with the hypothesis that shared risk alleles contributing to either excess risk of loss of GFR, excess risk of diabetic or hypertensive nephropathy or yet undescribed genetic risk factors may underlie the observed excess risk of ESRD and death in first-degree relatives of ESRD patients.

GWAS studies may also be of interest in a Fabry population, as other genetic risk factors may play a part in the heterogeneous clinical spectrum of Fabry disease. Identifying such additional risk factors may help to stratify patients with respect to risk of progressive disease, and aid the decision-making process of when to start Fabry specific treatment. With the advent of chaperone therapy (157) and other Fabry specific treatments such as new forms of ERT (259), combination therapy with chaperone and ERT or substrate reduction therapy in combination with ERT (260), it would be of interest to follow cohorts with serial kidney biopsies, and compare the findings to those treated with recombinant agalsidase based therapy. Certainly, new Fabry specific treatments that come to the market should provide robust data concerning efficacy on relevant clinical parameters as well as nephropathology parameters.

The pathophysiology behind Fabry vasculopathy has yet to be fully elucidated. Vasculopathy is a major concern in Fabry disease, and Fabry patients are more likely to die from cardiac disease than renal disease. Further research into the underlying mechanisms of Fabry vasculopathy and identification of reliable biomarkers is needed.

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