Early Manifestations of Anderson Fabry Disease

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I, Praveen Jeevaratnam confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

This thesis examines some early renal and neurological manifestations in Anderson Fabry disease (AFD). First, estimating glomerular filtration rate in AFD using serum creatinine (Cr) based equations was assessed in 106 AFD patients. The Modification in diet in renal disease (MDRD) and the Chronic kidney disease epidemiology collaboration (CKD-EPI) equations had the least bias and were the best methods of estimating glomerular filtration rates in AFD patients with chronic kidney disease (CKD) stage 1 to 3.

The monitoring of renal involvement in AFD use methods which assess glomerular function predominantly though there is evidence of renal tubular damage and atrophy on renal biopsy. We investigated possible urine markers of renal tubular dysfunction in AFD and 2 other proteins detectable in urine which have been shown to be markers of renal scarring and inflammation. Urine β-hexosaminidase (β-hex) and Monocyte chemoattractant protein-1 (MCP-1) were elevated in AFD patients compared with control demonstrating evidence of renal tubular involvement and possible renal inflammation.

Finally we investigated cardiac autonomic function, cardiac neuroendocrine function, sweat function and symptoms related to neuropathic and autonomic function in an AFD cohort. There was little evidence of sweat dysfunction, cardiac autonomic or cardiac neuroendocrine dysfunction, though there is significant evidence of neuropathic pain and autonomic symptoms.

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I dedicate this to my father and mother.

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List of Abbreviations

AFD   Anderson Fabry Disease

ANS   autonomic nervous system

α-Gal   α-Galactosidase A

β-Hex   β-Hexosaminidase

BMI   body mass index

BP   blood pressure

BSA   body surface area

CG   Cockcroft-Gault

CKD   chronic kidney disease

CKD-EPI   Chronic Kidney Disease Epidemiology Collaboration

COMPASS   composite autonomic symptom scale

Cr   creatinine

ECG   electrocardiogram

eGFR   estimated glomerular filtration rate

ER   endoplasmic reticulum

ERT   enzyme replacement therapy

FOS   Fabry outcome survey

GB3   globotriaosylceramide

GFR   glomerular filtration rate

iGFR   single point ^{52}Cr-EDTA radionuclide study

HR   heart rate

LANSS   Leeds assessment of neuropathic symptoms and signs

LVMI   left ventricular mass index

M   mean

MAP   mean arterial pressure

MCP-1   monocyte chemotactic protein 1

MDRD   Modification in Diet in Renal Disease

MSSI   Mainz severity score index

NGAL   neutrophil gelatinase associated lipocalin

QSART   quantitative sudomotor axon-reflex

RBP   retinol binding protein

SD	standard deviation of mean
TGF-β1	transforming growth factor-β1
UACR	urine albumin:creatinine ratio
UPCR	urine protein:creatinine ratio
24h U Cr Cl	24 hour urine creatinine clearance

Chapter 1. General Introduction

1.1   Introduction

Anderson – Fabry Disease (AFD) is an X-linked inherited lysosomal storage disease (Xq22.1) caused by a mutation in the gene encoding α-galactosidase A (α-Gal). AFD was first described independently in 1898 by Johannes Fabry in Germany and William Anderson in the UK, and characterised clinically by the presence of angiokeratomas, proteinuria, and lymphoedema[1, 2]. The gene encoding α-Gal has been isolated and sequenced[3, 4] and to date there are more than 500 mutations reported in the GLA gene in the Human Gene Mutation Database (Cardiff, UK: http://www.hgmd.cf.ac.uk/ac/gene.php?gene=GLA). AFD has been termed an orphan disease due to its low prevalence in the general population. Orphan diseases are diseases that are rare enough that there are no commercial incentives for research and the development of effective therapies to be carried out, without separate government legislation. In the United States, the Orphan Drug Act (1983) defines an orphan disease as a disease or condition considered to affect fewer than 200,000 individuals in the United States or has a prevalence of < 7.5 per 10,000 Americans[5]. In Europe, an orphan disease has been defined by the European Committee for Orphan Medicinal Products as a life-threatening or very serious disease affecting not more than 5 per 10,000 Europeans[6]. With these legislations enzyme replacement therapy (ERT) has been developed and approved to manage AFD individuals. Both agalsidase alfa (Replagal®, Shire Human Genetic Therapies Inc.), and agalsidase beta (Fabrazyme®, Genzyme Corp.), have been approved by the European Committee for Orphan Medicinal Products but due to orphan drug laws in the United States, only Fabrazyme has FDA approval. ERT has been available for more than a decade and has improved morbidity in AFD. ERT has been shown to stabilise and reduce the rate of decline of renal function[7-15], reduce left ventricular hypertrophy[8, 16-18], improve pain and peripheral neuropathy[8, 19-23], improve quality of life[8, 21, 24] and reduce abdominal pain[25, 26]. The improvement in cardiac and renal manifestations may ultimately decrease mortality in this orphan disease. Therefore the use of ERT and the potential efficacy of the treatment has increased awareness of the importance of diagnosing unrecognised AFD patients to ensure their optimal management.

1.2   Pathogenesis

The primary disease process is the pathological accumulation of neutral glycosphingolipids in lysosomes, predominantly globotriaosylceramide (GB3), due to a deficiency of the lysosomal enzyme hydrolase α-Gal, which catalyses the hydrolytic cleavage of the terminal galactose from GB3. Glycosphingolipids are the most abundant and diverse class of glycolipids in humans. They contain carbohydrate residues attached by glycosidic linkage to the C-1 hydroxyl group of a ceramide lipid moiety. The ceramide lipid moiety consists of a long-chain amino alcohol (sphingosine) in amide linkage to a fatty acid. The four principal classes of glycosphingolipids are the cerebrosides, sulfatides, globosides and gangliosides. A globoside is a type of glycosphingolipid with more than one sugar as the side chain, while a cerebroside has only one sugar as the side chain. Gangliosides are glycosphingolipids with one or more sialic acids linked on the sugar chain and sulfatides contain a sulphuric ester group. Fig 1.1 demonstrates the chemical structure of GB3 and Fig 1.2 illustrates the metabolic pathway of glycosphingolipid degradation and the defect in this metabolic pathway first described by Brady et al in 1967[27] resulting in AFD. The accumulation of GB3 occurs in multiple organs throughout the body most clinically significant in the cardiovascular, renal and neurological systems.
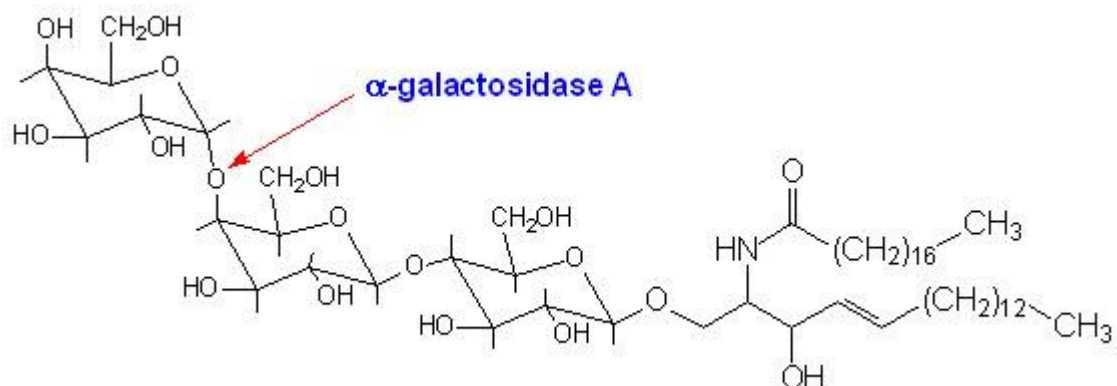
Fig 1.1. Chemical structure of globotriaosylceramide (GB3) and site of action of α-galactosidase A (α-Gal).
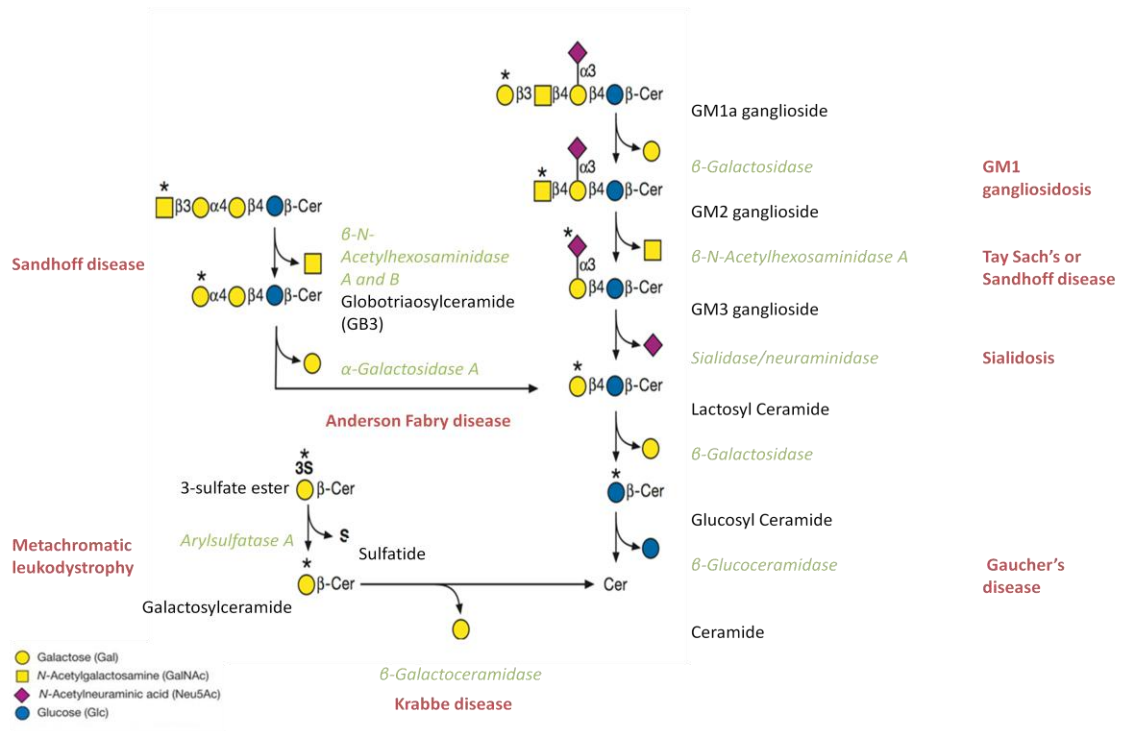
Fig 1.2. A schematic drawing of the possible enzyme defects in the catabolic pathways of sphingolipids. Names of glycosphingolipids (black), enzymes (green italic) and diseases caused by enzyme deficiency (red). Adapted from Chapter 41 Genetic Disorders of Glycan Degradation, Essentials of Glycobiology[28].

α-GAL is encoded by the GLA gene on the long arm of the X-chromosome and is synthesized on the endoplasmic reticulum (ER) bound ribosomes as a precursor form (429 amino acid residues). The enzyme is translocated into the lumen of the ER by the addition of N-linked oligosaccharides. The oligosaccharides are then trimmed in the ER, and the enzyme is transferred to the Golgi apparatus, where further modification of sugar chains and the addition of mannose 6-phosphate residues occur, and then transported to endosomes and subsequently lysosomes where it exerts its function as a mature form consisting of 398 residues.

In AFD, mutations in the GLA gene results in the loss of enzyme activity. The level of residual enzyme activity is determined by the type of mutation and the part of the gene affected. Usually non-functional, or a complete lack of the genetic product is caused by mutations affecting the active site of the enzyme[29]. Missense mutations on the other hand may result in a considerable loss of metabolic activity but still retain some residual activity enough to ameliorate clinical manifestations. These mutations are usually distant from the active site and result in small structural changes of the mutant enzyme. The mutant enzyme is post-translationally inactivated and rapidly degraded, but has

some appropriate residual activity which leads to milder clinical manifestations[30]. Mutations distant from the active site that adversely affects the folded state of the molecule, reduces the enzyme stability and this decreases the amount of active enzyme transported to the lysosome[31].

1.3   Clinical Manifestations

The initial description of AFD was of angiokeratomas, lymphoedema and proteinuria. With increasing awareness and diagnosis of this disease, it has been well documented that AFD patients also have cardiac, renal, cerebrovascular, neurological, gastrointestinal, ophthalmologic and auditory involvement. Ramaswami et al highlighted the main signs and symptoms in children, as neurological, gastrointestinal, ophthalmologic, auditory and skin involvement[32]. As AFD patients increase in age renal, cardiac and cerebrovascular involvement become more clinically relevant. Table 1.1 summarises the predominant signs and symptoms with age. Quality of life is impaired in AFD patients[33] and life expectancy reduced by approximately 20 years in males and 15 years in females[34-37].

It is well documented that AFD females (heterozygotes) can be as affected as males (homozygotes) despite being an X-linked inherited disorder. AFD females develop left ventricular hypertrophy, renal dysfunction, stroke, acroparaethesia, auditory involvement, skin involvement and gastrointestinal symptoms but usually at a older age[35, 38-44]. Dobyns et al[45] classified AFD females having a high penetrance (70%) but low severity (4%). The heterogeneity of disease manifestation in females depends on the degree to which the normal X-chromosome is inactivated[46-48]. This process whereby one copy of the X-chromosome is randomly inactivated in all cells of the female embryo is called lyonisation and partly explains heterogeneity in females. Another reason for phenotypic heterogeneity in females is the cross-correction mechanism or metabolic cooperation[49]. Lysosomal enzymes freely enter and leave lysosomes and are transferred from one cell to another via mannose-6-phosphate mediated endocytosis. This "sharing" of enzyme helps to correct for lack of enzyme in deficient cells. In AFD females, if the amount of active enzyme secreted is insufficient or not adequately taken up by cells, there might be reduced cross correction. The decreasing efficiency of this process through the years, could explain why females get more symptomatic with age[50].

Childhood and adolescence (≤16- years)

- Acroparaethesia/ Pain Crisis – chronic or episodic, burning sensation in the palms of hands or soles of feet, exacerbated by temperature changes, fever, stress, physical exercise and alcohol
- Angiokeratomas (Fig 1.2) – small, raised, dark red spots, develop slowly, found on the buttocks, genitalia, inner thighs, back and oral cavity
- Ophthalmologic abnormalities - cornea verticillata (whorl-shaped opacity), posterior subcapsular cataracts, torturous vascular lesions in the retina and conjunctiva
- Sensorineural hearing loss
- Hypohidrosis or Hyperhidrosis
- History of non specific bowel disturbances
- History of lethargy and tiredness

Early adulthood (17 – 30 years)

- More extensive angiokeratomas
- Proteinuria, lipiduria, haematuria
- Oedema
- Fever
- Hypohidrosis or anhidrosis
- Heat sensitivity
- Diarrhoea, abdominal pain

Later adulthood (age > 30 years)

- Prominence of acroparaethesia less prominent
- Heart disease – left and right ventricular hypertrophy, heart valve abnormalities and conduction disturbances
- Impaired renal function – including end stage renal failure needing renal replacement therapy with dialysis or renal transplantation
- Stroke or transient ischaemic attacks

Table 1.1. Summary of major signs and symptoms in AFD based on different age groups. There is an accumulation of symptoms from childhood to adulthood, with progressive end organ damage but acroparaethesia becomes a less prominent feature.

Fig 1.3. Angiokeratomas in the groin and male genitalia.

The prediction of organ involvement and progression in AFD is complicated by firstly the large number of different mutations described[51] in a small population, secondly the large phenotypic heterogeneity associated with the same mutation, both among patients in related and unrelated families[52]; and thirdly the difficulty that clinical features of AFD are frequently features of more prevalent diseases in the general population. Residual enzyme activity in affected individuals result in a delayed onset or less severe presentation of disease[53-55] and in females can range from normal to absent activity. Patients with the "cardiac variant" present usually in the 5th to 8th decades of life with left ventricular hypertrophy, arrhythmias, and/or cardiomyopathy[56-58] and with the "renal variant" present typically after the age of 50 years with proteinuria and later onset end stage renal failure[59, 60]. More recently Hughes et al developed a prognostic score, the Fabry International Prognostic Index, to demonstrate that it is possible to differentiate groups of patients with different outcome probabilities[61]. In terms of renal decline, Warnock et al showed that AFD patients with more significant proteinuria had a greater rate in decline in glomerular filtration rate[62].

Diagnostic methods are based on measuring plasma and leukocyte α-Gal activity and DNA mutation analysis. Once an index case is found, pedigree analysis and these methods are used to diagnose affected relatives. Classically affected hemizygotes have very low or undetectable enzymatic activity, but some heterozygotes, e.g. the N215S mutation may have residual enzyme activity in plasma and/ or leucocytes close to the normal range. In affected females enzymatic activity may range from low levels comparable to male, up to levels in the normal range. This means that heterozygote females, who may or may not be symptomatic, could have normal enzyme activity and be misdiagnosed if DNA mutation analysis is not performed. Therefore in heterozygotes enzymatic analysis alone may lead to misdiagnosis and DNA mutation analysis is essential. Fig 1.3 outlines a useful diagnostic algorithm.
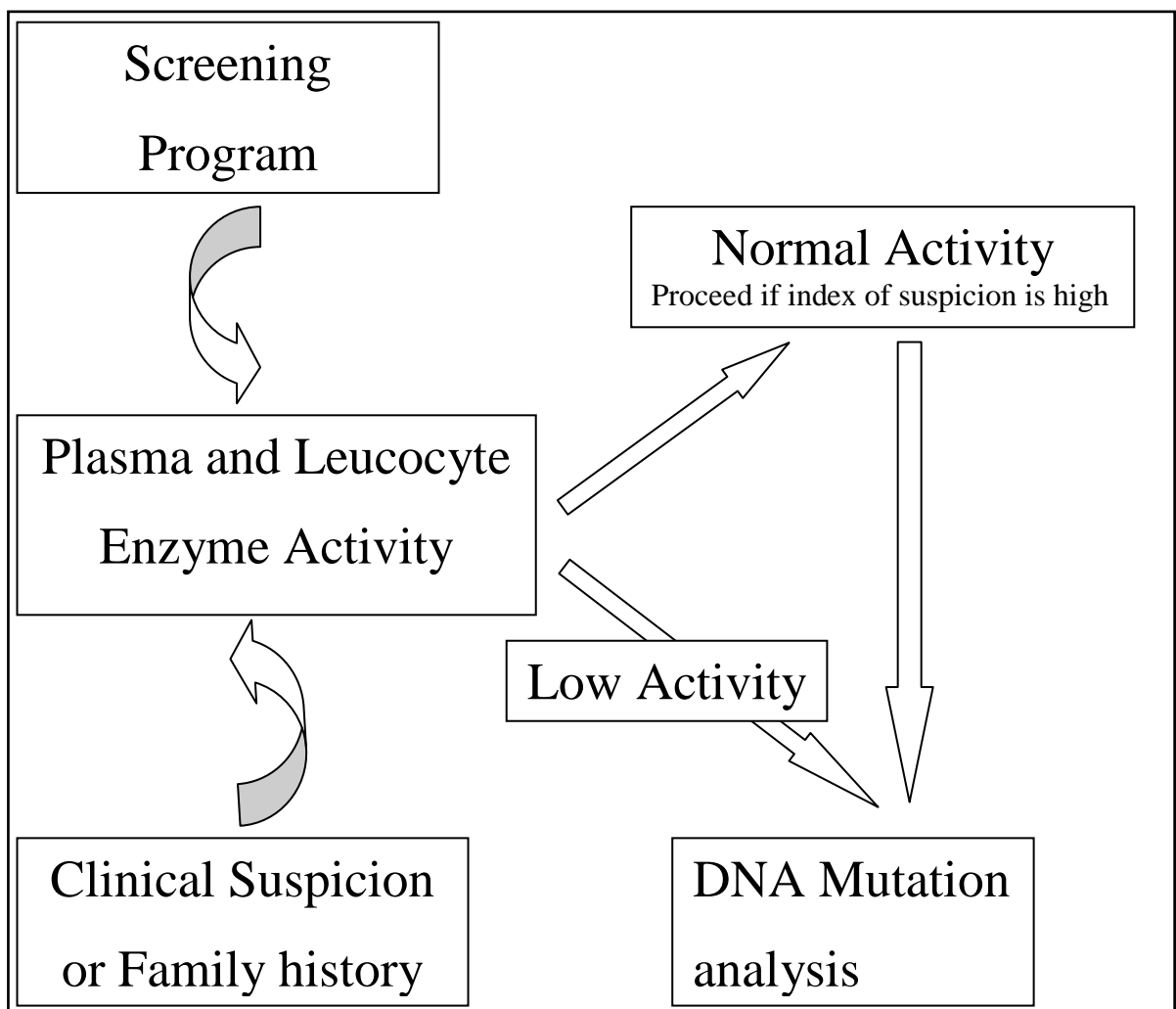
Fig 1.4. Diagnostic algorithm in AFD.

1.5   Prevalence of AFD

The prevalence of AFD has been reported at 1 in 117 000[63] (Australia), 1 in 468 000[64] (Netherlands) and 1 in 833 000[65] (Portugal), but the true prevalence maybe higher as highlighted by Italian and Taiwanese newborn screening studies. In the Italian study the incidence of α-Gal deficiency was 1 in 3100 (newborn Italian males) and if only known disease causing mutations were included the incidence was 1 in 4600[66]. In the Taiwanese screening study the incidence of α-Gal mutations were approximately 1 in 1250 newborn males and 1 in 40000 newborn females. The screening of high risk populations have shown an increased prevalence; 1 in 86[59] (chronic haemodialysis Japanese males), 1 in 621[60] (Austrian dialysis patients), 1 in 26[67] (German young adults with cryptogenic stroke) and 1 in 26[58] (males with hypertrophic cardiomyopathy). A recent systematic review by Linthorst et al of all screening studies for AFD in high risk groups showed a prevalence of 0.33% male dialysis patients, 0.1% female dialysis patients and at least 1% for patients with left ventricular hypertrophy[68]. Prevalence screening studies may inadvertently have falsely higher prevalence due to polymorphisms in particular D313Y[69]. In these polymorphisms, in vitro testing due to an artificial substrate shows low enzyme activity but further studies demonstrated normal in vivo activity.

Due to increased prevalence of AFD in high risk populations and the beneficial effects of ERT, strategies for recognising and diagnosing AFD are important.

1.6   Management of AFD

1.6.1 Multidisciplinary Approach

As AFD can present in so many varied ways and multiple organ involvement is usual, it is desirable that patients are treated by a multidisciplinary team with interested medical professionals from a number of different specialities. In the United Kingdom, the National Specialist Commissioning team has designated six centres to provide diagnosis, assessment and treatment of lysosomal storage diseases. Other key areas of provision of care would include genetic counselling and supportive care.

1.6.2 Enzyme replacement therapy (ERT) in AFD

Two preparations of α-galactosidase A have been tested and been approved for use; agalsidase alfa (Replagal®, Shire Human Genetic Therapies Inc.)[19] in Europe, and agalsidase beta (Fabrazyme®, Genzyme Corp)[24] in the USA and Europe (Table 1.2). Both these glycoproteins are identical but are produced in different cell lines, resulting in different glycosylation at the N-linked carbohydrate attachment sites. Replagal contains a greater amount of complex carbohydrate while Fabrazyme contains a higher fraction of sialylated and phosphorylated carbohydrate[70].

The clinical goals of ERT are to reduce symptoms and complications of GB3 deposition in the lysosomes of major organs, and where disease is already evident, stabilisation and possibly reversal of disease process. Clearance of microvascular endothelial deposits of GB3 from the kidneys, heart, and skin have been claimed[16, 19, 24, 71] and various studies and clinical trials have shown clinical benefit including improved neuropathic pain and peripheral neuropathy[8, 19-23], retarded or reduced progression of renal disease[7-15, 19], reduction in left ventricular mass or hypertrophy[8, 16-18], improved quality of life[8, 21, 24] and reduced abdominal pain[25, 26].

For agalsidase alfa, an analysis of 201 AFD patients (with CKD stage 2 or 3) in the Fabry outcome survey (FOS), who had been on treatment for up to 4.7 years, showed that the decline in estimated glomerular filtration rate (GFR) is halted when compared to the year before start of ERT[12]. Similarly ERT stabilised renal function in AFD patients with CKD stage 2 or 3[8]. Another single centre prospective open-label treatment trial of 25 adult male AFD patients initially in a 6 month randomized placebo-controlled study and subsequent open-label extension study, showed patients with CKD stage 1 and 2 had stable estimated GFR and in those with CKD stage 3, the slope of decline in GFR was reduced compared with historical controls[11]. For agalsidase beta, a double-blind, randomised, placebo-controlled phase III study, involving 58 AFD patients, median serum Cr and eGFR remained stable at up to 54 months[10].

ERT has been shown to be safe although infusion related reactions with fevers, chills, rigors, headaches, rhinitis and nausea can be easily treated with premedication (paracetamol, antihistamines and steroids) or by slowing the infusion rate. AFD patients develop antibodies toward both preparations of ERT, and the IgG antibodies produced when treated with either product are cross-reactive[72]. IgG antibodies were shown to reduce enzymatic activity in vitro and AFD patients who were IgG negative had

significantly decreased urinary GB3 compared with IgG positive AFD patients at 6 months of treatment, but with stable renal function[72] and the presence of IgG antibodies to ERT did not seem to affect clinical course or result in withdrawal of treatment[11]. Another multicenter 20 week phase 3 double blind, randomised and placebo controlled study and subsequent open-label extension study of 58 AFD patients, demonstrated approximately 90% of treated patients developed IgG antibodies with median time to seroconversion of 6 weeks and did not affect long-term efficacy of ERT[15].

Currently in the UK, agalsidase alfa, Replagal is licensed at a dose of 0.2mg/kg every 2 weeks and agalsidase beta, Fabrazyme is licensed at a dose of 1 mg/kg every 2 weeks (Table 1.2 summarises the administration, dosage and origin of agalsidase alfa and beta). There is only one study known comparing agalsidase alfa and agalsidase beta showing no difference in clinical end points with either preparation, but this study only had 34 patients enrolled with follow up of 24 months and agalsidase beta used at a lower dose than licensed for (0.2mg/kg compared to licensed dose of 1mg/kg)[73]. In the Canadian Fabry Disease Initiative study on of the aims is to determine the difference between efficacy of the two different enzyme preparations, unfortunately due to worldwide shortages in Fabrazyme this objective is not been able to be demonstrated currently.

	Agalsidase alfa, Replagal	Agalsidase beta, Fabrazyme
Dose	0.2mg/kg every 2 weeks	1.0mg/kg every 2 weeks
Origin	Human fibroblast cell line	Chinese hamster ovary cell line
Intravenous Administration	In 100mls Saline over 40mins	In 500mls Saline over 4 hours reducing to 90mins if tolerated

Table 1.2. Summary of available ERT

In the UK administration of ERT is conducted in a hospital environment and if no infusion reactions occur, it is usual for ERT to be administered in a local environment (home, work place, GP surgery, day hospital etc.). Administration by a home care team or patients themselves have been shown to be safe[11, 74]. The duties of the home care

team include intravenous cannulation, phlebotomy, drug delivery and preparation, as well as educating patients on drug administration and trouble-shooting home therapy problems.

The question of the effect of ERT in patients with end stage renal failure is not fully understood. It does not reverse end stage renal failure, but ERT in dialysis patients improves quality of life, appears to decrease progression of AFD cardiomyopathy and has been shown to be safe[75]. Others have reported favourable cutaneous, gastrointestinal, neurological and psychiatric response in dialysis dependent AFD patients[76]. A study of 10 AFD patients on haemodialysis showed there was no reduction in enzymatic activity with the administration of ERT during haemodialysis[77].

Current evidence shows there is probably is some benefit in ERT improving morbidity in AFD patients but long term data is needed to see if ERT would improve mortality.

1.7   Conclusion

AFD is a rare disease with multisystem involvement. Current therapies reduce morbidity but treatment is expensive, and timing of the initiation of this treatment is difficult to be precise with. In this thesis I hope to examine methods of identifying organ involvement at an earlier stage which maybe subclinical, to ensure that the future impairment of these organs could be prevented with appropriate early intervention.

Chapter 2. Assessment of glomerular filtration rate in AFD

2.1 Introduction

Proteinuria was one of the characteristics noted in AFD when first described in 1898 independently by two dermatologists, Johannes Fabry in Germany, and William Anderson in London, UK. This showed evidence of renal involvement in AFD from the onset of its description. Significant proteinuria is defined as ≥ 300mg/L of total protein in a 24 hour urine collection. Spot urine samples are now more reliable than 24 hour urine collections for estimating proteinuria. Two separate early morning urine samples, spaced by 1 to 2 weeks are needed to confirm significant proteinuria. Urine protein:creatinine ratio (UPCR) of ≥ 45 mg/mmol and urine albumin:creatinine ratio (UACR) of ≥ 2.5mg/mmol in males and ≥ 3.5 mg/mmol in females is significant[78]. Microalbuminuria refers to albumin excretion above the normal range but below the level of detection by tests for total urine protein. Increased excretion of albumin is a more sensitive marker than proteinuria for CKD in diabetes, glomerular diseases and hypertension.

2.1.1 Renal involvement in AFD

Renal involvement is usually apparent by the age of 30 years in affected males[34, 79-81], but in females there is more heterogeneity. Females may have no apparent renal disease to severe renal dysfunction requiring renal replacement therapy (dialysis or renal transplantation)[35, 82-85].

Branton et al in 2002[55] showed in a review of 105 male hemizygotes that all those who survived other complications of AFD developed end stage renal disease [defined as need for dialysis or renal transplantation or an estimated glomerular filtration rate (eGFR) less than or equal to 12 ml/min] by 55 years of age. Seventy-eight of the 105 (74%) male hemizygotes had proteinuria and/or chronic renal insufficiency [defined as sustained serum Cr more than or equal to 1.5mg/dL or 132.6µmol/L] by a median age 42 years. Fifty percent of patients developed proteinuria by age 35 years and a hundred percent by age 52 years.

Two large registries have reported the prevalence of renal involvement in AFD. Mehta et al in 2009[86] described data from the FOS in which 59% of 699 males (mean age 32.6 years) and 38% of 754 females (mean age 38.5 years) had renal symptoms and/or signs; also, 13% of males and 1% of females had reached end stage renal disease requiring renal replacement therapy. Wilcox et al in 2008[42]

from the Fabry Registry showed that 64% of males and 39% of females had significant proteinuria (≥300mg/24 hours), and 14% of males and 2% of females had reached end stage renal disease (mean ages 38.2 and 39.2 years respectively). In children and adolescents the predominant renal symptoms are proteinuria[32, 87] and occasional microscopic haematuria[32], but the prevalence of renal symptoms is generally low and other clinical manifestations of AFD are more apparent in this age group.

Current guidelines[88] for the initiation ERT for renal complications of AFD are:

i)   GFR < 80ml/min/1.73m^2;

ii)   Proteinuria > 300mg/24 hour;

iii)   Microalbuminuria with evidence of GB3 deposition on renal biopsy.

These are guidelines are based on expert consensus. Different measures of GFR will alter the patients who are initiated on ERT based on the above guidelines, as measures of GFR could be over or under estimated depending on test used.

2.1.2   Advantages and disadvantages of GFR measurements using serum creatinine

2.1.2.1   Serum creatinine

In muscle metabolism, Cr is synthesized endogenously from creatine and creatine phosphate at a fairly constant rate. Cr production and excretion varies among individuals especially children[89], the elderly[90], by ethnicity and sex, obesity[91], during pregnancy[92,93], and in severely ill patients with low muscle mass and poor nutritional status. Under conditions of normal renal function, Cr is excreted by glomerular filtration, and a small amount is actively secreted by the renal tubules. Cr clearance determinants are performed for the diagnosis and monitoring of renal function. In renal dysfunction, the serum Cr rises. Therefore, Cr levels have been used to estimate the GFR by a variety of methods. With a declining GFR, active tubular secretion of Cr plays a more significant role in Cr excretion from the body[94]. Serum Cr can be in the normal range even with GFR less than 60ml/min/1.73m^2, as there needs to be a marked decrease in functioning nephron mass, before the serum Cr is elevated. In severe renal impairment, extra-renal elimination of Cr may occur in the small bowel, where bacterial overgrowth causes degradation of up to two thirds of total daily Cr excretion[95]. Assays of Cr are technically difficult, hampered by interferences up

to 20% by oxidoreductive compounds and can be reduced by the enzymatic assay method[96]. Differences in specificity in different assays can make it difficult to compare values from different laboratories. Interference may also arise from certain medications (Flucytosine and some cephalosporins) or from ketones e.g. in diabetic ketoacidosis (increases serum Cr).

2.1.2.2   24 hour urine creatinine clearance (24h U Cr Cl)

Timed urine collections have been used to circumvent the problem of varied Cr production but not tubular secretion of Cr which can be doubled with declining GFR. Drugs such as cimetidine and trimethoprim can decrease tubular secretion of Cr so drugs administered during timed urine collections need to be identified. Also the result depends on accurately timed and complete urine collections, which can be difficult for some patients.

2.1.2.3   Equations estimating GFR based on serum creatinine

Cockcroft – Gault (CG)[97] equation

The CG equation was first derived in 1976 and based on 249 patients aged between 18 – 92 years, so as to predict Cr clearance using serum Cr and factoring in age, sex and weight. This formula was not normalised to body surface area (BSA) and was based on an outdated laboratory assay (Jaffe).

Modification in Diet in Renal Disease (MDRD)[98] equation

The MDRD equation was based originally on 1628 non-diabetic patients with CKD and it factored in age, sex, ethnicity, serum Cr, albumin and urea. Later a simplified 4 variable equation using age, sex, ethnicity and serum Cr was introduced for clinical use[99]. It is still unsuitable for healthy individuals and for GFRs above 60ml/min/1.73m^2 where the MDRD underestimates GFR. In GFRs below 20ml/min/1.73m^2 or in individuals with nephrotic range proteinuria it overestimates. In non-caucasian populations it is inaccurate but there is a correction for African-Americans (multiple eGFR of the MDRD equation by 1.2). The MDRD equation also has not been validated in acute renal failure.

Mayo Quadratic[100] equation

The Mayo quadratic equation was developed to improve estimates of GFR in healthy individuals, as well as in CKD, using serum Cr, age and sex. In developing this equation 320 subjects with CKD and 580 normal subjects were used, but the elderly and ethnic African-Americans were under-represented.

Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI)[101] equation

The CKD-EPI equation was recently developed to better estimate GFR in normal individuals with GFR more than $60ml/min/1.73m^2$. It included different ethnic groups, the elderly, and diabetics. The development of the equation used data from 8254 individuals and validation based on 3896 individuals. This formula has 8 different equations, depending on sex, serum Cr and ethnic group. Compared with MDRD it has less bias and increased accuracy in estimating higher GFR. However limitations are similar to the MDRD equation because of the small numbers of ethnic and elderly subjects.

2.1.3 Advantages and disadvantages of exogenous GFR markers

2.1.3.1 Radio-labelled or unlabelled polysaccharides

Inulin (MW 5.2kDa) is a polymer of fructose, is freely filtered at the glomerulus, and is neither reabsorbed nor secreted by renal tubules. It is also metabolically inert and cleared only by the kidney. These characteristics make it the gold standard for the estimation of GFR[102]. Using inulin clearance to measure GFR is expensive, involves time-consuming, labour intensive chemical analysis and has limited availability. It involves continuous administration of the intravenous marker to maintain plasma levels and once steady state has been achieved, plasma and timed urine collections (ideally via bladder catheterisation) is needed, therefore making it unsuitable for routine clinical use but used in a research setting.

2.1.3.2 Radio-labelled or unlabelled chelates

Cr^{51} EDTA, Tc^{99m} DTPA and Gd-DTPA GFR measurements are simpler to use than inulin clearance. Single intravenous bolus injection of a radio-labelled filtered marker is injected with a serum/plasma measurement of the marker at a

fixed time (usually 4 hours later) post-injection. Accuracy of the test could be increased with an increased number of serum/plasma measurements. Disadvantages are the complicated measures required to handle, store and dispose of radio-labelled waste. Skilled personnel are needed and some radiation is administered though in very small non-toxic amounts. Radio-labelled chelates are unsuitable in pregnant women and children.

2.1.3.3   Urographic contrast media (radio-labelled or non-labelled)

Urographic contrast media (Iothalamate/[131]Iothalamate or Iohexol) used as a GFR marker may have the added advantage of diagnostic imaging, such as urography, angiography or CT. Iohexol is readily available as a safe non-ionic low osmolar contrast agent. It is not secreted, metabolised or reabsorbed by the kidney and is eliminated exclusively without metabolism by the kidneys and has been increasingly been used as a GFR marker in adults and children. The only contraindication is an allergy to iodine and cautioned in severe asthma/eczema.

2.1.4   Current Studies of GFR measurement comparisons in AFD

Kleinert et al in 2005[103] retrospectively reviewed previous published studies and concluded that the MDRD equation overestimates GFR in AFD patients with normal or near normal serum Cr levels. In their cohort of 8 patients, using the MDRD formula, overestimated GFR in 5 patients and underestimated GFR in 3 patients when compared with ^{51}Cr EDTA radioisotope GFR studies.

Aakre et al in 2009[104] concluded in their cohort of 21 patients that the MDRD and CG equations overestimate GFR compared with ioxehol clearance in their male AFD patients with CKD stages 1-2 and in male AFD patients with a lower normal body mass index (BMI).

More recently Rombach et al[105] compared Cr, Cystatin C and beta-trace protein based GFR equations in 36 AFD patients on ERT and concluded that the Stevens equation (Cystatin C and Cr based equation) best most closely approximated the measured GFR based on [125]Iothalomate urinary clearance.

Unfortunately, studies to date comparing different methods of estimated GFRs in AFD patients have been small.

2.2 Rationale for study

A raised serum Cr alone is insufficient to diagnose renal involvement in AFD patients with GFR $\leq$ 80ml/min/1.73m^2. Our clinical practice needs to ensure that AFD patients with normal serum Cr, but reduced GFR are detected and managed because studies have shown that earlier treatment of AFD reduces the rate of decline in GFR[9-12, 14, 55, 106].
The gold standard test for measuring GFR in a research setting is inulin clearance and in a clinical setting radioisotope GFR estimates or iohexol clearance. These methods may not be available in some centres that manage AFD patients. Other methods of estimating GFR have their problems with under or overestimation of GFR, urine collection inaccuracies and reliability of test assays and when factoring in age, sex and BMI/BSA. Using a retrospective review of our single centre experience I wanted to determine the best method for estimating GFR in AFD and for monitoring progression of renal involvement.

2.3 Aims

i) To document the most appropriate method of measuring GFR in AFD in a clinical setting.
ii) To determine which method of measuring GFR over or under estimated GFR in AFD
iii) To determine what proportion of patients might be inappropriately treated with or deprived of ERT.

2.4 Materials and methods

Patients who were diagnosed and/or followed in the Lysosomal storage disorders unit (Royal Free Hospital) from January 2004 until April 2008 were reviewed. Patients included in our analysis were $\geq$ 18 years of age and had a known mutation in the GLA gene encoding α-galactosidase A. Data from investigations carried out within a month of each patient's routine isotopic GFR estimate by single point ^{51}Cr-EDTA radionuclide study (iGFR) were collected. Patients were excluded if they did not have a 24h U Cr Cl

and serum Cr measurement within 1 month of their iGFR estimate. Only single paired measurements from individual AFD patients were used. The data collected and analysed are summarised in Table 2.1. Serum Cr was determined by the enzymatic Jaffé[107] method, using a Roche automated clinical chemistry analyser. Table 2.2 summarises the equations used to calculate BSA, and eGFR by different formulae. If BSA correction is not already included in an eGFR formula, they were corrected for BSA using the Mosteller formula[108].

In analysing the data, iGFR estimates were presumed to be the most accurate measure of the true GFR[109-113] and all eGFRs were compared with the corresponding iGFR. Demographic data were represented as mean (M), standard deviation of mean (SD), and range. The Mann-Whitney U test was used for statistical analysis of the demographic data in Table 2.1, unless otherwise stated. The mean and SD were calculated and shown graphically for all CKD stages, and subdivided into CKD stages 1, 2 and 3, according to the method of estimating GFR; statistical analysis was by the Wilcoxon matched pairs test.

Bland-Altman plots[114] were used to analyse the agreement between iGFR and the different methods of calculating eGFR. These plots show the mean difference between iGFR and eGFR method depicted, 95% limit of agreement (mean difference of iGFR and eGFR method depicted ± 1.96SD), bias and Spearman's rank correlation coefficient between the absolute differences and the average.

The percentage of eGFR within ±30% of the iGFR (P_{30}) and the number and percentage of missed or early treated patients were calculated when the eGFR was used instead of the iGFR to decide when to start ERT. Missed patients were defined as patients who received ERT on the basis of an iGFR $< 80\text{ml/min}/1.73\text{m}^2$, but who had a matched eGFR of $\geq 80\text{ml/min}/1.73\text{m}^2$ (false negative), and early treated patients were those who had an iGFR of $\geq 80\text{ml/min}/1.73\text{m}^2$, but had a matched eGFR of $< 80\text{ml/min}/1.73\text{m}^2$ (false positive).

Characteristics	Males (n=45)			Females (n=61)			p values
	Mean	SD	Range	Mean	SD	Range	
Mean Age, years	44.70	± 13.57	20 - 75	45.57	± 14.96	18 – 80	NS
On Enzyme Replacement Therapy, n [%]	43 [95.56%]			38 [62.30%]			p < 0.0001 (Fishers exact test)
Height, m	1.77	± 0.05	1.64 – 1.89	1.60	± 0.06	1.50 – 1.73	p < 0.0001
Weight, kg	77.91	± 15.43	51.0 – 123.8	66.35	± 10.93	42.3 – 86.9	p = 0.0002
Body Surface Area, m^2	1.95	± 0.20	1.58 – 2.45	1.71	± 0.16	1.34 – 2.03	p < 0.0001
Body Mass Index, kg/m^2	24.9	± 4.8	16.7 – 40.9	25.9	± 4.0	17.6 – 34.1	NS
Serum Creatinine, μmol/L	89.60	± 21.45	63 - 187	72.38	± 13.54	47 – 123	p < 0.0001
iGFR, ml/min/$1.73m^2$	84.11	± 21.53	33.0 – 122.0	92.05	± 23.24	39.0 – 150.0	NS (p = 0.0890)
MDRD eGFR, ml/min/$1.73m^2$	90.42	± 21.11	37.1- 132.5	84.59	± 18.37	40.7 – 130.4	NS (p = 0.1004)
Cockcroft Gault eGFR, ml/min/$1.73m^2$	93.27	± 22.91	45.0 – 138.6	93.96	± 22.76	38.7 – 138.4	NS
24h U Cr Cl, ml/min/$1.73m^2$	92.33	± 35.24	40.2 – 193.0	93.55	± 24.39	34.9 – 160.8	NS
Mayo Quadratic eGFR, ml/min/$1.73m^2$	113.2	± 25.49	38.3 – 145.0	102.1	± 15.63	50.9 – 126.6	p = 0.0004
CKD-EPI eGFR, ml/min/$1.73m^2$	92.32	± 21.27	37.9 – 125.1	89.47	± 19.86	38.4 – 123.7	NS
24 hour Urine Protein, g/24 hour	0.25	± 0.28	0.00 – 0.99	0.18	± 0.25	0.05 – 1.50	p = 0.0271
Spot Urine Protein: Creatinine Ratio, mg/mmol	19.54	± 36.30	1.49 – 174.5	22.94	± 32.83	0.01 – 143.9	NS
Spot Urine Albumin: Creatinine Ratio, mg/mmol	17.63	± 29.17	0.31 – 119.2	9.13	± 20.08	0.25 – 109.4	p = 0.0327

Table 2.1. Demographic and patient characteristic data (p values calculated by Mann-Whitney U test unless stated).

Investigation	Formula			
Body Surface Area [m^2]	=	$\{[\text{Height(cm)} \times \text{Weight(kg)}]/3600\}^{1/2}$		
24 hour Urine Creatinine Clearance [ml/min/1.73m^2]	=	$\dfrac{\text{Urine Cr (µmol/L)} \times 24 \text{ hour Urine Volume (ml)} \times 1.73}{\text{serum Cr (µmol/L)} \times 24 \times 60 \times \text{BSA (m}^2)}$		
MDRD eGFR [ml/min/1.73m^2]	=	$[\text{serum Cr (µmol/L)}/88.4]^{-1.154} \times 186 \times \text{Age (years)}^{-0.203} \times 1.21(\text{if black}) \times 0.742 \text{ (if female)}$		
Cockcroft Gault eGFR [ml/min/1.73m^2]	=	$\dfrac{[140 - \text{Age (years)} \times \text{Weight(kg)}] \times 0.85 \text{ (if female)} \times 1.73}{\text{serum Cr (µmol)} \times 0.8136 \times \text{BSA}}$		
Mayo Quadratic eGFR [ml/min/1.73m^2]	=	$\exp\{1.911 + 5.249/\text{serum Cr [mg/dL]} - 2.114/(\text{serum Cr [mg/dL]})^2 - 0.00686 \times \text{Age} - 0.205 \text{ (if female)}\}$. (If serum Cr < 0.8mg/dL use 0.8mg/dL for serum Cr)		

CKD-EPI	**Race and Sex**	**Serum Cr (µmol/L)**	**Equation**
	Black		
	Female	≤ 62	eGFR [ml/min/1.73m^2] = 166 x (S Cr/0.7)$^{-0.329}$ x (0.993)$^{\text{Age}}$
		> 62	eGFR [ml/min/1.73m^2] = 166 x (S Cr/0.7)$^{-1.209}$ x (0.993)$^{\text{Age}}$
	Male	≤ 80	eGFR [ml/min/1.73m^2] = 163 x (S Cr/0.9)$^{-0.411}$ x (0.993)$^{\text{Age}}$
		>80	eGFR [ml/min/1.73m^2] = 163 x (S Cr/0.9)$^{-1.209}$ x (0.993)$^{\text{Age}}$
	White or Other		
	Female	≤ 62	eGFR [ml/min/1.73m^2] = 144 x (S Cr/0.7)$^{-0.329}$ x (0.993)$^{\text{Age}}$
		> 62	eGFR [ml/min/1.73m^2] = 144 x (S Cr/0.7)$^{-1.209}$ x (0.993)$^{\text{Age}}$
(Note S Cr denotes serum Cr)	Male	≤ 80	eGFR [ml/min/1.73m^2] = 141 x (S Cr/0.9)$^{-0.411}$ x (0.993)$^{\text{Age}}$
		>80	eGFR [ml/min/1.73m^2] = 141 x (S Cr/0.9)$^{-1.209}$ x (0.993)$^{\text{Age}}$

Table 2.2. Formulae used for calculating body surface area (BSA) and estimated glomerular filtration rate (eGFR)

2.5   Results

One hundred and thirty five AFD patients were reviewed: 5 patients were excluded as they were less than 18 years of age at the time of iGFR and 24 were excluded because matched investigations were not completed within 1 month of an iGFR measurement; 106 individual AFD patient investigations were included in this analysis. There were more females (61) than males (45), with no significant differences in age and BMI. Men had a significantly higher BSA, serum Cr, 24-hour urine protein excretion and microalbuminuria. A larger proportion of males compared to females were on ERT (95.56% vs 62.30%). All 106 patients included in this study were Caucasian.

All CKD stages (Figure 2.1 and Table 2.3)

Comparing eGFRs, there were significant differences between the CG equation, 24h U Cr Cl, and Mayo Quadratic equation when compared with iGFR; all 3 overestimated GFR in AFD patients when compared with iGFR. The mean differences between iGFR and eGFR of CG, 24h U Cr Cl, and Mayo Quadratic were 5.0±16.2, -4.4±21.8 and -18.1±17.7 ml/min/1.73m^2, respectively. The Mayo Quadratic equation gave the greatest mean difference, while the MDRD and CKD-EPI eGFR were not significantly different from iGFR. When analysed according to sex, in males all methods of eGFR calculation overestimated GFR compared with iGFR (-6.3 to -29.1 ml/min/1.73m2), but in females only the Mayo Quadratic equation overestimated eGFR (-10.1±15.7 ml/min/1.73m^2), and the MDRD significantly underestimated eGFR (7.5±17.5 ml/min/1.73m^2).

CKD stage 1 (Figure 2.2, Table 2.3)

According to the iGFR in CKD stage 1, the MDRD equation underestimated eGFR in males and females (8.7±16.8 ml/min/1.73m^2) and underestimated eGFR in females only (13.4±18.0 ml/min/1.73m^2), but not in males only (0.3±10.3 ml/min/1.73m^2). The Mayo Quadratic equation significantly overestimated eGFR in all CKD stage 1 patients (-11.2±17.0 ml/min/1.73m^2), but sub-analysis by sex showed significant overestimation in males (-26.1±9.1 ml/min/1.73m^2), although not in females (-2.9±14.5 ml/min/1.73m^2). The CKD-EPI equation significantly underestimated eGFR only in

females (6.6 ± 15.9 ml/min/1.73m^2). There was no significant difference in CG estimates of eGFR and 24h U Cr Cl compared with iGFR in CKD stage 1.

CKD stage 2 (Figure 2.3, Table 2.3)

In CKD stage 2, CG, MDRD, Mayo Quadratic and CKD-EPI equations all overestimated eGFR; only 24hr U Cr Cl was similar to iGFR. Sub-analysis by sex revealed that the eGFR overestimates were predominantly in males (-10.4 to -32.8 ml/min/1.73m^2) and in females only the Mayo Quadratic equation overestimated eGFR (-16.6 ± 9.9 ml/min/1.73m^2).

CKD stage 3 (Figure 2.4, Table 2.3)

In CKD stage 3, all equations and 24h U Cr Cl overestimated GFR compared with iGFR, from a mean difference of -9.5 ml/min/1.73m^2 for 24h U Cr Cl to -27.0 ml/min/1.73m^2 for the Mayo Quadratic equation. Sub-analysis by sex, showed that in females there was no significant difference between eGFR measurements and iGFR, except for the Mayo Quadratic equation, which significantly overestimated eGFR (-27.8 ± 16.2 ml/min/1.73m^2); however, all equation-based methods of calculating eGFR, but not 24h U Cr Cl, overestimated eGFR in males.

Agreement between iGFR and eGFR (Figure 2.5 and Table 2.4)

Using Bland-Altman plots the Mayo Quadratic equation has the most bias in over-estimating eGFR (-18.14) and the MDRD and CKD-EPI equations having the least bias (+1.62 and +2.00, respectively). The CKD-EPI had the highest P_{30} value at 93.4%, followed by the CG and the MDRD equations. The Mayo Quadratic equation had the lowest P_{30} value at 64.2%. If divided by GFR <60ml/min/1.73m^2 (CKD stage 3), the CKD-EPI equation performed the best with a P_{30} of 83.3% and the Mayo Quadratic equation the worst with a P_{30} value of 16.7%.

Treatment accuracy (Tables 2.5 and 2.6)

Based on a GFR estimate and criterion for treatment of < 80ml/min/1.73m^2, 16 males and 16 females would have received ERT, if treated according to iGFR values.

Comparing the matched eGFRs with the corresponding iGFRs, the Mayo Quadratic equation gave the highest number of 'missed' (untreated) patients (20), while the MDRD, 24h U Cr Cl, and CKD-EPI estimates each resulted in 8 'missed' patients. Conversely, 29 males and 45 females would not be treated on the basis of their iGFRs only. Compared with the corresponding iGFR, the MDRD (16 patients) and CKD-EPI (18 patients) eGFRs would have resulted in the greatest number of 'early treated' patients.
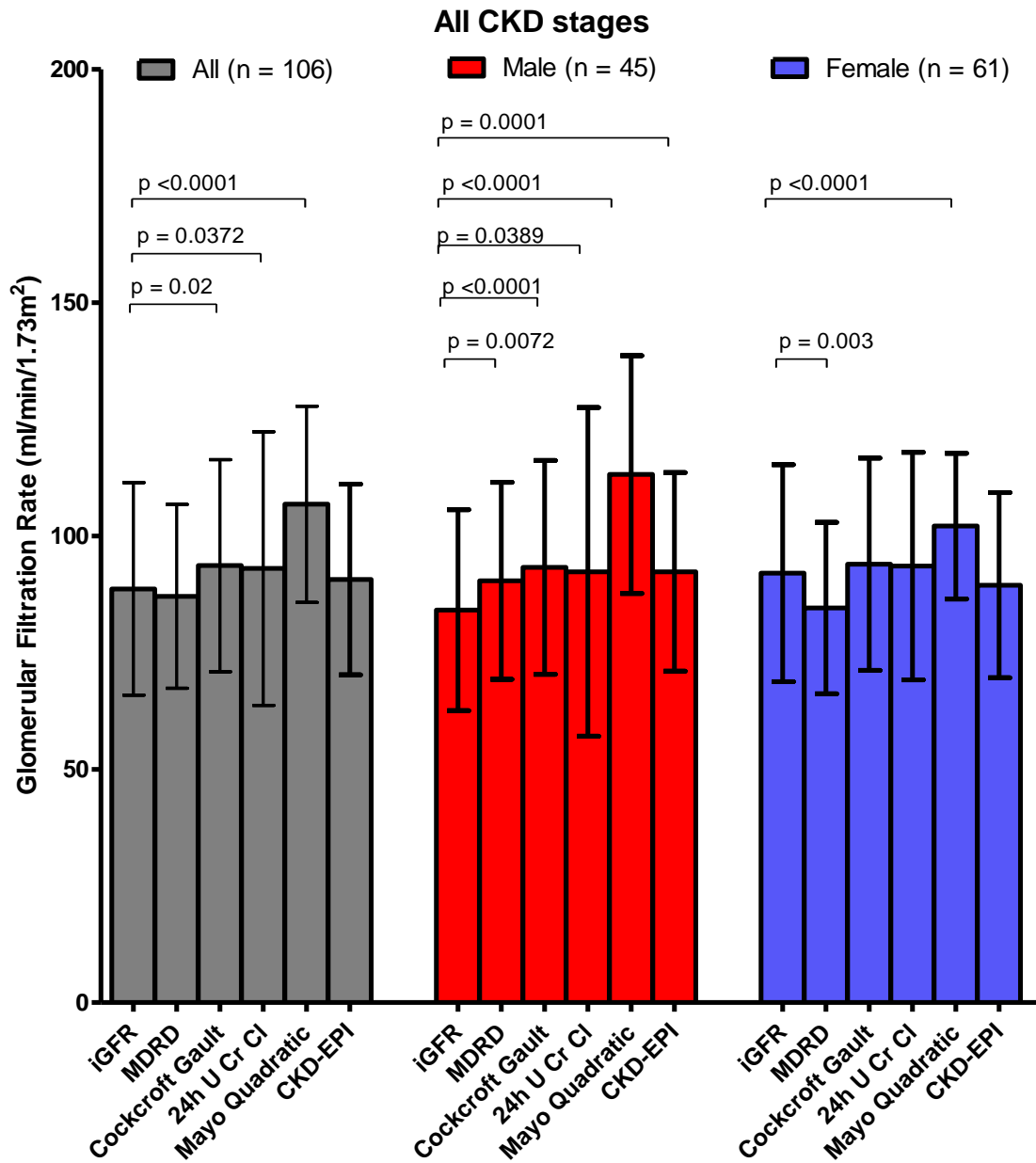
Fig 2.1. Comparison of iGFR, MDRD equation, CG equation, 24h U Cr Cl, Mayo Quadratic equation and CKD-EPI equation in AFD males and females for all stages of CKD. Wilcoxon matched pairs test used for statistical analysis.
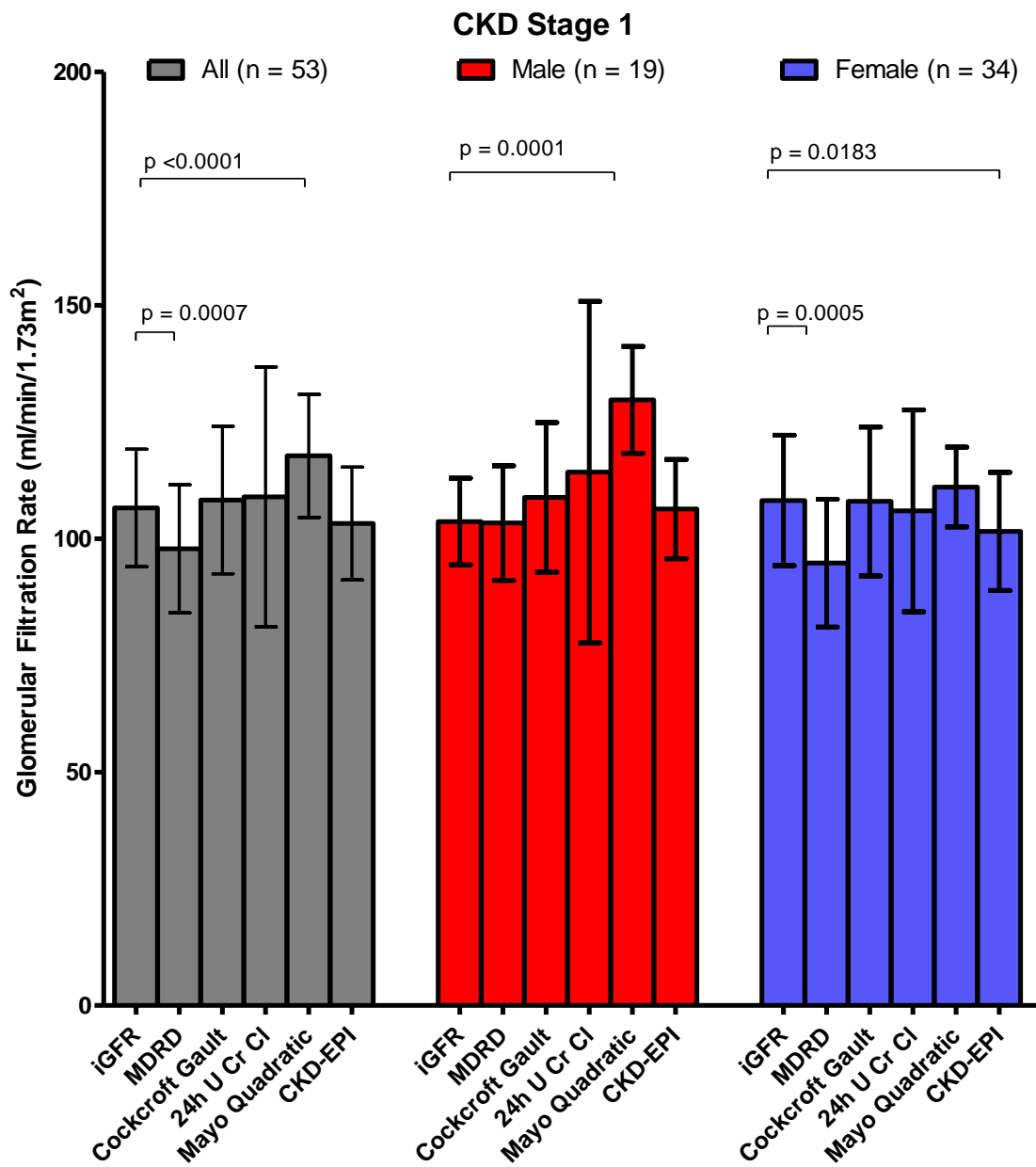
Fig 2.2 Comparison of iGFR, MDRD equation, CG equation, 24h U Cr Cl, Mayo quadratic equation and CKD-EPI equation in AFD males and females for CKD stage 1. Wilcoxon matched pairs test used for statistical analysis.
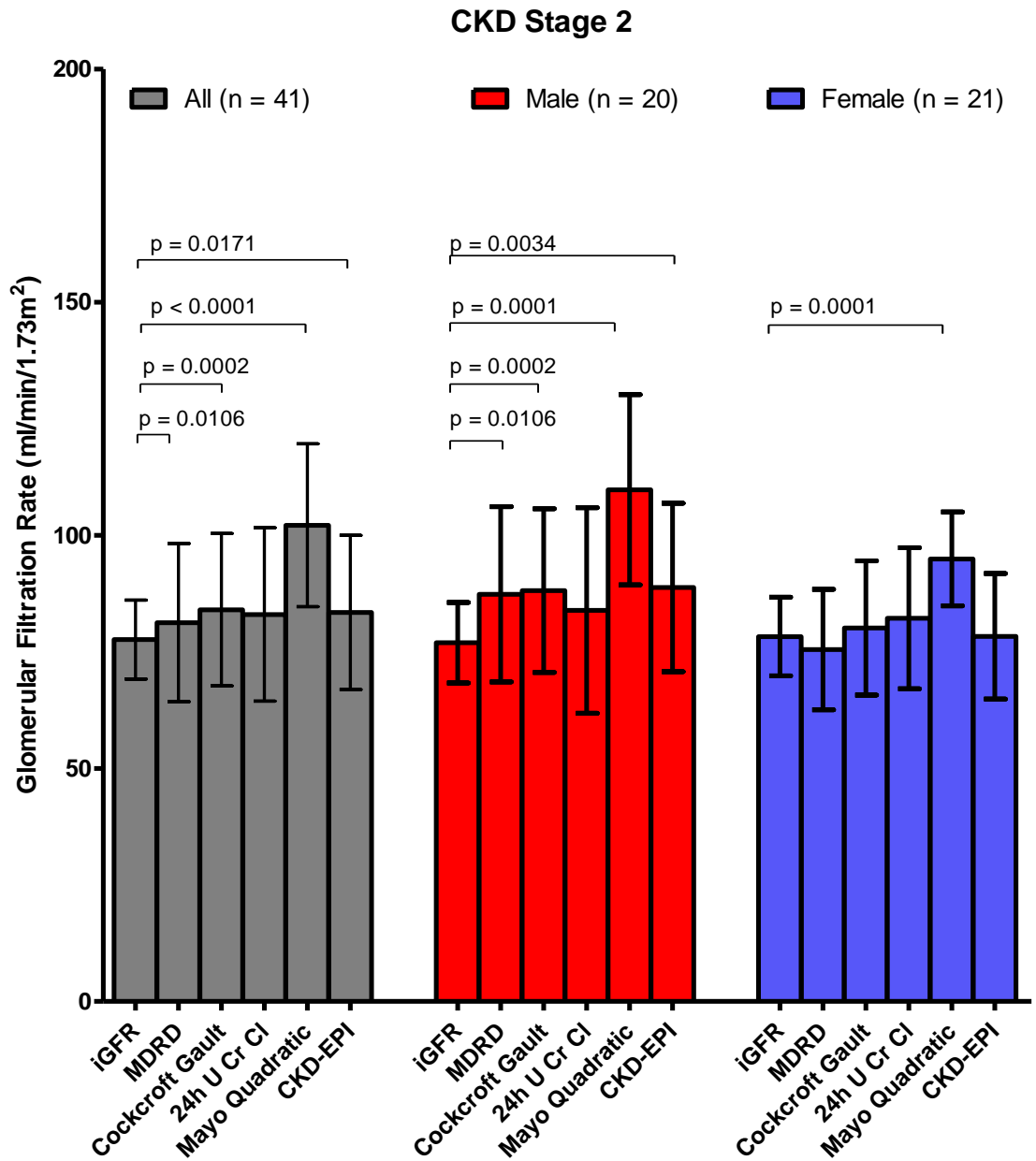
Fig 2.3. Comparison of iGFR, MDRD equation, CG equation, 24h U Cr Cl, Mayo quadratic equation and CKD-EPI equation in AFD males and females for CKD stage 2. Wilcoxon matched pairs test used for statistical analysis.
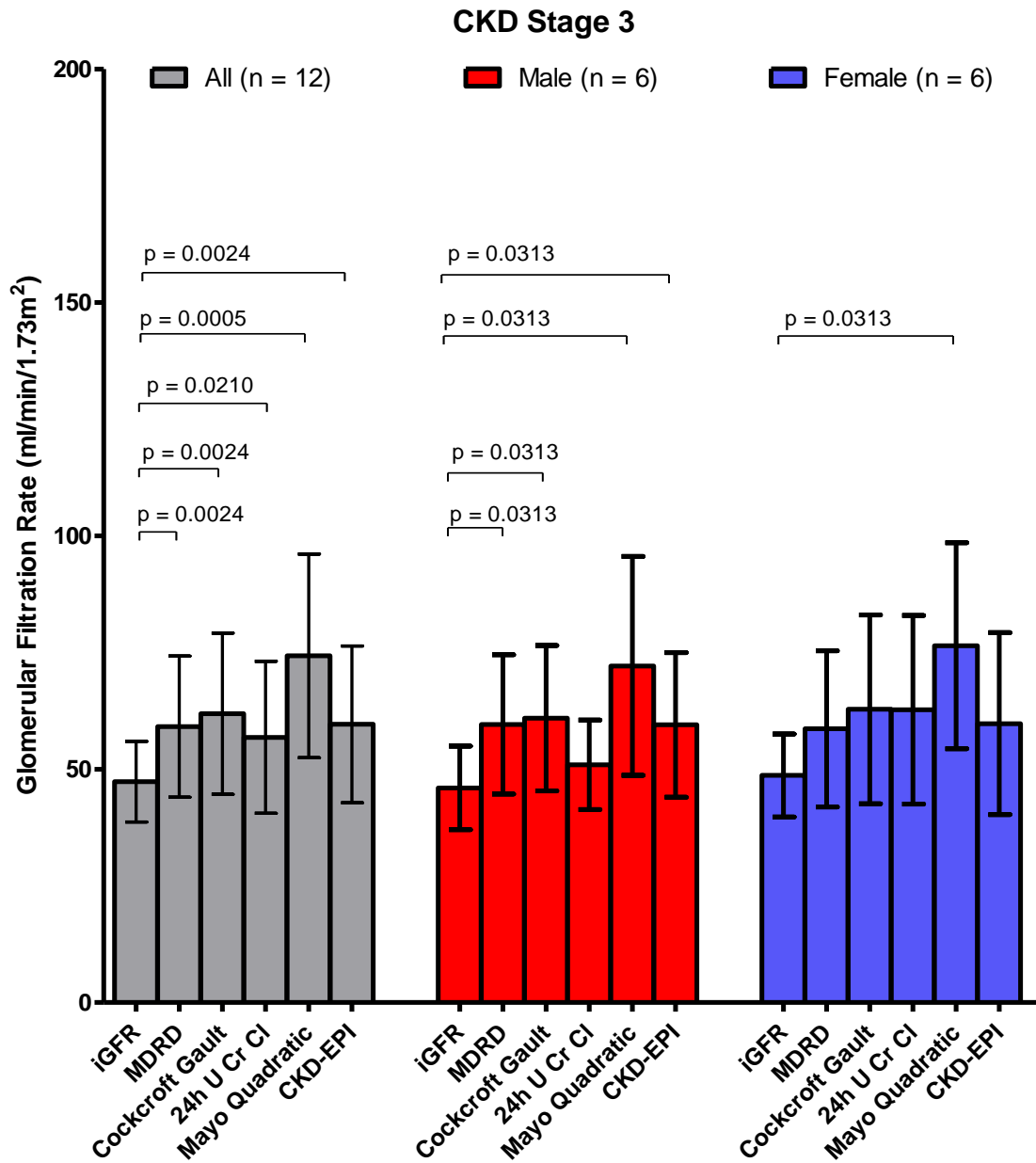
Fig 2.4. Comparison of iGFR, MDRD equation, CG equation, 24h u Cr Cl, Mayo quadratic equation and CKD-EPI equation in AFD males and females for CKD stage 3. Wilcoxon matched pairs test used for statistical analysis.

(a)	All (n = 106)							
Investigation, ml/min/1.73m^2	**All Stages**		**CKD 1**		**CKD 2**		**CKD3**	
	M	**SD**	**M**	**SD**	**M**	**SD**	**M**	**SD**
MDRD eGFR	1.6	±17.4	8.7	±16.8	-3.7	±15.8	-11.8	±9.8
Cockcroft Gault eGFR	-5.0	±16.2	-1.7	±17.4	-6.4	±14.3	-14.6	±13.5
24 hour Urine Creatinine Clearance	-4.4	±21.8	-2.4	±25.9	-5.4	±17.8	-9.5	±11.8
Mayo Quadratic eGFR	-18.1	±17.7	-11.2	±17.0	-24.5	±15.8	-27.0	±15.6
CKD-EPI eGFR	-2.0	±15.3	6.5	±15.3	-5.8	±14.9	-12.3	±11.6

(b)	Males (n = 45)							
Investigation, ml/min/1.73m^2	**All Stages**		**CKD 1**		**CKD 2**		**CKD3**	
	M	**SD**	**M**	**SD**	**M**	**SD**	**M**	**SD**
MDRD eGFR	-6.3	±13.8	0.3	±10.3	-10.4	±15.6	-13.6	±8.2
Cockcroft Gault eGFR	-9.2	±13.5	-5.2	±13.3	-11.2	±13.5	-14.9	±13.1
24 hour Urine Creatinine Clearance	-8.2	±27.1	-10.6	±36.3	-6.9	±20.6	-4.9	±6.9
Mayo Quadratic GFR	-29.1	±14.0	-26.1	±9.1	-32.8	±16.7	-26.1	±16.5
CKD-EPI eGFR	-8.2	±12.5	-2.7	±8.4	-11.9	±14.7	-13.5	±9.7

(c)	Females (n = 61)							
Investigation, ml/min/1.73m^2	**All Stages**		**CKD 1**		**CKD 2**		**CKD3**	
	M	**SD**	**M**	**SD**	**M**	**SD**	**M**	**SD**
MDRD eGFR	7.5	±17.5	13.4	±18.0	2.8	±13.2	-10.0	±11.7
Cockcroft Gault eGFR	-1.9	±17.4	0.2	±19.2	-1.9	±13.9	-14.2	±15.0
24 hour Urine Creatinine Clearance	-1.5	±16.5	2.2	±16.7	-3.9	±15.2	-14.1	±14.5
Mayo Quadratic eGFR	-10.1	±15.7	-2.9	±14.5	-16.6	±9.9	-27.8	±16.2
CKD-EPI eGFR	2.6	±15.6	6.6	±15.9	-0.1	±13.0	-11.1	±14.0

Table 2.3. Mean of differences between matched investigations eGFR compared with iGFR, negative values indicate overestimation, and positive values underestimation of GFR. (a) All, (b) Males and (c) Females.
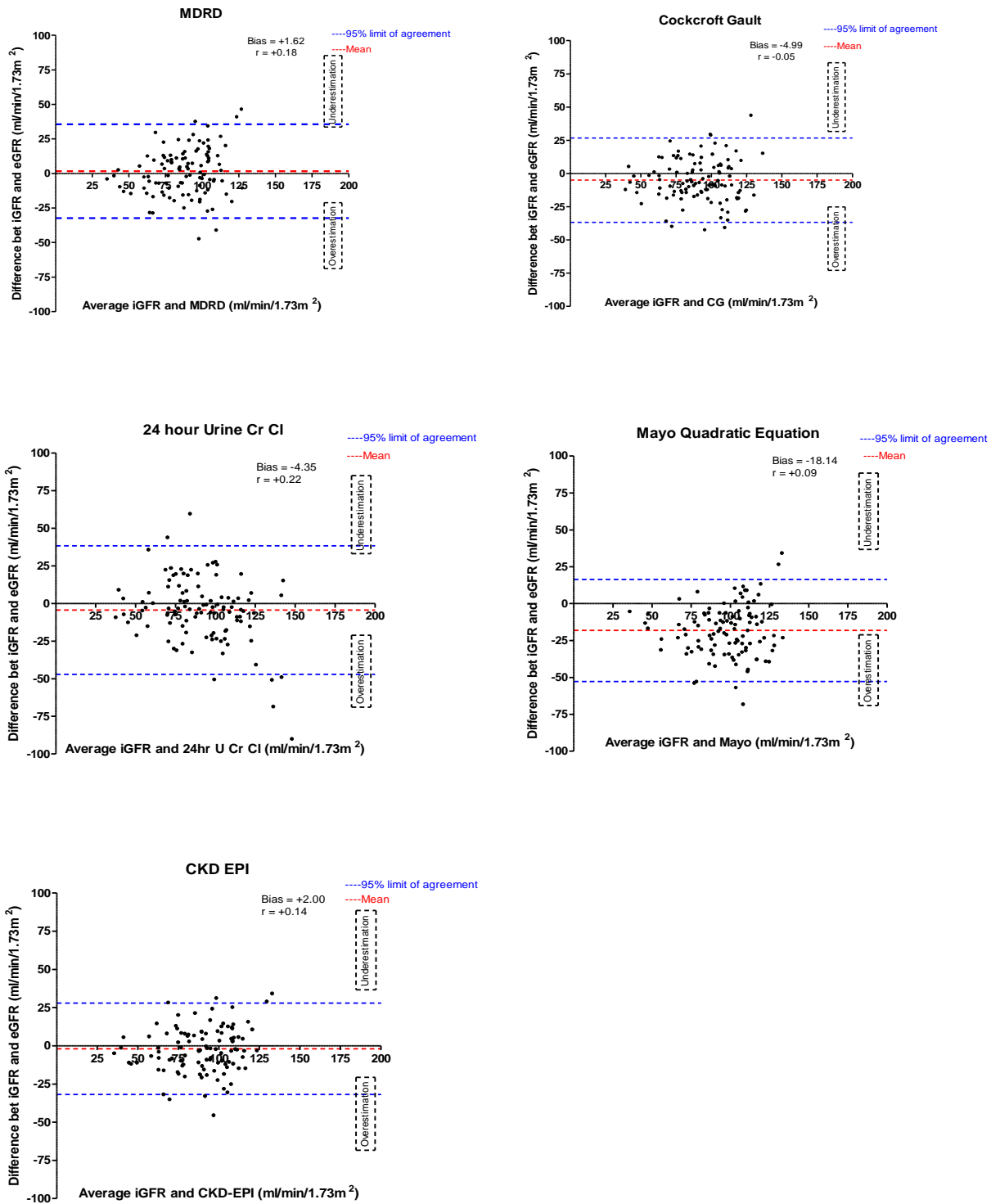
Fig. 2.5. Bland Altman plots of iGFR (gold standard) compared with different methods of eGFR.

P$_{30}$, %	All patients	< 60 ml/min/1.73m^2	≥ 60 ml/min/1.73m^2
MDRD	88.7%	66.7%	91.5%
Cockcroft Gault	92.5%	50%	97.9%
24h U Cr Cl	83%%	58.3%	86.2%%
Mayo Quadratic	64.2%	16.7%	70.3%
CKD-EPI	93.4%	83.3%	94.7%

Table 2.4. Percentage of matched eGFR within 30% of iGFR (P$_{30}$).

ml/min/1.73m^2	iGFR		MDRD		Cockcroft Gault		24h U Cr Cl		Mayo Quadratic		CKD-EPI	
	M	F	M	F	M	F	M	F	M	F	M	F
≥ 80	29 (64.4%)	45 (73.8%)	32 (71.1%)	35 (42.6%)	35 (77.8%)	46 (75.4%)	26 (57.8%)	42 (68.9%)	39 (86.7%)	56 (91.8%)	35 (77.8%)	43 (70.5%)
< 80	16 (35.6%)	16 (26.2%)	13 (28.9%)	26 (57.4%)	10 (22.2%)	15 (24.6%)	19 (42.2%)	19 (31.1%)	6 (13.3%)	5 (8.2%)	10 (22.2%)	18 (29.5%)

Table 2.5. Classification of those who meet criteria for ERT based on different measures of GFR; numbers (%)

	MDRD	Cockcroft Gault	24h U Cr Cl	Mayo Quadratic	CKD-EPI
Missed Females	3 (18.8%)	7 (43.8%)	4 (25.0%)	12 (75.0%)	5 (31.3%)
Early treated Females	14 (31.0%)	6 (13.3%)	8 (17.8%)	1 (2.2%)	7 (15.6%)
Missed Males	5 (31.3%)	7 (43.8%)	4 (25%)	8 (50.0%)	3 (18.8%)
Early treated Males	2 (6.9%)	1 (3.4%)	7 (24.1%)	2 (6.9%)	11 (37.9%)

Table 2.6. Summary of missed or early treated patients if using other GFR estimates compared with iGFR; numbers (%).

2.6   Discussion

In view of the low prevalence, it is difficult to obtain large numbers of AFD patients for study. With increasing awareness and increasing diagnosis of the "late-onset" phenotype from screening studies[66, 68], there is need to understand the disease process and effectiveness of current therapies in managing AFD. An increasing number of AFD patients are diagnosed at an earlier age due to family screening and before the onset of clinically apparent end-organ damage. Genetic polymorphisms should caution us in diagnosing AFD without evidence of organ involvement. Renal involvement is one facet of disease-related progression that needs to be monitored closely; if there are signs a renal dysfunction, ERT should be introduced early to prevent or slow the progression to end stage renal failure.

GFR measurements vary in method and accuracy, and from centre to centre, which could lead to premature or delayed initiation of ERT. The present study is the largest to assess the accuracy of different eGFR methods used to monitor renal function in AFD patients. The study population had more females than males, but there were no significant differences between age and BMI. AFD males had a higher BSA and serum Cr than females and as affected males have earlier and more severe organ involvement, more males than females were on ERT. Males had more proteinuria and microalbuminuria compared with females, as reported in the FOS[86] and Fabry registry[42]. There were no significant difference between males and females using iGFR or eGFR, except for the Mayo Quadratic equation, where the mean eGFR was higher in males than females. As with any study involving AFD, numbers are limited. In our study this was true for CKD stage 3 (according to iGFR), for which we had only 12 patients and our study population did not include patients in CKD stages 4 and 5.

The MDRD equation has been validated for GFRs < 60ml/min/1.73m^2, but current guidelines for starting ERT in AFD use a value of < 80ml/min/1.73m^2. The majority of our study patients were in CKD stages 1 and 2. In this study, the MDRD formula, when compared with iGFR, showed no significant difference overall, but when divided by sex, it overestimated eGFR in males and underestimated it in females. Sub-dividing by stage of CKD, the MDRD eGFR underestimated eGFR in CKD stage 1 and overestimated it in CKD stages 2 and 3. This shift from underestimation to overestimation is more apparent when sub-divided by sex: in males there was no

difference in CKD stage 1, but a significant overestimation in CKD stages 2 and 3, and in females there was an underestimation in CKD stage 1, but no difference in CKD stages 2 and 3. The MDRD equation had the least bias compared with iGFR, and approximately 90% of eGFRs were within 30% of their corresponding iGFRs. Also, males with CKD stages 2 and 3 had an overestimate of eGFR, suggesting more significant renal disease than evident from their MDRD-based eGFR. However, in females we must be careful that underestimation of eGFR by MDRD in stage 1 CKD may lead to initiation of ERT too early.

The CG equation overestimated eGFR more in those with poorer renal function (CKD stages 2 and 3), which was more apparent in males than females. Using this equation could potentially result in a significant number of patients being excluded from ERT. Thus, it is not recommended for monitoring renal function in AFD.

24h U Cr Cl overestimated of GFR in all patients, although when subdivided by CKD stage, this was only significant in CKD Stage 3. Compared with the other methods, only 83% of 24h U Cr Cl eGFRs were within 30% of iGFRs. 24h u Cr Cl had the largest standard deviation of the mean, most notable in males with CKD stage 1. This could reflect incomplete or inaccurately timed urine collections, a common problem, and a disadvantage using 24h U Cr Cl to estimate GFR. Therefore it is not recommended for monitoring renal function in AFD patients.

However, the Mayo Quadratic equation was the least reliable when compared with iGFR; it significantly overestimated GFR in all categories of eGFR, except for females with CKD stage 1. Only 64.2 % of eGFRs were within 30% of their corresponding iGFRs. Using this equation there would be the highest number of 'missed' patients for ERT, and it is not recommended for use in AFD patients.

The CKD-EPI equation is probably the best non-invasive method of calculating eGFR in all stages of CKD, including CKD stage 1, as there were no significant differences when compared with iGFR. However, there was significant underestimation of eGFR in females in CKD stage 1, similar to the MDRD equation, although not as large a difference. It also significantly overestimated eGFR in males with CKD stages 2 and 3, but had the highest percentage of eGFRs within 30% of their corresponding iGFRs. Therefore the CKD-EPI equation would be our recommended method for calculating eGFR in AFD patients.

In a clinical setting, because of the small numbers of AFD patients, and the need for early treatment to prevent progression of disease, simple methods for the reliable detection, assessment, and monitoring of organ involvement needs to be easily available. The best methods of estimating GFR in a clinical setting would be isotopic GFR measurements, but they are not available in all hospitals and they are unsuited to regular monitoring, because of the risks of repeated radiation exposure and cost. Iohexol has been increasingly used in research and clinical practice as another measure of GFR but is more invasive and expensive than standard eGFR calculations. An easier and cost efficient method of estimating GFR is needed, since the majority of patients are now diagnosed and monitored through screening, or family tracing, when early renal involvement may not be evident[115] and most patients have normal renal function or CKD stages 1 and 2. ERT is expensive and life-long, but it can potentially slow disease progression and prevent the development of renal failure, if started early enough[7].

Still the most widely used measure of renal function is serum Cr with all its limitations. Cr production and excretion varies among individuals especially by ethnicity, sex, age, in obesity[91], during pregnancy[93], and in severely ill patients with low muscle mass and poor nutritional status. Under conditions of normal renal function, Cr is excreted by glomerular filtration, and a small amount is actively secreted by the renal tubules. With a declining GFR, active tubular secretion of Cr plays a more significant role in Cr excretion from the body[94]. In severe renal impairment, extra-renal elimination of Cr may occur in the small bowel, where bacterial overgrowth causes degradation of up to two thirds of total daily Cr excretion[95]. Assays of Cr are technically difficult, hampered by interferences up to 20% by oxidoreductive compounds and can be reduced by the enzymatic assay method[96]. Differences in specificity in different assays can make it difficult to compare values from different laboratories. Therefore serum Cr is a poor measure of renal dysfunction in early kidney injury.

Equations have been developed to estimate GFR more accurately based on serum Cr to improve stratification of CKD, but the widely used MDRD equation has not been validated in CKD stages 1 and 2[99], and the new CKD-EPI equation may be more reliable in CKD stages 1 and 2[101].

A review of previously published studies[103] concluded that the MDRD equation overestimated GFR in AFD patients with normal or near normal serum Cr levels. Another study[104] with a cohort of 21 patients, the MDRD and CG equations

overestimated GFR compared with ioxehol clearance in male AFD patients with CKD stages 1-2 and lower normal BMI. The present study shows that the CKD-EPI equation in AFD patients is the best method for calculating eGFR, especially in CKD stages 1 and 2, followed by the MDRD equation. More recently, Rombach et al[105] recommended the use of the Stevens equation (based on serum Cystatin C concentration and serum Cr) in AFD; however, serum Cystatin C assays are not widely available in routine clinical practice and this recommendation was based on a small study population (n=36).

Currently in the UK, most pathology laboratories report eGFR based on the MDRD equation. Changing this current practice to incorporate the CKD-EPI equations in all biochemistry laboratories, would be time consuming and increase costs. Unless the CKD-EPI equation was to be used for monitoring all renal diseases or renal dysfunction, streamlining current pathology systems to monitor AFD would not be cost efficient. But as we have shown, the CKD-EPI equation is a fairly simple to calculate, individual clinicians involved in the management of AFD patients could calculate this with basic databases and spreadsheets, and therefore not need wholesale changes to pathology departments reporting systems.

Currently there are two large international registries for AFD patients; the Fabry Registry and the FOS. Incorporating the CKD-EPI equation in these registries, would provide a more accurate monitoring of renal function of AFD patients. This could be done on retrospective and prospective data, thus providing more longitudinal data and larger numbers to determine the accuracy of the CKD-EPI equation compared to the MDRD eGFR, radioisotopic GFRs or iohexol GFRs.

2.7   Limitations

As with any study involving AFD, small numbers will always be a limitation. This is especially true for CKD stage 3, where we had only 12 patients (based on iGFR). We also had no patients in CKD Stage 4 and 5 and we cannot comment on the reliability of eGFR in AFD for these stages of CKD.

2.8   Conclusions

In a clinical setting in which GFR may be the only criterion for determining the initiation of ERT, eGFR based on the CKD-EPI or MDRD equations may still not be sufficient and an exogenous marker-based estimate of GFR is required. However, based on our findings, we would recommend the CKD-EPI equation as the best method for estimating GFR and for monitoring renal function in AFD patients.

Chapter 3. Urine proteins as biomarkers of renal function and overall review in AFD

3.1   Introduction

Current methods of assessment and monitoring of renal involvement in AFD predominantly look at glomerular function, and evidence of non-glomerular renal involvement would need invasive tissue diagnosis based on renal biopsy. Renal biopsies although invasive have been used as the primary means of diagnosing AFD and in clinical trials to monitor efficacy of therapeutic interventions. Renal biopsies have also been used to ensure that there is no second pathology occurring in AFD patients with renal dysfunction such as diabetic nephropathy[116], IgA nephropathy[117-122], focal segmental glomerular sclerosis[123], minimal change glomerulonephritis[124], crescenteric glomerulonephritis[125, 126] and systemic lupus erythematosus nephropathy[127-129].

3.2   Renal histopathology in AFD

Renal histopathological changes in AFD has been described based on autopsies and renal biopsies, in male[130-133] and female[130, 134] patients. Characteristic renal pathological findings are vacuolisation of podocytes (Fig 3.1), tubular epithelial cells and vascular endothelial cells (GB3 deposition)[115, 116, 122, 135, 136], mesangial expansion[115, 116, 135], segmental and global glomerulosclerosis[115, 116, 122, 135, 137], tubular atrophy[115, 116, 122, 135, 137], interstitial fibrosis[115, 116, 122, 135, 137], vascular medial thickening and chronic inflammatory infiltrates. Females can be as affected as males[115, 116, 122] and renal histopathological changes are present even with normal renal function and minimal or no proteinuria[115, 138].

Ultrastructural changes include inclusion bodies in the cytoplasm of all types of renal cells, characteristic onion skin or zebra appearance due to concentric lamellation (Fig 3.2). These osmiophillic, myelinic bodies are found in tubular epithelial cells, all glomerular cells especially the podocytes, and in endothelial vascular cells[116, 135, 136, 139, 140]. Other diseases that can mimic these pathological changes seen in AFD are silicon nephropathy[141] and chloroquine induced phospholipidosis[142, 143]. Podocyte effacement is only apparent on electron microscopy when there is overt proteinuria[115, 116, 122] suggesting preservation of foot processes and slit diaphragms early on when proteinuria is absent.

Fig 3.1. Renal pathology in AFD from Alroy et al[135]. (A) Glomerulus showing extensive inclusion bodies of glycolipid in podocytes (arrowhead), and mild mesangial widening (PAS stain; magnification, _80). (B) Plastic embedded tissue showing in-site deposition of glycolipid in glomerular podocytes (arrowhead; toluidine blue stain; magnification, _80). (C) Plastic embedded renal tissue demonstrating glycolipid inclusion bodies in distal tubules (asterisk), with relative sparing of proximal tubules, and interstitial fibrosis (toluidine blue stain; magnification, _80). (D) Deposition of glycolipid in endothelial cells of peritubular capillaries (asterisk; toluidine blue stain; magnification, _200). (E) Urine showing vacuolated urinary epithelial cells (oval fat bodies) in a Fabry patient (Papanicolaou stain; magnification, _160). Figure modified with permission from Branton et al[55].
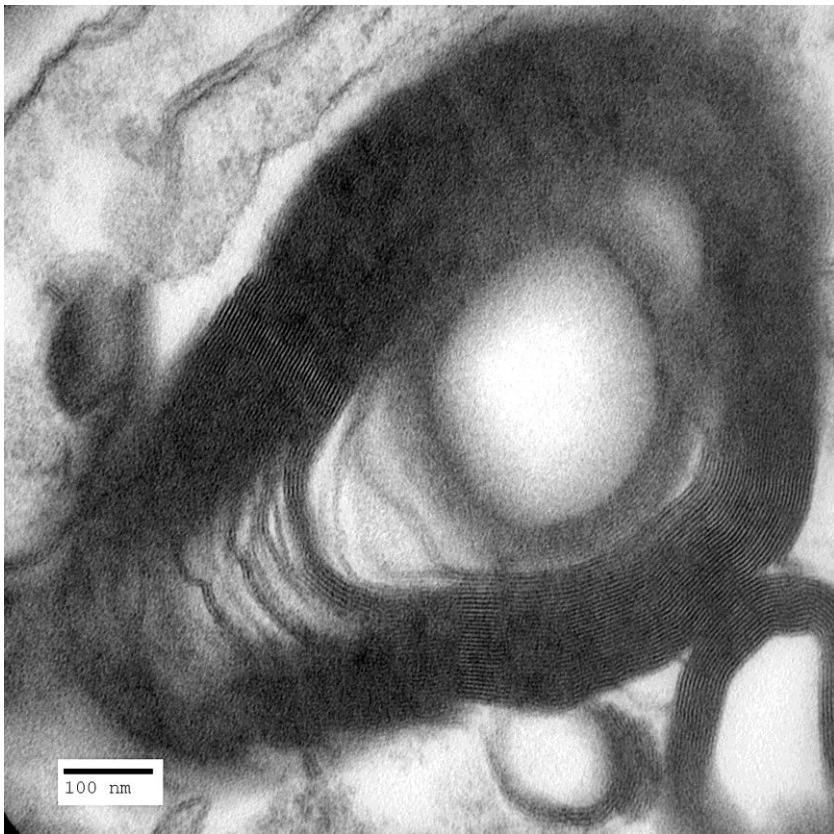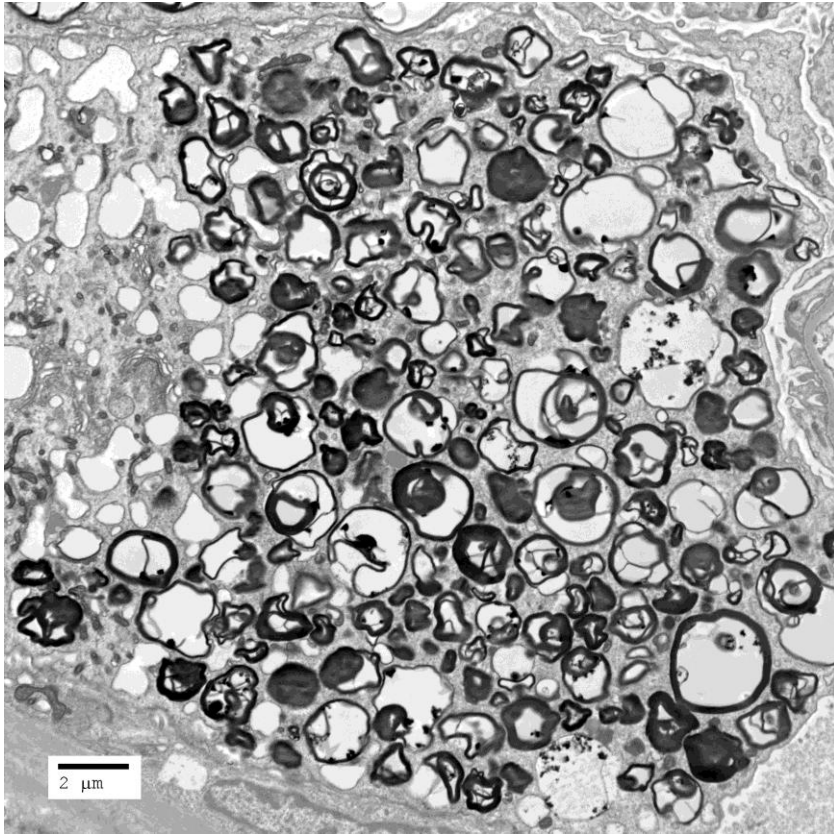
Fig 3.2. Electron microscopy showing concentric lamellar inclusion bodies called Zebra bodies (Images courtesy of Jackie Lewin, Head Clinical scientist, EM Unit, UCL Medical School, Royal Free Campus).

Age has been shown to correlate with composite glomerular pathology scores but not tubulointerstial scores or glycolipid inclusions[55]. Glomerular pathology and tubulointerstial scores were also higher in patients with undetectable plasma α-Gal activity (<1%) but glycolipid scores were similar regardless of plasma α-Gal activity[55]. In males glomerular segmental or global sclerosis is the only significant pathological association with proteinuria in early AFD renal disease[115]. Tondel et al described renal lesions in children with normal GFR values but with slightly elevated urine albumin but could not correlate GB3 inclusion scores with age[138]. The International Study Group of Fabry Nephropathy also reported no relationship between light microscopy scoring system for renal lesions and renal fucntion[144]. More recently Najafian et al has shown in children (median age 12 years) the progressive accumulation of GB3 deposits in podocytes with normal GFR and absent or low-grade proteinuria. Using quantitative stereological electron microscopy methods, progressive accumulation of podocytes GB3 inclusions can be correlated with increasing age[145]. The progressive accumulation of GB3 in podocytes is thought to be because podocytes are terminally differentiated and proliferate poorly in response to injury or loss[146].

3.3   Renal pathogenesis in AFD

Ischaemic tissue damage[115, 135, 137] from microvascular endothelial disease and/or necrosis of vascular smooth muscles and/or pericyte injury[147-149] maybe the cause of these non-specific renal pathological findings. Injury from GB3 overloaded podocytes maybe another mechanism of glomerulosclerosis[135, 150] and membranofibrillary deposits seen in cytoplasm[115, 116, 122] are remnants of GB3 inclusions of dead cells. Mesangial cell necrosis[115], direct toxic injury to tubular cells[135] and glomerular proteinuria causing tubular injury as it passes down the lumen[151] are thought to play roles in progressive renal dysfunction in AFD.

3.4   Rationale for study

i) Lipid laden distal tubular epithelial cells desquamate and can be detected in urinary sediment[152] indicating possible renal tubular damage. These cells

containing glycosphingolipids (mainly GB3) in the urine are visualised as oval fat bodies with light microscopy and as maltese crosses when viewed by polarised light[136, 140].

ii) Currently non-invasive renal investigations in AFD and indications to start ERT do not account for renal tubular damage.

iii) GB3 may be causing direct or indirect renal damage which eventually leads to renal scarring and fibrosis.

iv) Serum Cr and estimation of GFR based on serum Cr may be insufficient to detect early renal dysfunction.

3.5   Aims

i) To investigate urine α-galactosidase A activity in AFD patients and healthy controls

ii) To investigate if lysosomal enzymuria is an earlier marker of renal dysfunction or correlates with end organ damage in AFD

iii) To determine if other markers of tubular dysfunction are an earlier marker of renal involvement in AFD

iv) To determine if urine markers of renal fibrosis are elevated in AFD

3.6   Hypothesis

Urine biomarkers of glomerular or tubular dysfunction or renal fibrosis can be detected prior to clinically apparent renal disease.

3.7   Rationale for urine proteins tested

3.7.1   α-Galactosidase A

Human α-Galactosidase A (α-Gal) is a lysosomal glycohydrolase that catalyzes the hydrolysis of terminal α-galactosyl residues from glycoproteins and glycolipids[153]. Human α-Gal is a homodimeric glycoprotein[31] with a molecular

weight of 110 kDa. It is encoded by the GLA gene localised to the chromosomal region Xq22.1[3, 4, 154, 155]. Reduced or absent activity of this enzyme results in a X-linked lysosomal storage disorder called AFD[27, 153]. This leads to the accumulation of galactosylated substrates, primarily GB3.

Christensen et al[156] described the distribution of α-Gal in the normal kidney. In the renal cortex α-Gal is predominantly present in the proximal convoluted tubules, thick ascending limb of the Loop of Henle and in interstitial cells, and in the renal medulla present in the collecting ducts and thin limb of the loop of Henle. α-Gal was not demonstrated in the glomerular cells including podocytes, parietal epithelial cells of Bowman's capsule and vascular endothelial cells. This was an unexpected finding as there is GB3 accumulation in these cell types in AFD patients, suggesting that there should be normal α-Gal activity and α-Gal present in these cell types in normal humans. The authors explained this finding a by saying that enzyme analysis may have been below the detection limit of their method used. The authors also showed increased urine α-GAL activity in AFD mice, after ERT infusions and concluded there was glomerular filtration of the recombinant enzyme[156] but due to its relatively large molecular weight α-Gal is more likely to be secreted from lysosomes of tubular cells[157]. Immunolabelled recombinant enzyme accumulated in renal proximal tubules, interstitial cells and glomerular podocytes but not in renal vascular endothelial cells.

To date there are few studies measuring urine α-GAL activity. Urine α-GAL activity has been shown to remain stable at 4°C, 25°C and 37°C over a 4 hour period and at -70°C over 1 month[158]. There is a trend in a decrease of the fractional excretion of α-GAL (non-significant) and a decrease in urine α-GAL activity in urine (significant) as age increases, but without any correlation to inulin clearance[159] (gold standard for estimating true GFR). Also the fractional excretion of α-GAL decreases in pregnancy[160] and in sickle cell patients[161] . There is no difference in urine α-GAL activity in diabetics compared to non-diabetics[162] and between bladder cancer patients and matched controls[163], but urine α-GAL activity increases in severely malnourished children[164] and in preeclampsia[165].

In AFD there have been 3 reported studies of urine α-GAL. Berty et al measured fractional excretion of α-GAL in an AFD male with a functioning renal transplant in relation to induced acute acidosis and alkalosis[166]. Hamers et al

demonstrated that in AFD females, urine α-GAL activity was within the normal range[167] while Kitigawa et al showed AFD males and females had significantly lower urine α-GAL activity than controls[168].

3.7.2   β-Hexominidase

β-Hexominidase (β-Hex) is a 130 kDa lysosomal enzyme which hydrolyses the terminal non-reducing N-acetyl-D-hexosamine residues in N-acetyl-beta-D-hexosaminides. It is also known as β-N-acetylhexosaminidase or N-acetyl-beta-glucosaminidase (NAG). Functional β-Hex is dimeric in structure and made up of 3 isoenzymes with either α or β subunits. β-Hex A (α/β heterodimer) is able to cleave GM2 gangliosides, GA2 gangliosides, globosides, and hexosamine oligosaccharides and β-Hex B (β/β homodimer) is able to cleave all the above except for GM2 gangliosides. Deficiency of β-Hex A causes Tay-Sachs disease and deficiencies of both β-Hex A and B causes Sandhoff disease.

β-Hex is present in tissues other than the kidney and is rapidly cleared by the liver as shown in rat experiments[169, 170]. β-Hex is not normally filtered at the glomeruli (MW 130 kDa) and is secreted from lysosomes of tubular cells[171]. Immunohistochemistry has located β-Hex to the renal proximal tubules[172]. Urinary excretion of β-Hex is relatively constant with minimal diurnal changes[173, 174], and is stable against changes of temperature and pH. In urine of healthy humans, β-Hex is present in similar amounts throughout adulthood[173] with isoenzyme A to isoenzyme B ratio of 4:1 (kidney) to 10:1(urine)[175]. The fractional excretion of β-Hex decreases in pregnancy[160] and increases with age[159], and this is related to either reduced tubular reabsorption or increased tubular secretion as changes were independent of glomerular filtration measured by inulin clearance. Increase in urinary β-Hex activity indicates damage to tubular cells or interstitial renal damage[171, 173, 176-178] but can also reflect increased lysosomal activity without cellular damage.

Urinary β-Hex activity has been used as a non-invasive, sensitive and reliable indicator of renal damage in a variety of conditions; vesicoureteric reflux[179], diabetic nephropathy[180-182], polycystic kidney disease[183], Henoch-Schonlein purpura[184], hypertensive nephropathy[185, 186], pre-eclampsia[161, 165], obstructive uropathy[187], hypercalciuria[188], nephrotoxic drugs related nephropathy[189-192] and protein energy malnutrition[164].

To our knowledge only two studies have investigated urine β-Hex in AFD patients. First was in an AFD male with a functioning renal transplant demonstrating changes in urinary lysosomal enzymuria with induced acid – base changes[166] and by Rietra et al, showing that urine β-Hex:α-Gal ratios are raised in AFD males and females[193].

3.7.3 Chitotriosidase

Chitotriosidase is a 39-50 kDa hydrolase and member of a family of glycosylhydrolases called chitinases and has the capability to hydrolyse chitin. It is mainly expressed by activated macrophages[194] and neutrophils[195]. This macrophage derived enzyme had been discovered to be grossly elevated in untreated patients with Gaucher's disease[196] and modestly increased in other inherited lysosomal storage disorders, especially sphingolipidoses such as Niemann Pick[197] GM1-gangliosidosis and Krabbe's disease[198, 199]. Its level decreases with successful ERT[200]. It is therefore a very useful biomarker for Gaucher's disease[201, 202] and measurement of chitotriosidase activity is a reliable and easy way of monitoring therapy. Increased chitotriosidase activity has been observed in serum of patients with atherosclerosis[203-205], β-thalassaemia[206] and acute Plasmodium falciparum malaria[207]. More recently chitotriosidase has been thought to be important during immunological response and inflammatory processes[208-213].

Urinary chitotriosidase is elevated in Gaucher patients and corrects with treatment[214] and elevated in neonates with fungal and bacterial infection reflecting phagocyte activity[215]. In AFD two studies have shown that plasma chitotriosidase levels are elevated in male hemizygotes but not females, and in male hemizygotes elevated plasma chitotriosidase levels reduce with ERT[216, 217].

3.7.4 Retinol binding protein

Retinol binding protein (RBP) is a 21kDa plasma protein of the lipocalin superfamily first described by Kanai et al in 1968[218]. RBP is synthesized in the liver and requires the binding of retinol to trigger its secretion[219]. In the plasma RBP binds to the larger protein transthyretin with a resulting molecular mass of 80 kDa preventing loss of RBP by glomerular filtration[220]. Unbound RBP is filtered at the glomeruli, reabsorbed in the proximal tubules after binding to

megalin[221] where it is catabolised[220]. RBP production is relatively constant, and the protein is very stable across whole range of urinary pH[222]. RBP serves as an important molecule in regulation and mobilisation of vitamin A from the liver to cell surfaces.

Plasma levels of RBP are reduced in liver disease[223, 224], inadequate dietary vitamin A intake[225-228] and inflammation[229, 230], and plasma RBP is increased in CKD[223, 224, 231-234]. Urine RBP levels are increased in renal insufficiency[231, 232, 235] and has been shown to be a urinary marker of tubular dysfunction[231, 236-238]. More specifically it is increased in tubulo-interstitial nephropathy[239], in heart transplant patients which predicted increased risk of renal failure over 5 years follow up[240] and in renal transplants which predicted graft dysfunction [241, 242].

More recently plasma RBP levels have been reported to be elevated in insulin resistant subjects and in subjects with obesity and/or type 2 diabetes[243-245] due to upregulation of RBP in adipocytes[243] but may more likely be related to impaired glomerular filtration manifested as decreased GFR[234, 246, 247] or microalbuminuria[248] rather than type 2 diabetes.

To our knowledge in the AFD population, only 1 author has shown an increase in urine RBP by western blotting method (2 out 12 AFD patients)[156].

3.7.5   Transforming growth factor-β1

Transforming growth factor-β1 (TGF-β1) is a 25kDa, disulfide-linked, non-glycosylated homodimer, secreted by most cell types, generally in a latent form, requiring activation before exerting biological effect. It is an important endogenous mediator of growth, maintenance and repair processes in the developing of the embryo, neonate and adult. Major activities are to inhibit the proliferation of most cells but can stimulate the growth of some mesenchymal cells, exert immunosuppressive effects and enhance the formation of extracellular matrix.

Increased levels of TGF-β1 has been described in animal models of renal disease, especially associated with renal scarring[249-251]. TGF-β1 is the principal mediator of diabetic renal complications and is important in the development of hypertrophy and accumulation of extracellular matrix[252, 253]. Neutralising TGF-β1 has been to shown to reduce renal scarring and diminish the loss of renal function[254-256].

Urinary TGF-β1 levels were shown to be raised in patients with diabetes[257-263], focal glomerular sclerosis[264, 265], IgA nephropathy[261, 265], systemic lupus erythematosus[261], membranous nephropathy[266] and crescenteric glomerulonephritis[267]. Sato et al also showed urinary TGF-β1 was higher in patients with increased mesangial expansion and higher HbA1c, but did not correlate with serum TGF-β1, serum Cr or tubular proteinuria[259]. This probably reflects increased urinary TGF-β1 was due to increased production in the kidney of TGF-β1 rather than increased filtration or reduced tubular reabsorption. Higher urinary TGF-β1 levels have be shown by other authors to be associated with mesangial proliferation[261, 265]. In IgA nephropathy high urinary TGF-β1 levels reduced after treatment with steroid therapy[268] and higher baseline urinary TGF-β1 in crescenteric glomerulonephritis was associated with a poorer renal outcome[267]. Therefore urinary TGF-β1 levels may be a means of predicting or monitoring treatment response.

3.7.6 Monocyte chemotactic protein 1

Monocyte chemotactic protein 1 (MCP-1/CCL2) is a 13kDa[269], potent monocyte attractant, also known as monocyte chemotactic and activating factor (MCAF), and is a member of the CC subgroup of chemokine superfamily[270]. MCP-1 is produced by many cell types including epithelial, endothelial, smooth muscle, fibroblasts, astrocytes, monocytes and microglia cells[271-273], however the major source of MCP-1 are monocytes and macrophages[274, 275]. MCP-1 is thought to be an important player in the inflammatory processes. Blocking MCP-1 can suppress models of endotoxaemia, delayed-type sensitivity reactions, and inflammatory arthritis, and overexpression enhances the recruitment of monocytes and lymphocytes in vivo[276-279]. Elevated MCP-1 levels in human have been associated with sepsis[280], Crohn's disease[281], lupus nephritis[282], amyotrophic lateral sclerosis[283], multiple sclerosis[284], rheumatoid arthritis[285] and artherosclerosis[286]. It is also upregulated in several cancers including gastric carcinoma[287], oesophageal squamous cell carcinoma[288], malignant glioma[289] and ovarian[290], bladder[291] and breast cancers[292].

In the kidney, MCP-1 is produced by a variety of mesenchymal cells including glomerular cells[293-295]. MCP-1 has the potential to drive the process of renal fibrosis indirectly by macrophage recruitment and also via direct induction of

fibrotic response in glomerular mesangial cells[296]. Over expression of MCP-1 has been found in various proteinuric conditions, such as diabetic, hypertensive and membranous nephropathies[297-299]. Urinary MCP-1 has been shown to be significantly higher in lupus nephritis[300-304], diabetic nephropathy[305-310], IgA nephropathy[307, 311, 312], inflammatory glomerulopathies[313], acute renal allograft rejection[314, 315], urolithiasis[316, 317], renal vasculitis[318, 319] and crescenteric glomuerulonephritis[320].

3.8   Materials and methods

Patients were recruited from the lysosomal storage disorders unit at the Royal Free Hospital from Nov 2006 till Feb 2009. Study was approved by the NHS Research Ethics Committee (REC reference 07/Q0501/81). Patients recruited had to give informed and signed consent and have a documented mutation in the α-Galactosidase A gene. Controls were recruited from healthy volunteers working at the Royal Free Hospital Haematology department. They provided random morning urine samples only.

3.8.1   Sample collection and storage

Two approximately 10-15 ml random morning urine samples were collected. The first sample was analysed by the pathology department of the Royal Free Hospital for urine albumin, urine protein and urine Cr as part of the patients' routine clinical assessment. The second sample was aliquoted into 1ml containers and frozen at -80˚C and stored for future analysis of various urine proteins. When analysis was ready to be carried out, 1ml aliquots were defrosted at room temperature and analysed within 1 hour. Samples were not refrozen and stored again once defrosted.

3.8.2   Urine protein analysis

The following urine proteins analysed were carried out by me at the haematology research laboratory at the Royal Free hospital.

3.8.2.1 <u>α-Galactosidase A (α-Gal)</u>

<u>Principle</u>[321, 322]:

4-methylumbelliferyl-α-D-galactopyranoside

⇓   α-Galactosidase A at Acid pH 4.8

4-methylumbelliferone (4MU) + Galactose

Adding an alkaline buffer stops the enzyme reaction and causes 4-methylumbelliferone (4MU) to fluorescence at different wavelength from the unhydrolysed substrate. α-Galactosidase A contributes 95% of total activity in leucocytes and α-Galactosidase B may contribute significantly to total α-galactosidase activity in other cells and is inhibited by N-acetyl-D-galactosamine. A relatively large sample is needed as there is decreased fluorescence due to quenching especially in haemolysed or jaundiced samples and increased fluorescence due to presence of other fluorescence material e.g. drugs. This is corrected by use of Substrate blank, Standard and Standard Blank.

<u>Reagents:</u>

1. **Buffer: 0.5M acetate pH 4.8**

   Solution X - Prepare a 0.5M solution in distilled water of sodium acetate.

   Solution Y - Prepare a 0.5M solution in distilled water of glacial acetic acid

   Mix 56.6ml of Solution X and 43.4ml of Solution Y to give 0.5M acetate pH 4.8.

2. **Substrate: 4-methylumbelliferyl-α-D-galactopyranoside 250mg (MWt 338) Sigma Aldrich.**

   Solution A - Dissolve 250mg substrate in 74ml of 0.5M acetate buffer by warming to 80°C (which can be stored indefinitely at -20°C but will need warming to dissolve when removed from the freezer). Store in 11.3ml aliquots.

3. **Inhibitor: N-acetyl-D-galactosamine 500mg (MWt 221.2) Sigma Aldrich.**

4. **Substrate+Inhibitor:**

Solution B is the substrate/inhibitor working solution. Dissolve 500mg of inhibitor in 11.3ml of substrate solution A (Can be stored at -20°C indefinitely but will need warming to dissolve when removed from the freezer).

5. **Standard: 4-methylumbelliferone (MWt 176) Sigma Aldrich.**

Stock standard solution - Dissolve 176mg in 1ml methanol then make up to 1 litre with distilled water

Working standard - Dilute 100μl stock standard solution in 19.9ml distilled water to give 1nmol per 200ul.

Both stock and working solutions are stable indefinitely when stored at -20°C.

6. **Stopping Reagent: 1M glycine buffer pH 10.4.**

Solution C - Dissolve 75g glycine and 58g sodium chloride in 1 litre of distilled water.

Solution D - 1M sodium hydroxide solution.

Add 55.7ml of solution C to 44.3ml solution D to give 1M glycine buffer pH 10.4.

Methodology:

A 24 well microtitre plate is divided into 4 rows of 5 wells as in Fig 3.3. Each plate can test 4 different samples. Into the 4th and 5th wells (samples in duplicate) of each row, pipette 100μl sample urine and 100ul solution B (substrate + inhibitor solution) and mix well. Into the 3rd well (Substrate Blank) pipette 100μl solution B. Incubate the microtitre plate at 37°C for 2 hours. Add 1.0ml stopping reagent into wells 3, 4 and 5 and mix. Add 100μl sample urine to well 1 (Standard), well 2 (Standard Blank) and well 3 (Substrate Blank). Pipette 200μl working standard and 0.9ml stopping reagent into well 1. Pippette 200μl distilled water and 0.9ml stopping reagent into well 2. Mix well. The total volume of each well is 1.2ml. Fluorescence is read using a flurometer from Fluostar Galaxy, BMG Lab Technologies, using an excitation wavelength of 360nm and emission wavelength 460nm.

	1	2	3	4	5
Empty	Standard 1	Standard Blank 1	Substrate Blank 1	S1	S1
Empty	Standard 2	Standard Blank 2	Substrate Blank 2	S2	S2
Empty	Standard 3	Standard Blank 3	Substrate Blank 3	S3	S3
Empty	Standard 4	Standard Blank 4	Substrate Blank 4	S4	S4

Fig 3.3. Schematic layout of 24 well microtitre plate for α-Gal analysis.

Calculating activity:

For each sample, Standard – Standard blank = fluorescence of 1nmol 4MU.

Average the test reading for duplicate of samples = T

Standard = St

Standard Blank = StB

Substrate Blank for each sample = SB

Activity of α-Galactosidase A

$$= \frac{(T - SB)}{(St - StB)} \times \frac{60}{120} \times \frac{1000}{100} \quad (\text{nmol/hr/ml urine})$$

[2 hr correction]   [conversion to per ml urine]

If the sample has to be diluted multiply the reading by the dilution factor before subtracting the blank reading. These sample values are then standardised for urine Cr.

3.8.2.2 β-Hexosaminidase (β-Hex)

Principle[323, 324]:

4-methylumbelliferyl-N-acetyl-β-D-glucosaminide

$$\Downarrow \quad \text{β-N-acetylhexosaminidase at Acid pH 4.6}$$

4-methylumbelliferone (4MU) + N-acetyl-D-hexosamine

Adding an alkaline buffer stops the enzyme reaction and causes 4-methylumbelliferone (4MU) to fluorescence at different wavelength from the unhydrolysed substrate. A relatively large sample is needed as there is decreased fluorescence due to quenching especially in haemolysed or jaundiced samples and increased fluorescence due to presence of other fluorescence material e.g. drugs. This is corrected by use of Substrate blank, Standard and Standard Blank.

Reagents:

1. **Buffer: 0.1M Citrate Phosphate Buffer (McIlvaine Solution) pH 4.5**
   Prepare a 0.1M solution in distilled water of Citric Acid (Solution X). Prepare a 0.1M solution in distilled water of $Na_2HPO_4.2H_2O$ (Solution Y). Mix 55ml of solution X and 45 ml of solution Y to give 0.1M Citrate Phosphate Buffer pH 4.2.

2. **Substrate: 0.1mM 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide (MWt 379.4) Sigma Aldrich**
   Dissolve 3.8mg substrate in 100 ml in 0.1M Citrate Phosphate Buffer (McIlvaine Solution) pH 4.5 by warming to 80^oC. Store at -20^oC in 5ml aliquots.

3. **Standard: 4-methylumbelliferone (MWt 176) Sigma Aldrich.**
   Stock standard solution - Dissolve 176mg in 1ml methanol then make up to 1 litre with distilled water
   Working standard - Dilute 100μl stock standard solution in 19.9ml distilled water to give 1nmol per 200μl.
   Both stock and working solutions are stable indefinitely when stored at -20^oC.

4. **Stopping Reagent: 1M glycine buffer pH 10.4.**

   Solution C - Dissolve 75g glycine and 58g sodium chloride in 1 litre of distilled water.

   Solution D - 1M sodium hydroxide solution.

   Add 55.7ml of solution C to 44.3ml solution D to give 1M glycine buffer pH 10.4.

Methodology:

A 24 well microtitre plate is divided into 4 rows of 5 wells as in Fig 3.4. Each plate can test 4 different samples. Into the 4th and 5th wells (samples in duplicate) of each row, pipette 100μl sample urine and 100μl substrate solution and mix well. Into the 3rd well (Substrate Blank) pipette 100μl substrate solution only. Incubate microtitre plate at 37°C for 15 minutes. Add 1.0ml stopping reagent into wells 3, 4 and 5 and mix. Add 100μl sample urine to well 1 (Standard), well 2 (Standard Blank) and well 3 (Substrate Blank). Pipette 200μl working standard and 0.9ml stopping reagent into well 1. Pipette 200μl distilled water and 0.9ml stopping reagent into well 2. Mix well. Total volume of each well is 1.2ml. Fluorescence is read using a flurometer from Fluostar Galaxy, BMG Lab Technologies, using an excitation wavelength of 360nm and emission wavelength 460nm.

	1	2	3	4	5
Empty	Standard 1	Standard Blank 1	Substrate Blank 1	S1	S1
Empty	Standard 2	Standard Blank 2	Substrate Blank 2	S2	S2
Empty	Standard 3	Standard Blank 3	Substrate Blank 3	S3	S3
Empty	Standard 4	Standard Blank 4	Substrate Blank 4	S4	S4

Fig 3.4. Schematic layout of 24 well microtitre plate for β-Hex analysis

Calculating activity:

For each sample, Standard − Standard blank = fluorescence of 1nmol 4-MU.

Average the test reading for duplicate of samples = T

Standard = St

Standard Blank = StB

Substrate Blank for each sample = SB

Activity of β-Hexosaminidase

$$= \frac{(T - SB)}{(St - StB)} \times \frac{60}{15} \times \frac{1000}{100} \quad (\text{nmol/hr/ml urine})$$

[15 mins correction]   [conversion to per ml urine]

If the sample has to be diluted multiply the reading by the dilution factor before subtracting the blank reading. These sample values are then standardised for urine Cr.

3.8.2.3 <u>Chitotriosidase</u>

<u>Principle</u>[196]:

$$\text{4-methylumbelliferyl-}\beta\text{-D-N,N}^i\text{,N}^{ii}\text{-triacetylchitotriose}$$

⇓ Chitotriosidase at pH 5.2
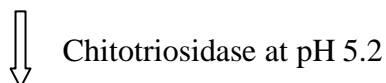
4-methylumbelliferone (4MU) + Chitotrioside

Adding an alkaline buffer stops the enzyme reaction and causes 4-methylumbelliferone (4MU) to fluorescence at different wavelength from the unhydrolysed substrate.

<u>Reagents:</u>

1. **Buffer: McIlvaine Citrate Phosphate Buffer 0.15M pH 5.2**

   Prepare a 0.1M solution in distilled water of Citric Acid (Solution X). Prepare a 0.2M solution in distilled water of $Na_2HPO_4.2H_2O$ (Solution Y). Mix 46.8ml of solution X and 53.1 ml of solution Y to give 0.15M Citrate Phosphate Buffer pH 5.2.

2. **Substrate: 4-methylumbelliferyl-chitotrioside 1mg (MWt 786) Sigma Aldrich.**

   Dissolve 1mg of substrate in 57.8ml of 0.15M citrate phosphate buffer pH 5.2 (McIlvaine buffer) by warming to 80°C. Store at -20°C in 5ml aliquots.

3. **Standard: 4-methylumbelliferone (MWt 176) Sigma Aldrich.**

   Dissolve 176mg in 1 litre of distilled water by warming to 80°C – stock standard solution. Dilute 100μl stock standard solution in 19.9ml distilled water to give working standard – 1nmol per 200μl. Both stock and working solutions are stable indefinitely when stored at -20°C.

4. **Stopping Reagent: 1M glycine buffer pH 10.4.**

   Solution C - Dissolve 75g glycine and 58g sodium chloride in 1 litre of distilled water.

   Solution D - 1M sodium hydroxide solution.

   Add 55.7ml of solution C to 44.3ml solution D to give 1M glycine buffer pH 10.4.

Methodology:

A 24 well microtitre plate is divided into 4 rows of 6 wells as in Fig 3.5. Each plate can test 4 different samples. In the 3rd and 4th wells add 5μl deionised water with 100μl substrate. In the 5th and 6th wells add 5μl sample with 100μl substrate. Incubate microtitre plate at 37°C for 1 hour. Add 1.0ml stopping reagent into wells 3, 4, 5 and 6. In the 1st and 2nd wells add 200μl of standard and 0.9ml of stopping reagent. Fluorescence is read using a flurometer from Fluostar Galaxy, BMG Lab Technologies, using an excitation wavelength of 360nm and emission wavelength 460nm.

1	2	3	4	5	6
Standard 1	Standard 1	Substrate Blank 1	Substrate Blank 1	S1	S1
Standard 2	Standard 2	Substrate Blank 2	Substrate Blank 2	S2	S2
Standard 3	Standard 3	Substrate Blank 3	Substrate Blank 3	S3	S3
Standard 4	Standard 4	Substrate Blank 4	Substrate Blank 4	S4	S4

Fig 3.5. Schematic layout of 24 well microtitre plate for chitotriosidase analysis.

Calculating activity:

For each sample Standard – Substrate blank = fluorescence of 1nmol 4MU.

Average the test reading for duplicate of samples = T

Average of Standard = St

Average of Substrate Blank = SB

Activity of Chitotriosidase

$$= \frac{(T - SB)}{(St - SB)} \times \frac{1.105}{1.100} \times \frac{1000}{5} \quad (\text{nmol/hr/ml urine})$$

[volume correction]   [conversion to per ml urine]

If the sample has to be diluted multiply the reading by the dilution factor before subtracting the blank reading. These sample values are then standardised for urine Cr.

3.8.2.4 <u>Retinol Binding Protein (RBP)</u>

<u>Principle:</u>

A double antibody sandwich ELISA (Enzyme-Linked Immunosorbent Assay) from Immunology Consultants Laboratory, for quantitative determination of RBP in biological samples, was used. In the first incubation step, RBP in samples react with the anti-RBP antibodies which have been adsorbed to the surface of polystyrene microtitre wells. After removal of unbound sample proteins by washing, anti RBP antibodies was conjugated with horseradish peroxidise that had been added. These enzyme-labelled antibodies form complexes with previously bound sample RBP. After another washing step, the enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate. The quantity of bound enzyme varies directly with the concentration of RBP. A dose response curve of absorbance unit (optical density at 450nm) versus concentration is generated using values obtained from standard. RBP present in the patient samples is determined directly from this curve.

<u>Reagents:</u>

1. **Diluent:**

    50ml of 5x concentrated phosphate buffered saline (PBS) solution containing bovine serum albumin, 0.25% Tween, and 0.1% Proclin300 as a preservative. Diluted 1:5 with deionised water, stored at 4-8˚C for up to 1 week.

2. **Wash solution concentrate:**

    50ml of 10x concentrated PBS solution containing 0.5% Tween. Warm concentrate to 30-35˚C before dilution. Dilute 1:10 with deionised water, stored 4-8˚C for up to 1 week.

3. **Enzyme-Antibody conjugate:**

    200μl of 100x concentrated affinity purified anti-Human RBP antibody conjugated with horseradish peroxidase in a stabilising buffer. Dilute 100μl of 100x concentrated enzyme-antibody conjugate with 10ml of diluent, stable for 1 day.

4. **Chromogen-substrate solution:**

12ml of 3,3',5,5'-tetramethbenzidine (TMB) and hydrogen peroxide in citric acid buffer at pH 3.3

5. **Stop Solution:**

12ml 0.3M sulphuric acid

6. **Anti-Human RBP ELISA microplate:**

96 microwell plate. Each well coated with affinity purified anti-Human RBP

7. **Human RBP calibrator:**

Lyophilized Human RBP. Add 1.0 ml of deionised water to the Human RBP calibrator, mix gently until dissolved. Concentration now is at 4.25μg/ml. This can be frozen and stored at -20˚C in aliquots. Human RBP standards need to be prepared immediately prior to use.

Methodology:

1. Dilute urine samples 1:20 with diluent.
2. Prepare standards as follows

Standard	Concentration (ng/ml)	Volume of calibrator or standard added	Volume of Diluent added
1	250	50 μl of calibrator	800 μl
2	125	250 μl of Standard 1	250 μl
3	62.5	250 μl of Standard 2	250 μl
4	31.25	250 μl of Standard 3	250 μl
5	15.625	250 μl of Standard 4	250 μl
6	7.8125	250 μl of Standard 5	250 μl
7	3.90625	250 μl of Standard 6	250

3. Set up RBP ELISA microplate as follows [Diluent (D), Standard (S) and urine samples (U)], adding 100ul of diluent, standard or diluted urine sample to corresponding well, in duplicate.

D	D	U1	U1	U9	U9	U17	U17	U25	U25	U33	U33
S1	S1	U2	U2	U10	U10	U18	U18	U26	U26	U34	U34
S2	S2	U3	U3	U11	U11	U19	U19	U27	U27	U35	U35
S3	S3	U4	U4	U12	U12	U20	U20	U28	U28	U36	U36
S4	S4	U5	U5	U13	U13	U21	U21	U29	U29	U37	U37
S5	S5	U6	U6	U14	U14	U22	U22	U30	U30	U38	U38
S6	S6	U7	U7	U15	U15	U23	U23	U31	U31	U39	U39
S7	S7	U8	U8	U16	U16	U24	U24	U32	U32	U40	U40

4. Incubate at room temperature (22˚C) for 60 minutes (± 2 minutes).

5. Aspirate contents of well and wash with wash buffer four times.

6. Pipette 100μl of diluted enzyme-antibody conjugate to each well.

7. Incubate in the dark at room temperature for 10 minutes (± 2 minutes).

8. Aspirate contents of well and wash with wash buffer four times.

9. Pipette 100μl of TMB substrate into each well.

10. Incubate in the dark at room temperature for exactly 10 minutes.

11. Add 100μl of stop solution into each well.

12. Determine the absorbance at 450 nm.

Calculating Results:

The absorbance for each sample is calculated by subtracting the average absorbance of the diluents (background value) from the average of duplicate samples.

Absorbance Sample 1 = [Average U1 – Average D]

Using results from the observed standards, a Standard Curve is constructed. Sample values are interpolated from the standard curve and multiplied by 20 to correct for dilution of original sample. These sample values are then standardised for urine Cr.

3.8.2.5 <u>Transforming Growth Factor β1 (TGF-β1)</u>

<u>Principle:</u>

A quantitative sandwich enzyme immunoassay technique from R&D systems is used. A monoclonal antibody specific to TGF-β1 has been pre-coated onto a microplate. Standards and samples are pipette into wells and any TGF-β1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TGF-β1 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops proportional to the amount of TGF-β1 bound in the initial step. This colour development is stopped and the intensity of the colour is measured.

<u>Reagents:</u>

1. **Calibrator Diluent (RD5-53) concentrate:**
   21ml/vial of concentrated buffered protein base with preservatives. Dilute 1:4 with deionised water. Maybe stored for up to 1 month at 2-8˚C.

2. **Assay Diluent (RD1-21):**
   12.5ml/vial of a buffered protein solution with preservatives. Maybe stored for up to 1 month at 2-8˚C.

3. **Wash Buffer concentrate:**
   50ml/vial of 25x concentrated solution of buffered surfactant with preservatives. Warm to room temperature and mix gently with deionised water at a dilution of 1:25. Maybe stored for up to 1 month at 2-8˚C.

4. **TGF-β1 conjugate:**
   12.5ml/vial of polyclonal antibody against TGF-β1 conjugated to horseradish peroxidase with preservatives. Maybe stored for up to 1 month at 2-8˚C.

5. **Colour Reagent A:**
   12.5ml/vial of stabilised hydrogen peroxide. Maybe stored for up to 1 month at 2-8˚C.

6. **Colour Reagent B:**
   12.5ml/vial of stabilised chromogen (tetramethylbenzidine). Maybe stored for up to 1 month at 2-8˚C.

7. **Stop Solution:**

   23ml/vial of a diluted hydrochloric acid solution. Maybe stored for up to 1 month at 2-8˚C.

8. **TGF-β1 microplate:**

   96 well polystyrene microplate coated with a monoclonal antibody specific for TGF-β1. May be stored for up to 1 month at 2-8˚C.

9. **TGF-β1 standard:**

   4.0ng/vial of recombinant human TGF-β1 in a buffered protein base with preservatives; lyophilized. Reconstitute with 2.0 ml of diluted calibrator diluent RD5-53, giving a concentration of 2000pg/ml. Unused Standard to be discarded.

10. **1N HCl:**

    9.8ml of 10.2 N HCL (32%) added to 90.2ml deionised water.

11. **1.2N NaOH/0.5M HEPES:**

    20G of NaOH + 50ml deionised water = 10N NaOH

    12ml of 10N NaOH + 75ml deionised water + 11.9g HEPES making final volume to 100ml with deionised water.

Methodology:

1. Activate latent TGF-β1 in urine samples. To 100μl urine sample add 20μl of 1N HCL. Incubate 10 minutes at room temperature. Neutralise by adding 20μl of 1.2N NaOH/0.5M HEPES.

2. Prepare standards as follows

Standard	Concentration (pg/ml)	Volume of calibrator or standard added	Volume of Diluent (RD5-53) added
1	1000	200μl of reconstituted TGF-β1 standard	0μl
2	500	200μl of Standard 1	200μl
3	250	200μl of Standard 2	200μl
4	125	200μl of Standard 3	200μl
5	62.5	200μl of Standard 4	200μl
6	31.25	200μl of Standard 5	200μl
7	15.625	200μl of Standard 6	200μl

3. Set up TGF-β1 ELISA microplate as follows by adding 50μl of Assay diluents RD1-21 to each well, followed by adding 50μl of diluent, standard or urine sample to corresponding well, in duplicate [Diluent (D), Standard (S) and urine samples (U)].

D	D	U1	U1	U9	U9	U17	U17	U25	U25	U33	U33
S1	S1	U2	U2	U10	U10	U18	U18	U26	U26	U34	U34
S2	S2	U3	U3	U11	U11	U19	U19	U27	U27	U35	U35
S3	S3	U4	U4	U12	U12	U20	U20	U28	U28	U36	U36
S4	S4	U5	U5	U13	U13	U21	U21	U29	U29	U37	U37
S5	S5	U6	U6	U14	U14	U22	U22	U30	U30	U38	U38
S6	S6	U7	U7	U15	U15	U23	U23	U31	U31	U39	U39
S7	S7	U8	U8	U16	U16	U24	U24	U32	U32	U40	U40

4. Incubate at room temperature (22˚C) for 2 hours.
5. Aspirate contents of well and wash with wash buffer four times.
6. Add 100μl of TGF-β1 Conjugate to each well.
7. Incubate at room temperature for 2 hours.
8. Aspirate contents of well and wash with wash buffer four times.
9. Mix Colour Reagents A and B together in equal volumes to make substrate solution and use within 15 minutes.
10. Add 100μl of substrate solution to each well.
11. Incubate in the dark at room temperature for 30 minutes.
12. Add 100μl stop solution into each well.
13. Determine the optical density within 30 minutes at 450 nm, with wavelength correction at 540nm or 570nm.

Calculating Results:

The optical density for each sample is calculated by subtracting the average optical density of the diluents (background value) from the average of duplicate samples.

Absorbance Sample 1 = [Average U1 – Average D]

Using results from the observed standards, a Standard Curve is constructed. Sample values are interpolated from the standard curve and multiplied by 1.4 to correct for dilution of original sample (activation step). These sample values are then standardised for urine Cr.

3.8.2.6 Monocyte Chemoattractant Protein-1 (MCP-1)

Principle:

A quantitative sandwich enzyme immunoassay technique from R & D systems is used. A monoclonal antibody specific to MCP-1 has been pre-coated onto a microplate. Standards and samples are pipetted into wells and any MCP-1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for MCP-1 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops proportional to the amount of MCP-1 bound in the initial step. This colour development is stopped and the intensity of the colour is measured.

Reagents:

1. **Calibrator Diluent (RD5L) concentrate:**

   21ml/vial of concentrated buffered protein base with preservatives. Dilute 1:5 with deionised water. Maybe stored for up to 1 month at 2-8˚C.

2. **Wash Buffer concentrate:**

   21ml/vial of 25x concentrated solution of buffered surfactant with preservatives. Warm to room temperature and mix gently with deionised water at a dilution of 1:25. Maybe stored for up to 1 month at 2-8˚C.

3. **MCP-1 conjugate:**

   21ml/vial of polyclonal antibody against MCP-1 conjugated to horseradish peroxidase with preservatives. Maybe stored for up to 1 month at 2-8˚C.

4. **Colour Reagent A:**

   12.5ml/vial of stabilised hydrogen peroxide. Maybe stored for up to 1 month at 2-8˚C.

5. **Colour Reagent B:**

   12.5ml/vial of stabilised chromogen (tetramethylbenzidine). Maybe stored for up to 1 month at 2-8˚C.

6. **Stop Solution:**

   6ml/vial of 2N sulphuric acid. Maybe stored for up to 1 month at 2-8˚C.

7. **MCP-1 microplate:**

96 well polystyrene microplate coated with a mouse monoclonal antibody against human MCP-1. Maybe stored for up to 1 month at 2-8˚C.

8. **MCP-1 standard:**

10ng/vial of recombinant human MCP-1 in a buffered protein base with preservatives; lyophilized. Reconstitute with 5.0ml of diluted calibrator diluent RD5L, giving a concentration of 2000pg/ml. May be stored in aliquots at -20˚C for up to 1 month.

Methodology:

1. Dilute urine samples 1:2 with diluted calibrator diluent RD5L.
2. Prepare standards as follows

Standard	Concentration (pg/ml)	Volume of calibrator or standard added	Volume of Diluent (RD5L) added
1	1000	500μl of reconstituted MCP-1 standard	500μl
2	500	500μl of Standard 1	500μl
3	250	500μl of Standard 2	500μl
4	125	500μl of Standard 3	500μl
5	62.5	500μl of Standard 4	500μl
6	31.25	500μl of Standard 5	500μl
7	15.625	500μl of Standard 6	500μl

3. Set up MCP-1 ELISA microplate as follows by adding 200μl of diluent, standard or diluted urine sample to corresponding well, in duplicate [Diluent (D), Standard (S) and urine samples (U)].

D	D	U1	U1	U9	U9	U17	U17	U25	U25	U33	U33
S1	S1	U2	U2	U10	U10	U18	U18	U26	U26	U34	U34
S2	S2	U3	U3	U11	U11	U19	U19	U27	U27	U35	U35
S3	S3	U4	U4	U12	U12	U20	U20	U28	U28	U36	U36
S4	S4	U5	U5	U13	U13	U21	U21	U29	U29	U37	U37
S5	S5	U6	U6	U14	U14	U22	U22	U30	U30	U38	U38
S6	S6	U7	U7	U15	U15	U23	U23	U31	U31	U39	U39
S7	S7	U8	U8	U16	U16	U24	U24	U32	U32	U40	U40

4. Incubate at room temperature (22˚C) for 2 hours.

5. Aspirate contents of well and wash with wash buffer three times.

6. Add 200μl of MCP-1 Conjugate to each well.

7. Incubate at room temperature for 1 hour.

8. Aspirate contents of well and wash with wash buffer three times.

9. Mix Colour Reagents A and B together in equal volumes to make Substrate solution and use within 15 minutes.

10. Add 200μl of Substrate solution to each well.

11. Incubate in the dark at room temperature for 20 minutes.

12. Add 50μl stop solution into each well. Colour should change from blue to yellow.

13. Determine the optical density at 450 nm, with wavelength correction at 540nm or 570nm.

Calculating Results:

The optical density for each sample is calculated by subtracting the average optical density of the diluents (background value) from the average of duplicate samples.

Optical Density Sample 1 = [Average U1 – Average D]

Using results from the observed standards, a Standard Curve is constructed. Sample values are interpolated from the standard curve and multiplied by 2 to correct for dilution of original sample. These sample values are then standardised for urine Cr.

3.8.3 Analysing results

The mean, standard deviation and range for age, height, weight, BSA (Monsteller formula[108]) and BMI, for AFD patients and/or controls were calculated and compared with the Mann-Whitney U test. Also the mean, standard deviation and range for serum Cr, eGFR, iGFR, left ventricular mass index (LVMI)[325], Mainz Severity Score Index (MSSI)[326] and number and percentage of AFD patients on ERT were calculated for each cohort.

All activities of urine proteins tested were corrected for urine concentration by correcting for urine Cr and compared between AFD patients and controls, based on sex and age. The AFD patients were then categorised based on renal parameters of microalbuminuria, significant total proteinuria, serum Cr, eGFR and iGFR, and the 6 urine proteins tested were compared. Next the AFD patients were categorised according to the type of mutation, baseline plasma and leucocyte α-gal levels and disease severity based on LVMI and MSSI scores, and the 6 urine proteins were compared. The statistical analysis used was the Mann-Whitney U test to compare 2 groups and the Kruskal-Wallis to compare more than 2 groups. The Mann Whitney U test and Kruskal-Wallis tests were used as they compare non-parametric statistical hypothesis of independent samples.

As there was a significant difference in urine β-Hex and urine MCP-1 activities in AFD compared to controls, the corresponding urine protein activities or levels within the 95% confidence intervals of the controls were then considered to be the "normal" range. AFD patients who had urine protein activities or levels that were higher than the presumed normal range were compared with AFD patients who had activities in the presumed normal range based on age, sex, urine UACR, UPCR, serum Cr, eGFR, iGFR, type of mutation, baseline plasma and leucocyte α-Gal activity, MSSI scores and LVMI.

Multiple regression analysis of urine β-Hex and urine MCP-1 using age, sex and type of mutation were analysed.

3.9 Results

3.9.1 Demographics

In all groups, female controls were significantly younger than AFD females, except in the TGF-β1 cohort probably due to smaller numbers. There was no statistically significant difference in age between the control males and females, control males and AFD males or the AFD males and females for all urine protein cohorts tested (Table 3.1). AFD males were taller, heavier and had a larger surface area than AFD females but there was no significant difference in their BMI (Table 3.2). There was no difference in iGFR for AFD males and females but AFD males had a significantly higher LVMI and MSSI score compared to females in all of the urinary protein cohorts tested (Table 3.3). In the α-Gal, β-Hex, RBP and MCP-1 cohorts there were significantly more males than females treated with ERT (Table 3.3).

Age (years)	Control Male			Control Female			AFD Male			AFD Female			*
	M	SD	R	M	SD	R	M	SD	R	M	SD	R	
α-Gal	40.2	±11.3	25-60	32.4	±5.9	25-44	44.9	±14.4	18-77	44.6	±16.7	16-79	p = 0.0306
β-Hex	40.2	±11.3	25-60	32.7	±5.8	25-44	44.4	±14.0	18-77	44.6	±16.6	16-79	p = 0.0103
Chitotriosidase	39.0	±11.9	25-60	33.0	±7.2	25-44	45.2	±14.3	26-68	53.2	±12.2	28-74	p = 0.0011
RBP	39.6	±11.0	25-60	33.7	±5.4	25-44	43.3	±13.4	20-74	46.0	±14.8	16-73	p = 0.0054
TGF-β1	36.0	±11.2	25-54	35.2	±6.8	25-44	45.9	±16.6	18-69	41.2	±15.8	16-74	ns
MCP-1	41.4	±12.0	25-60	33.3	±5.9	25-44	46.4	±14.5	18-77	44.8	±15.9	16-79	p = 0.0325

Table 3.1. Age differences between the different urine protein cohorts tested. M – Mean, SD – standard deviation, R – Range. * Mann-Whitney U test comparing age between the control female and AFD female groups for each cohort. No statistically significant difference in age between control male and AFD male, AFD male and AFD female, and control male control female groups in each cohort.

	α-Gal			β-Hex			Chitotriosidase			RBP			TGF-β1			MCP-1		
	M	SD	R	M	SD	R	M	SD	R	M	SD	R	M	SD	R	M	SD	R
Height (m)	*			*			*			*			*			*		
AFD M	1.75	±0.07	1.58-1.93	1.76	±0.07	1.60-1.93	1.75	±0.07	1.58-1.83	1.76	±0.06	1.60-1.90	1.77	±0.09	1.58-1.93	1.76	±0.07	1.64-1.93
AFD F	1.62	±0.07	1.50-1.88	1.62	±0.07	1.50-1.88	1.60	±0.06	1.50-1.73	1.62	±0.06	1.50-1.74	1.64	±0.05	1.56-1.74	1.62	±0.06	1.50-1.74
Weight (kg)	*			*			*			*			*			*		
AFD M	76.9	±15.6	54.0-124.4	77.1	±15.1	54.0-124.4	73.2	±18.6	53.8-124.4	75.7	±15.5	54.0-124.4	83.4	±18.5	55.0-124.4	77.2	±15.0	55.0-124.4
AFD F	67.0	±11.9	42.3-98.0	66.8	±11.7	42.3-98.0	65.5	±10.6	45.5-90.3	67.1	±11.9	49.0-98.0	67.0	±11.1	50.2-98.0	66.3	±11.9	42.3-98.0
BSA (m^2)	*			*			*			*			*			*		
AFD M	1.93	±0.21	1.55-2.45	1.93	±0.20	1.59-2.45	1.88	±0.24	1.55-2.45	1.92	±0.21	1.59-2.45	2.03	±0.25	1.55-2.45	1.93	±0.20	1.60-2.45
AFD F	1.73	±0.17	1.34-2.18	1.73	±0.17	1.34-2.18	1.70	±0.16	1.39-2.08	1.73	±0.17	1.44-2.18	1.74	±0.15	1.53-2.18	1.72	±0.17	1.34-2.18
BMI (kg/m^2)																		
AFD M	25.1	±4.6	17.5-41.1	24.9	±4.5	17.5-41.1	23.7	±5.6	18.9-41.1	24.5	±4.6	17.5-41.1	26.9	±5.5	19.7-41.1	24.9	±4.7	18.9-41.1
AFD F	25.5	±4.3	18.0-34.8	25.4	±4.2	18.0-32.9	25.6	±2.9	19.4-30.4	25.6	±4.2	18.2-32.6	25.1	±4.2	18.0-32.4	25.2	±4.4	18.0-32.6

Table 3.2. Demographic data for different urine protein cohorts, M – mean, SD – standard deviation, R – range. Mann-Whitney U test statistical analysis used, * p < 0.05.

	α-Gal			β-Hex			Chito			RBP			TGF-β1			MCP-1		
	M	**SD**	**R**	**M**	**SD**	**R**	**M**	**SD**	**R**	**M**	**SD**	**R**	**M**	**SD**	**R**	**M**	**SD**	**R**
Serum Cr (µmol/L)	*			*			*			*			*			*		
AFD M	89.0	±26.1	56-206	89.6	±25.5	57-206	86.0	±17.4	60-119	90.2	±25.9	56-206	90.6	±17.7	57-119	86.1	±16.9	57-119
AFD F	66.7	±14.2	44-119	66.8	±14.4	44-119	69.1	±13.3	53-106	66.9	±14.1	44-111	65.5	±12.1	49-106	68.1	±15.2	49-119
eGFR (ml/min/1.73m²)							*						*					
AFD M	93.9	±27.5	32.8-156.0	92.8	±26.1	32.8-143.8	94.1	±22.2	56.1-128.0	92.4	±26.6	32.8-155.9	89.5	±25.2	56.1-140.7	94.2	±25.8	56.2-143.8
AFD F	94.5	±23.1	41.8-155.3	94.6	±23.7	41.8-155.3	85.8	±18.4	49.4-111.7	93.6	±23.2	49.4-143.2	97.7	±24.0	49.4-155.3	92.9	±24.0	41.8-155.3
iGFR (ml/min/1.73m²)																		
AFD M	82.8	±24.2	27-125	82.5	±23.4	27-128	82.4	±16.7	63-116	80.6	±21.6	27-122	90.2	±21.4	63-125	82.5	±21.5	42-125
AFD F	89.3	±23.1	41-133	90.5	±22.6	41-133	82.90	±20.6	46-117	88.6	±22.1	45-133	96.1	±22.4	46-133	86.5	±22.7	41-133
LVMI (g/m²)	*			*			*			*			*			*		
AFD M	128.5	±40.3	73.3-277.1	126.2	±36.7	76.3-228.7	133.0	±36.4	81.4-189.2	126.2	±38.6	76.3-228.7	130.8	±40.5	76.6-203.1	123.8	±33.2	81.4-228.7
AFD F	83.8	±33.6	47.1-225.8	83.3	±32.6	47.1-225.8	103.3	±32.7	58.1-159.3	85.2	±28.6	47.1-159.3	81.4	±29.3	47.1-159.3	79.3	±32.9	47.1-225.8
MSSI	*			*			*			*			*			*		
AFD M	23.2	±9.6	3 - 51	23.1	±9.4	3 - 51	25.7	±11.1	8 - 51	23.4	±9.6	4 - 51	21.3	±10.0	3-39	24.1	±10.0	3-51
AFD F	13.5	±9.3	1 - 42	13.7	±9.3	1 - 42	17.9	±9.8	1 - 40	13.9	±8.2	1 - 40	11.0	±6.7	1-24	13.4	±10.0	1-42
AFD on ERT	no	%		no	%		no	%		no	%		no	%		no	%	
	*			*						*						*		
AFD M	40	87.0		43	87.8		14	93.3		32	91.4		11	73.3		25	80.6	
AFD F	27	48.2		27	48.2		10	66.7		22	57.9		11	45.8		17	39.5	

Table 3.3. Demographics based on parameters of organ involvement data, all statistics based on Mann-Whitney U test; M – mean, SD – standard deviation, R – range, no – number of patients on ERT. * p < 0.05.

3.9.2 Different urine protein levels tested and compared with renal and other AFD parameters

As expected urine α-Gal levels were significantly lower in AFD than control subjects. AFD subjects also had a significantly higher urine β-Hex and MCP-1 levels compared with controls (Fig 3.6). Urine α-Gal, Chitotriosidase, RBP and TGF-β1 activity/levels were not significantly different based on renal parameters of microalbuminuria, proteinuria, serum Cr, eGFR or iGFR, but urine β-Hex activity was significantly higher in AFD patients with raised UACR and UPCR, and urine MCP-1 was higher in AFD patients with raised UACR (Table 3.5). Urine α-Gal, β-Hex, RBP, TGF-β1 and MCP-1 activity/levels were not significantly different based on type of AFD mutation but urine chitotriosidase activity was the only urine protein to be higher in non-missense mutations (Table 3.6). As expected urine α-Gal activity was lower in AFD patients with lower plasma and/or leucocyte α-Gal activity but urine β-Hex, RBP, chitotriosidase, TGF-β1 and MCP-1 activity/levels were not statistically different based on the baseline plasma or leucocyte α-Gal activity (Table 3.6). Looking at the subgroups, urine β-Hex activity was significantly higher if baseline plasma α-Gal activity of < 1 nmol/hr/ml was compared with > 4 nmol/hr/ml and urine MCP-1 levels were higher if baseline leucocyte α-Gal activity of < 5 nmol/hr/ml were compared with >30 nmol/hr/ml (Table 3.6, Fig 3.7). Urine α-Gal activity was significantly lower and urine β-Hex activity was significantly higher in AFD patients with a higher MSSI score and LVMI.
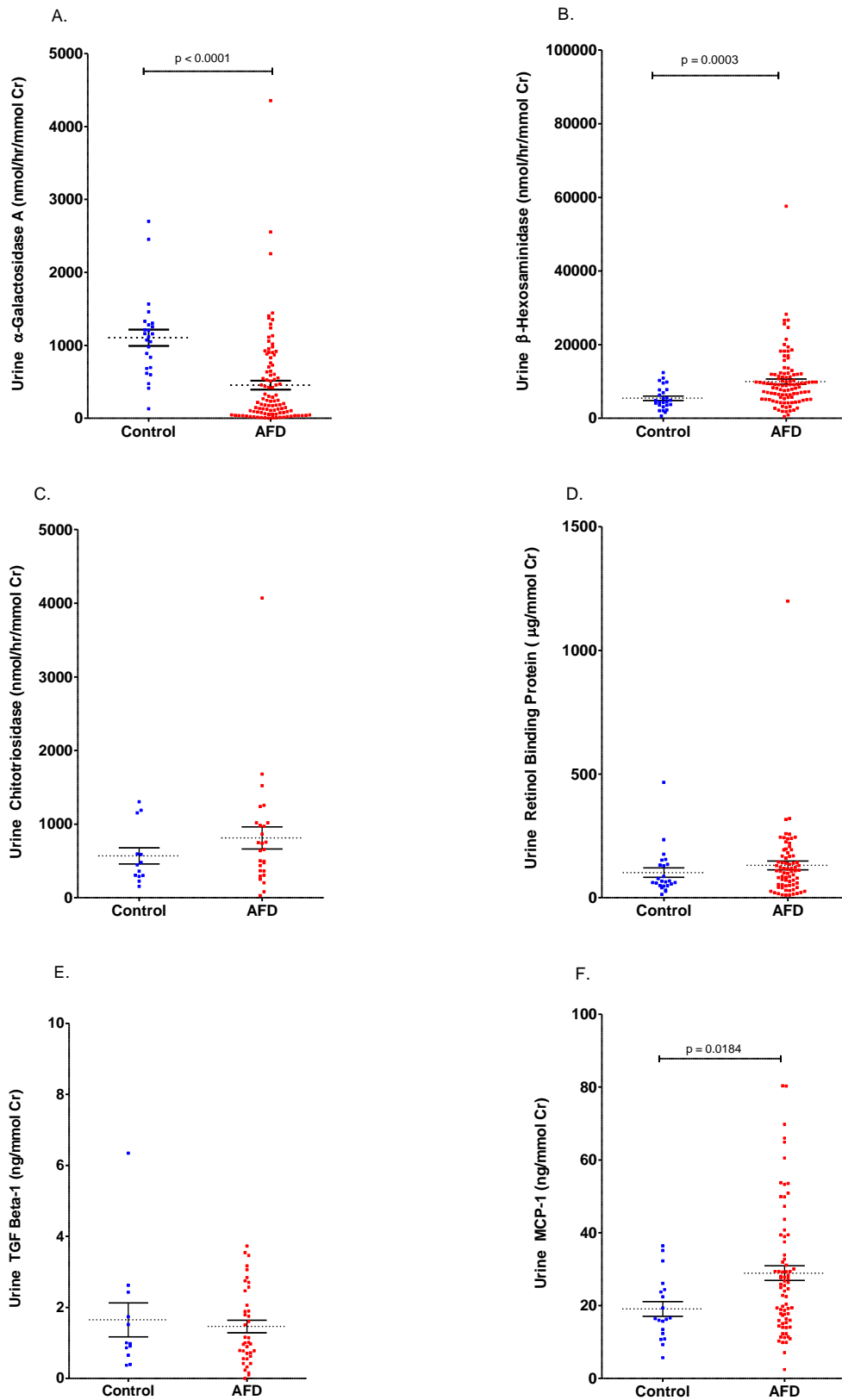
Fig 3.6. Summary of Urine Protein levels in AFD vs Control. Statistical analysis used is Mann-Whitney U test.

		Male	Female		Age < 40 years	Age ≥ 40 years	
α-Gal	Control (n=25)	1151 ± 503.8	1055 ± 644.2	ns	997.7 ± 571.2	1380 ± 480.8	ns
(nmol/hr/mmol Cr)	AFD (n=102)	124.1 ± 134.8	727.1 ± 731.3	p < 0.0001	382.1 ± 495.8	504.3 ± 697.9	ns
		p < 0.0001	p = 0.0403		p < 0.0001	p = 0.0006	
β-Hex	Control (n=26)	4446 ± 1922	6452 ± 3808	ns	5390 ± 3487	5609 ± 2060	ns
(nmol/hr/mmol Cr)	AFD (n=105)	11847 ± 9276	8319 ± 5026	p = 0.0224	7753 ± 4055	11441 ± 8824	p = 0.0338
		p < 0.0001	ns		p = 0.0362	p = 0.0188	
Chitotriosidase	Control (n=18)	687.8 ± 495.9	427.8 ± 170.9	ns	454.4 ± 345.5	823.0 ± 411.8	ns
(nmol/hr/mmol Cr)	AFD (n=30)	630.3 ± 365.0	981.0 ± 1009	ns	773.4 ± 498.9	828.4 ± 878.8	ns
		ns	ns		ns	ns	
RBP	Control (n=26)	81.31 ± 43.39	121.2 ± 126.8	ns	104.8 ± 107.7	90.56 ± 41.86	ns
(μg/mmol Cr)	AFD (n=73)	160.7 ± 197.8	103.4 ± 75.4	ns	121.8 ± 70.67	136.8 ± 184	ns
		ns	ns		ns	ns	
TGF-β1	Control (n= 12)	0.9719 ± 0.7681	2.606 ± 2.149	p = 0.0303	1.167 ± 0.6249	3.111 ± 1.740	ns
(ng/mmol Cr)	AFD (n=39)	1.057 ± 0.8389	1.720 ± 1.146	ns	1.561 ± 1.168	1.375 ± 1.006	ns
		ns	ns		ns	ns	
MCP-1	Control (n=19)	16.34 ± 6.982	22.90 ± 9.913	ns	17.59 ± 8.251	21.69 ± 9.584	ns
(ng/mmol Cr)	AFD (n=74)	34.15 ± 20.10	25.12 ± 14.18	p = 0.0486	26.70 ± 17.62	30.16 ± 17.30	ns
		p = 0.0022	ns		ns	ns	

Table 3.4. Showing difference between sex and age for various urinary proteins. Statistical test used is Mann-Whitney U test and results are shown as mean and SD.

	α-Gal (nmol/hr/mmol Cr) n = 102	β-Hex (nmol/hr/mmol Cr) n = 105	Chitotriosidase (nmol/hr/mmol Cr) n = 30	RBP (μg/mmol Cr) n = 73	TGF-β1 (ng/mmol Cr) n = 39	MCP-1 (ng/mmol Cr) n = 74
Urine Albumin:Cr (mg/mmol/Cr)						
<3.5	425.1 ± 435.2	7731 ± 3913	613.2 ± 413.9	110.3 ± 77.20	1.418 ± 1.115	24.34 ± 13.70
>3.5	500 ± 842.8	12850 ± 10061	1284 ± 1198	158.1 ± 209.9	1.540 ± 1.047	37.03 ± 20.33
Mann-Whitney	ns	p = 0.0040	ns	ns	ns	p = 0.0036
Urine Protein:Cr (mg/mmol Cr)						
< 30	410.2 ± 461.6	8577 ± 7142	657.4 ± 417.6	132.6 ± 83.42	1.508 ± 1.119	26.51 ± 15.22
>30	604.9 ± 979.0	14076 ± 7663	1624.0 ± 1714	189.1 ± 270.8	1.270 ± 1.024	38.00 ± 23.46
Mann-Whitney	ns	p = 0.0002	ns	ns	ns	ns
Serum Cr (μmol/L)						
< 100	495.5 ± 658.1	9495 ± 7295	866.0 ± 827.1	123.3 ± 79.80	1.506 ± 1.117	29.05 ± 17.22
≥ 100	198.1 ± 239.7	13268 ± 8574	502.1 ± 231.5	183.6 ± 383.5	1.280 ± 0.926	28.03 ± 20.10
Mann-Whitney	ns	ns	ns	ns	ns	ns
eGFR (ml/min/1.73m^2)						
CKD 1	403.4 ± 523.7	9484 ± 8451	667.8 ± 457.3	129.0 ± 78.25	1.531 ± 1.041	29.64 ± 16.02
CKD 2	561.9 ± 766.9	10093 ± 6026	1093.0 ± 1131.0	110.3 ± 83.35	1.335 ± 1.187	27.66 ± 17.56
CKD 3	250.6 ± 291.9	12387 ± 8469	547.5 ± 260.8	230.7 ± 429.5	1.659 ± 1.062	32.44 ± 28.53
Kruskal-Wallis	ns	ns	ns	ns	ns	ns
iGFR (ml/min/1.73m^2)						
CKD 1	384.1 ± 534.9	8352 ± 4949	644.8 ± 502.2	126.6 ±77.10	1.744 ± 1.155	28.81 ± 16.35
CKD 2	485.1 ± 762.8	10971 ± 9115	733.8 ± 423.2	116.0 ± 83.85	1.128 ± 0.874	29.08 ± 17.76
CKD3	484.9 ± 498.1	13234 ± 8815	1676.0 ± 2079.0	221.3 ± 399.1	1.768 ± 1.386	27.28 ± 22.69
Kruskal-Wallis	ns	ns	ns	ns	ns	ns

Table 3.5.Showing urine protein levels compared with renal parameters in AFD patients. Statistical analysis used is Mann-Whitney U-test or Kruskal-Wallis as stated.

	α-Gal (nmol/hr/mmol Cr) n = 102	β-Hex (nmol/hr/mmol Cr) n = 105	Chitotriosidase (nmol/hr/mmol Cr) n = 30	RBP (μg/mmol Cr) n = 73	TGF-β1 (ng/mmol Cr) n = 39	MCP-1 (ng/mmol Cr) n = 74
Type of mutation						
Missense	474.3 ± 660.5	9827 ± 8030	561.2 ± 395.1	112.4 ± 79.22	1.306 ± 0.9694	28.71 ± 18.74
Non-missense	407.1 ± 531.8	10296 ± 6144	1263 ± 1136	171.0 ± 237.3	2.548 ± 1.263	29.31 ± 14.48
Mann-Whitney	ns	ns	p = 0.0208	ns	ns	ns
Plasma α-Gal (nmol/ml/hr)						
< 1	138 ± 156.9	10575 ± 6058*	697.2 ± 342.0	161.5 ± 224.7	0.924 ± 0.809	30.10 ± 18.06
1 – 4	617.2 ± 808.1	8863 ± 5732	647.5 ± 479.6	105.9 ± 79.24	1.750 ± 1.187	25.64 ± 14.53
≥ 4	836.8 ± 606.9	7307 ± 4849*	2151.0 ± 1693	112.4 ± 69.79	1.312 ± 1.151	19.93 ± 8.910
Kruskal-Wallis	p < 0.0001	ns	ns	ns	ns	ns
Leucocyte α-Gal (nmol/hr/mg Prot)						
< 5	84.02 ± 71.57	11187 ± 6649	447.1 ± 326.7	202.3 ± 263.3	0.803 ± 0.677	33.45 ± 17.08[+]
5-30	337.5 ± 261.3	13479 ± 14322	520.8 ± 323.0	111.5 ± 66.27	1.714 ± 1.503	35.06 ± 25.83
≥ 30	956.8 ± 190.5	8599 ± 4537	1540 ± 1360	102.9 ± 77.49	1.484 ± 1.027	21.74 ± 11.28[+]
Kruskal Wallis	p < 0.0001	ns	ns	ns	ns	ns
MSSI						
<20	611.5 ± 752.7	7922 ± 4633	693.0 ± 496.7	122.2 ± 75.05	1.627 ± 1.191	27.63 ± 15.92
≥ 20	237.4 ± 330.9	12732 ± 9421	862.2 ± 874.7	144.1 ± 203.7	1.069 ± 0.746	31.43 ± 19.60
Mann-Whitney	p = 0.0021	p = 0.0022	ns	ns	ns	ns
LVMI (g/m²)						
Normal	516.3 ± 552.4	8248 ± 5074	752.1 ± 427.2	126.1 ± 80.77	1.594 ± 1.180	25.99 ± 15.13
Abnormal	388.8 ± 753.7	12612 ±9762	877.5 ± 956.2	144.4 ± 209.2	1.233 ± 0.936	34.79 ± 20.76
Mann-Whitney	p = 0.0362	p = 0.0071	ns	ns	ns	ns

Table 3.6. Showing urine protein levels compared with other disease severity markers. *between cohorts <1 and >4, Mann-Whitney U-test p = 0.0391; [+]between cohorts <5 and > 30, Mann-Whitney U test p = 0.0292.
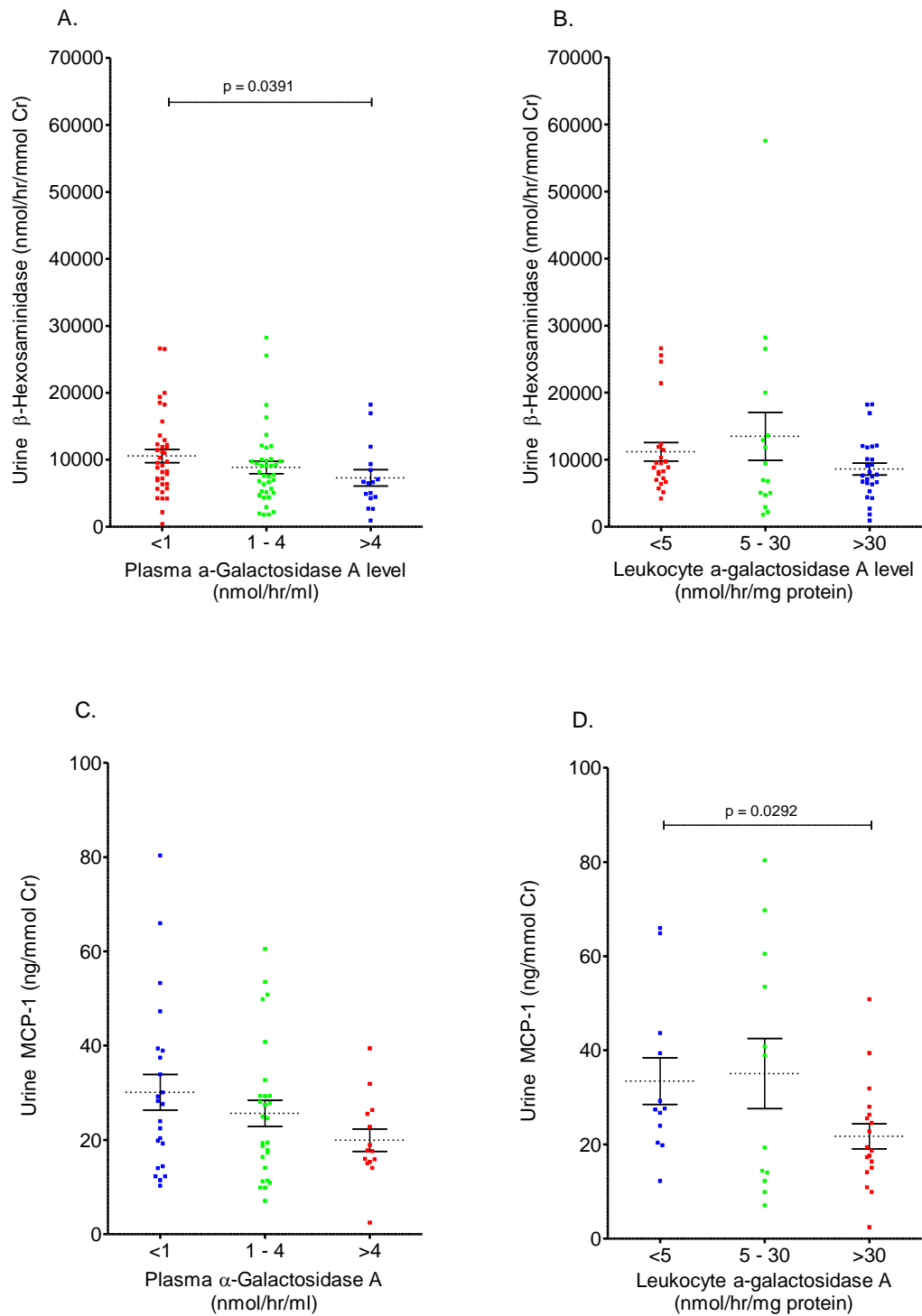
Fig 3.7. Graphs showing (A) Urine β-Hex vs Plasma α-Gal levels, (B) Urine β-Hex vs Leucocyte α-Gal levels, (C) Urine MCP-1 vs α-Gal levels, (B) Urine MCP-1 vs Leucocyte α-Gal levels

3.9.3 AFD patients with normal urine β-Hex or MCP-1 activity compared with raised urine β-Hex or MCP-1 activity

AFD patients with urine β-Hex activity more than the 95% confidence intervals of urine β-Hex activity of controls, were older, had higher UACR, higher urine UPCR, higher MSSI scores and higher LVMI compared with AFD patients with urine β-Hex activity within the normal range (95% confidence intervals of urine β-Hex activity of controls). For AFD patients using the urine MCP-1 levels in the same comparison, only a higher LVMI was found in AFD patients with a higher urine MCP-1 activity (Table 3.7).

	Normal or raised urine protein activity or level	B-Hex			MCP-1		
		M	SD	R	M	SD	R
Age (years)	Normal	42.5	±14.9	16 - 73	43.9	± 15.0	16 – 71
	Raised	50.3	±15.6	24 – 79	49.8	± 15.3	31 - 79
		p = 0.0397			ns		
Sex	Normal	32 M	46 F		20 M	35 F	
	Raised	17 M	10 F		11 M	8 F	
		*ns			*ns		
Urine Albumin:Cr (mg/mmol Cr)	Normal	8.0	±16.5	0.04 – 97.7	9.3	±27.7	0.04 – 178.1
	Raised	20.3	±36.8	0.3 – 178.1	14.0	±21.6	0.3 – 72.5
		p = 0.0091			ns		
Urine Protein:Cr (mg/mmol/Cr)	Normal	19.5	±24.3	1.6 – 154.3	19.9	±26.5	1.6 – 154.3
	Raised	40.9	±39.6	6.7 – 182.8	34.2	±43.3	3.7 – 182.8
		p < 0.0001			ns		
Serum Cr (µmol/L)	Normal	75.4	±18.1	44 – 121	75.9	±18.4	49 - 119
	Raised	83.1	±33.6	51 – 206	74.1	±17.8	44 - 116
		ns			ns		
eGFR (ml/min/1.73m^2)	Normal	94.6	±24.3	41.8 – 155.3	92.1	±25.1	41.8 – 155.3
	Raised	91.4	±26.5	32.8 – 143.1	97.3	±23.3	56.3 – 140.7
		ns			ns		
iGFR (ml/min/1.73m^2)	Normal	88.4	±21.8	41 – 133	84.4	±22.6	41 - 133
	Raised	81.7	±26.5	27 - 120	85.6	±20.9	42 - 120
		ns			ns		
Type of mutation	Normal	18 non-missense	37 missense		23 non-missense	55 missense	
	Raised	6 non-missense	13 missense		8 non-missense	19 missense	
		*ns			*ns		
Plasma α-Gal (nmol/ml/hr)	Normal	2.3	±2.0	0.0 – 7.4	2.6	±2.1	0.0 – 7.4
	Raised	1.7	±1.7	0.1 – 5.4	1.6	±1.6	0.0 – 5.2
		ns			ns		
Leucocyte α-Gal (nmol//hr/mg Prot)	Normal	27.4	±25.4	0.1 – 88.0	32.4	±25.7	0.2 – 88.0
	Raised	18.8	±21.2	0.4 – 58.0	15.4	±19.3	0.5 – 60.0
		ns			ns		
MSSI	Normal	16.4	±9.4	1 – 39	17.2	±10.8	1 - 40
	Raised	23.2	±11.8	5 - 51	20.3	±12.4	1 - 51
		p = 0.0123			ns		
LVMI (g/m^2)	Normal	96.0	±34.1	48.8 – 189.3	90.2	±31.0	47.1 – 161.8
	Raised	122.8	±50.6	47.1 – 228.7	117.8	±52.3	49.6 – 228.7
		p = 0.0118			p = 0.0420		

Table 3.7. Comparing urinary β-Hex and MCP-1activity or levels in AFD patients within the normal range (95% confidence interval of corresponding results from controls) with AFD patients with raised activity or levels (> 95% confidence interval of controls). Statistical analysis used was Mann-Whitney U-test unless otherwise stated. * Fishers exact test

3.9.4 Multiple regression analysis

Significant variables in multiple regression analysis for urine β-Hex and urine MCP-1 were age and sex of subjects but not the type of mutation (Table 3.8).

Variable	p value	Significant	Correlation Coefficient
Urine α-Gal			
Age	0.0544	No	0.1710
Sex	<0.0001	Yes	0.4827
Missense	0.7793	No	0.0488
Urine β-Hex			
Age	0.0011	Yes	0.2992
Sex	0.0117	Yes	-0.2359
Missense	0.4289	No	-0.0287
Urine MCP-1			
Age	0.0420	Yes	0.2414
Sex	0.0317	Yes	-0.2583
Missense	0.6786	No	-0.0164

Table 3.8. Multiple regression analysis for urine β-Hex and MCP-1.

3.10   Discussion

This is the largest study looking at urine α-Gal activity in urine. Urine α-Gal activity is significantly lower in AFD patients than controls (Fig 3.6) as expected and confirms Kitigawa et al's findings. Urine α-Gal activity is also lower in AFD males compared with AFD females. Lower baseline plasma and leucocyte α-Gal activity was also significantly associated with urine α-Gal activity. Multiple regression analysis showed that sex was a significant variable but not age and type of mutation for urine α-Gal activity. Lower urine α-Gal activity was associated with more significant end organ damage reflected by an increased LVMI and higher MSSI scores but not by renal parameters of eGFR, iGFR, microalbuminuria or proteinuria which are reflection of glomerular function rather than renal tubular function.

Two other lysosomal enzymes β-Hex and Chitotriosidase were analysed in the urine of AFD patients. Urine β-Hex activity was significantly higher in AFD male patients compared with male controls and AFD females. Also urine β-Hex activity was higher in AFD males at a younger age (<40) and at an older age (>40) when compared with controls. Multiple regression analysis showed age and sex were important variables for urine β-Hex activity in AFD patients but the type of mutation was not a significant variable. But urine β-hex levels were significantly higher in AFD patients with a lower baseline plasma α-Gal levels. These results were not reflected in urine chitotriosidase activity where there is no difference comparing AFD patients with controls eventhough plasma chitotriosidase activity is reported to be higher in AFD males[216]. Of note urine chitotriosidase activity was significantly higher in patients with non-missense mutations. Looking at other measures of end organ involvement, urine β-hex is significantly higher in AFD patients with raised UACR and UPCR but not different based a staging of CKD with eGFR or iGFR. Urine β-hex activity was also higher in AFD patients with higher MSSI scores and raised LVMI. This suggests that urine β-hex activity is increased in AFD patients with evidence of cardiac, renal and systemic organ involvement. We then used 95% confidence interval values of urine β-hex in controls to be the normal range and presumed that >95% confidence interval value of urine β-hex activity in controls as being abnormally raised activity. AFD patients with an abnormal urine β-hex activity were older, had increased microalbuminuria and proteinuria, higher

MSSI scores and raised LVMI. This further suggests that urine β-hex activity appears to be raised in AFD patients with significant end organ damage.

This increase in urine β-hex activity maybe a reflection of reduced tubular reabsorption or increased tubular secretion due to damage to renal tubular cells or interstitial damage or increased lysosomal activity in renal cells, but the absence of concurrent increased chitotriosidase activity suggests that increased lysosomal activity is less likely. Sample size for urine chitotriosidase cohort were smaller (controls = 18, AFD = 30) compared to the urine β-hex cohort (controls = 26, AFD = 102) therefore significant differences may be difficult to elicit. Also because of glomerular filtration of the smaller protein chitotriosidase compared to β-hex which is not filtered due to its larger molecular mass, could mean that the proportion of activity of urine chitotriosidase could predominantly be from filtered chitotriosidase rather than chitotriosidase from tubular damage or increased lysosomal activity. Therefore if there was increased urine chitotriosidase from renal tubular damage or lysosomal activity this may not significantly increase the overall total urine chitotriosidase activity. Previously it had been shown that plasma chitotriosidase activity was elevated in male AFD hemizygotes and this reduced with ERT[216, 217]. Since chitotriosidase would be freely filtered due to its small molecular weight (39-50kDa) it would be expected that urine chitotriosidase activity of male AFD hemizygotes should be similarly be increased compared to females as plasma chitotriosidase activity, though we could not show this in our data.

In our study we have shown that urine RBP levels are not elevated in AFD patients. RBP which is freely filtered at the glomerulus and reabsorbed at the proximal tubules has been shown to be a marker of renal tubular dysfunction. Considering that urine β-hex activity is increased and urine RBP levels are normal, more likely that tubular reabsorption function in our group of patients is preserved but there is increased lysosomal enzymuria excretion from renal tubules (indicated by raised urine β-hex activity). The reasons for preserved tubular reabsorptive function but not glomerular function could be because firstly recombinant α-Gal is filtered despite having a molecular weight of 110kDa and via megalin receptors accumulate in the proximal tubules[156] and secondly the proximal tubular cells have a relatively fast turnover[327]. Combination of these 2 factors could mean that tubular cells are less susceptible or regenerate faster than glomerular cells and therefore maintain function. Analysing our data further (not shown) AFD patients not on ERT (3 AFD males and 16 AFD females)

did not have significantly different mean RBP levels compared to controls (12 males and 12 females; note the small numbers) possibly indicating that tubular reabsorptive function is preserved in AFD.

TGFβ-1, a mediator of renal scarring is not increased in urine of AFD patients. MCP-1 a chemokine important in the inflammatory process is raised in AFD patients compared to controls. Urine MCP-1 levels are significantly higher in AFD males compared with controls or AFD females, but not affected by age. It is higher in AFD patients with microalbuminuria but not associated with other markers of end organ damage and appears to be higher in AFD patients with lower leucocyte α-Gal levels. Again we used the 95% confidence intervals of urine MCP-1 levels of controls as the normal range and presumed that >95% confidence interval value of urine MCP-1 levels of controls as being abnormal. Only AFD patients with an abnormal LVMI had a significantly higher urine MCP-1 level. Multiple regression analysis also showed that age and sex were significant variables for urine MCP-1 levels in AFD. This may indicate that the biological pathway for renal fibrosis and damage may be via MCP-1 and not TGF-β1. Again we would be cautious in saying that TGFβ-1 is not a mediator for renal scarring in AFD since the sample size was again small (AFD = 39, controls =12).

Proposed mechanisms of renal injury in AFD are
1) The development of microvascular disease due to the deposition on GB3 in arterial vessel walls which then result in subsequent vascular compromise. This was proposed by Gubler et al[115] due to the ischaemic changes of glomerulosclerosis, often with wrinkled and partially collapsed glomerular basement membranes, tubular atrophy, interstitial fibrosis and vascular thickening occurring in older patients found on renal biopsies.
2) The toxic accumulation of GB3 in podocytes[135]; podocytes are postmitotic and fail to proliferate under most pathological conditions and are generally not replaced[328].

Podocytes lost

↓

Denuded GBM contacts parietal epithelial cells

↓

Forms a synechia

↓

Activation and proliferation of cells (Mesangial)

Entry of immune cells (macrophages)

Accumulation of extracellular matrix protein

↓

Matrix expansion and subsequent collapse of capillary loop appears as a focus of solidification (Segmental glomerulosclerosis)
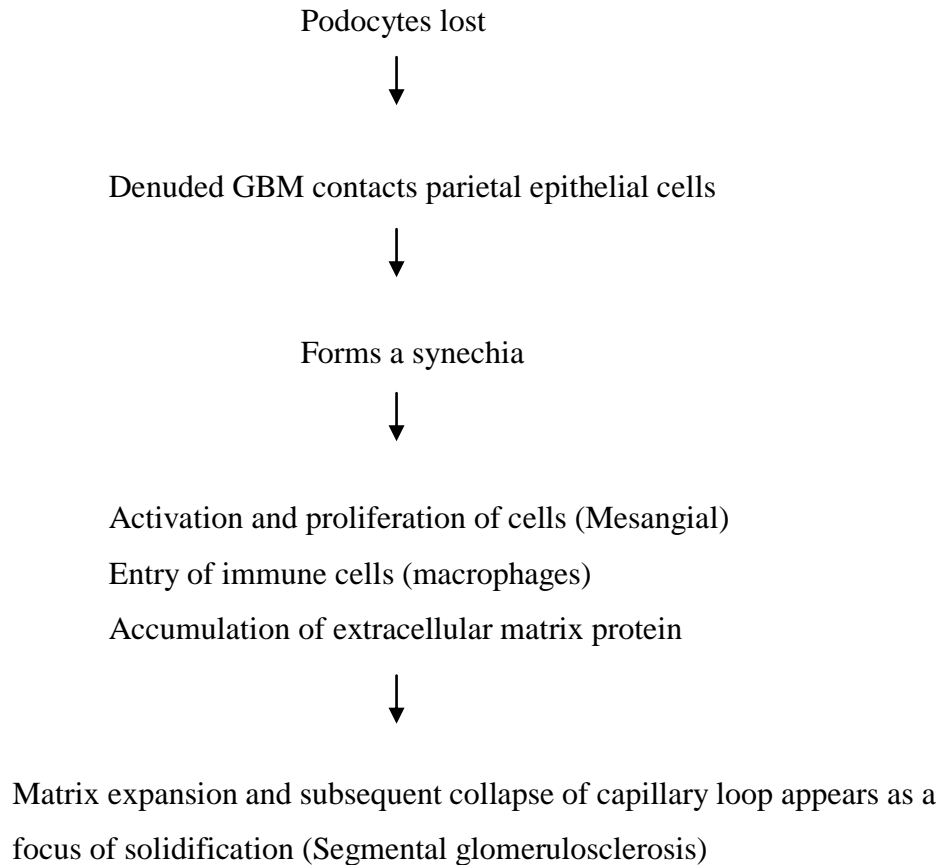
3) Deposition of GB3 within tubular epithelial cells may lead to focal tubular atrophy and interstitial fibrosis

It is well documented that glomerular function is affected in AFD due to significant microalbuminuria, proteinuria and reduced eGFR and iGFR. There has been no quantitative study on assessment of renal tubular function to date. From our study, a cross sectional investigation of AFD patients, we have shown that urine β-hex activity is increased with normal urine RBP levels, suggesting an increased renal tubular secretion without significant renal tubular reabsorptive dysfunction. This does not correlate with renal pathological findings that GB3 deposition and renal atrophy and scarring is present in most regions of the kidney. The well known difficulty with diagnosing and quantifying disease severity in AFD is due to the heterogeneity of the disease especially in females.

Current guidance for the use of ERT suggests that ERT should be initiated in AFD patients with impaired GFR and/or significant proteinuria implying glomerular dysfunction. Renal biopsies have been advocated in AFD patients with microalbuminuria but no other evidence of end organ damage prior to making a

decision on initiating on ERT. But it has been shown that even in individuals with normal renal function and no proteinuria, renal pathological findings of AFD are present[115, 138, 145]. The benefits of invasive tissue diagnosis from renal biopsies and prediction of progression have to be weighed with the risks of complications from the procedure. We would suggest that renal biopsies be reserved for patients where a second renal pathology is possible resulting in different therapeutic options (e.g. an immunological process) or in females with the α-Gal A mutation with minimal microalbuminuria, proteinuria or impaired GFR where renal disease could be due to AFD or other coexisting comorbidities (diabetes or hypertension).

Renal tubular dysfunction can lead to a variety of complications due to renal tubular acidosis. This may result in bone disorders (rickets, osteomalacia, osteopenia, and osteoporosis), Fanconi syndrome, renal stone disease and electrolyte disturbances. Currently there is no published literature demonstrating AFD patients with Fanconi syndrome, glycosuria, aminoaciduria, hyperuricosuria, hyperphosphaturia or metabolic acidosis. This makes renal tubular acidosis less likely in AFD. But studies have shown evidence of osteopenia and osteoporosis in AFD patients[329, 330] and proposed mechanisms of this maybe due to vitamin D deficiency, secondary hyperparathyroidisim, associated depression, carbamazepine use, reduced physical activity and GB3 deposition in bone macrophages[330]. Another potential mechanism of these bone disorders in AFD could be due to potential renal tubular acidosis form renal tubular dysfunction. We propose that a further study into screen AFD patients for renal tubular acidosis, vitamin D deficiency and hyperparathyroidism should be undertaken.

3.11   Limitations

Sample size was a major limitation in our study, with the largest cohort being 105 samples and smallest 30 samples. Also some of the patients were already on ERT but ideally the study should have been carried out on patients before the initiation of ERT and followed up prospectively, over a long period of time, ideally years rather than months. As AFD is a rare disease, patients in our unit travelling nationally and at most for 6 monthly follow up, it was difficult for a larger population size to be studied.

Even though in our study population there was a statistically younger control female cohort compared to AFD females, when older AFD females were removed from the

AFD cohort (ensuring no significant difference in age of the AFD females versus control females) the statistical significance of the data presented was not altered (data not shown). Therefore, to have a larger number, the data presented were from the original study population.

3.12   Conclusions

Currently predicting the progression of renal disease in AFD is difficult and the timing of the initiation of ERT to prevent end stage renal disease is challenging. Once significant proteinuria (>1G/24 hours) or glomerulosclerosis (>50%) has developed the therapeutic benefit of ERT is reduced[10]. A non-invasive biomarker in AFD, such a plasma chitotriosidase in Gaucher's disease, would be ideal for diagnosis, management and monitoring. We suggest that urine β-hex can be used with or without microalbuminuria as another marker for early evidence of renal damage in AFD. Unexpectedly urinary markers of renal fibrosis or scarring do not appear to be elevated in AFD (maybe due to small numbers or technical reasons) but an increased inflammatory process maybe present as evidence by raised urine MCP-1 levels.

Chapter 4. Assessment of autonomic function in AFD

4.1   Introduction

The autonomic nervous system (ANS) or visceral nervous system is the part of the nervous system that controls visceral functions and acts as a control system below the conscious level. It affects heart rate, blood pressure, gut motility and digestion, respiratory rate, salivation, perspiration, pupillary diameter, micturition and sexual arousal. Most of the ANS actions are involuntary and is divided into the parasympathetic and the sympathetic nervous systems. A variety of conditions can affect the ANS and is summarised in Table 4.1. Due to this variety of conditions, autonomic dysfunction may present in any age group or may have a significant family history if genetic in origin. Due to the varied functions of the ANS, symptoms of dysfunction are varied and are summarised in Table 4.2.

System	Disease / Disorder
Primary	Acute/ subacute dysautonomias – pure pandysautonomia, pandysautonomia with neurological features, pure cholinergic dysautonomia
	Chronic autonomic failure syndromes – pure autonomic failure, multiple system atrophy (Shy-Drager syndrome), autonomic failure with parkinson's disease
Secondary	Congenital – nerve growth factor deficiency
	Hereditary – autosomal dominant trait, familial amyloid neuropathy, autosomal recessive trait, familial dysautonomia: Riley-Day syndrome, dopamine β-hydroxylase deficiency
	Metabolic diseases – diabetes mellitus, chronic renal failure, chronic liver disease, alcohol induced
	Inflammatory – Guillain-Barŕe syndrome, transverse myelitis
	Infections – bacterial (tetanus), parasitic (Chagas' disease), viral (HIV)
	Neoplasia – brain tumours especially of the third ventricle or posterior fossa, paraneoplastic, systemic amyloidosis
	Surgery – vagotomy and drainage procedures
	Trauma – cervical and high thoracic spinal cord transection
Drugs, chemical toxins	By causing a neuropathy or direct effects; see Table 4.3

Table 4.1. Summary of the causes of autonomic dysfunction. Adapted from Mathias CJ. Disorders of the autonomic nervous system. In: Bradley WG, Daroff RB, Fenichel GM, Marsden CD. *Neurology in clinical practice*, 3rd ed. Boston: Butterworth-Heinemann, 2000:2131-65.

System	Symptoms
Cardiovascular	Orthostatic Hypotension
	- Cerebral hypoperfusion – dizziness, visual disturbance, syncope, cognitive deficits
	- Muscle hypoperfusion - paracervical and suboccipital ache, lower back/buttock ache
	Renal Hypoperfusion
	- Oliguria
	Spinal Cord hypoperfusion
	Non-specific
	- Weakness
	- Lethargy
	- Fatigue
	Falls
	Lability of blood pressure
	Tachycardia
	Supine Hypertension
	Paroxsymal hypertension
	Bradycardia
Sudomotor	Hypo- or anhidrosis
	Hyperhidrosis
	Gustatory sweating
	Hypothermia
	Hyperpyrexia
Gastrointestinal	Xerostomia
	Dysphagia
	Gastric stasis
	Dumping syndromes
	Constipation
	Diarrhoea
Urinary	Nocturia
	Frequency
	Urgency, retention
	Incontinence
Sexual	Erectile failure
	Ejaculatory failure
	Retrograde ejaculation
	Priapism
Eye	Pupillary abnormalities
	Ptosis
	Alachryma
	Abnormal lacrimation with food ingestion

Table 4.2. Summary of symptoms of autonomic nervous system dysfunction[331].

4.2 Rationale for study

Currently there is documented evidence for involvement of the central and peripheral nervous system in AFD. Patients suffer with symptoms of acroparaethesia, fever pain attacks, transient ischaemic attacks or strokes, altered sweating, postural hypotension and headaches. Extensive GB3 deposition are found in neurons of autonomic and dorsal root ganglia[332-334], with clinical evidence of impairment of autonomic function[335], also present in heterozygotes[336]. Small calibre myelinated and unmyelinated nerve fibres are more severely affected than thick myelinated nerve fibres[333, 337-340]. GB3 deposits have been found in perineurial cells, endothelial and periepithelial cells of small epineurial or endoneurial vessels in nerve axons and Schwann cells[339, 341-343]. GB3 deposition has also been found in sweat gland cells[339, 340]. To our knowledge there is only two published articles showing abnormal cardiac autonomic function (heart rate variability) in males[344, 345] which improved significantly over a 26 week period with ERT[345] but also 2 studies showing normal cardiac autonomic[346, 347] function. Sweat function also improved with ERT[20] though baseline skin moisture may not[348].

4.3 Aims

   i) To investigate cardiac autonomic dysfunction in AFD subjects.
   ii) To investigate plasma catecholamine levels in AFD subjects.
   iii) To investigate autonomic symptoms and neuropathic pain in AFD subjects.

4.4 Hypothesis

AFD patients have significant autonomic symptoms, neuropathic pain, evidence of cardiac autonomic involvement and abnormal plasma catecholamine response.

4.5 Materials and methods

I recruited patients from the lysosomal storage disorders unit at the Royal Free Hospital, UK from November 2006 until February 2009. Study was approved by the NHS Research Ethics Committee (REC reference 07/Q0501/81). Patients recruited had to give informed and signed consent and have a documented mutation in the α-Gal A gene. I performed the autonomic function tests, at the Pickering Unit, St Mary's Hospital, UK with the exception of the quantitative sudomotor axon-reflex test (QSART) which I carried out at the Royal Free Hospital. Subjects were also asked to complete the Leeds Assessment of Neuropathic Symptoms and Signs (LANSS) and Composite Autonomic Symptom Scale (COMPASS) questionnaires.

4.5.1 Cardiac autonomic function screening tests

These tests were carried out by me, at the Pickering Unit, St Mary's Hospital. Prior to conducting autonomic function screening tests, subjects were asked to refrain from caffeine, nicotine and alcohol for at least 3 hours before, and avoid drugs with adrenergic and anticholinergic properties (Table 4.3) for at least 48 hours before. Subjects with cardiac pacemakers and/or defibrillators, known to have autonomic dysfunction due to another disorder or disease process other than AFD, had proliferative diabetic retinopathy or if they were unable to avoid drugs as listed in Table 4.3, were excluded from this study.

Medication	Effect
Decreasing sympathetic activity	Centrally acting – clonidine, methyldopa, moxonidine, reserpine, barbiturates, anaesthetics Peripherally acting – sympathetic nerve endings (guanethidine, bethanidine), α adrenoceptor blockade (phenoxybenzamine), β adrenoceptor blockade (propanolol)
Increasing sympathetic activity	Amphetamines, releasing noradrenaline (tyramine), uptake blockers (imipramine), monoamine oxidase inhibitors (tranylcypromine), β adrenoceptor stimulants (isoprenaline)
Decreasing parasympathetic activity	Antidepressants (imipramine), tranquilisers (phenothiazines), antidysrhytmics (disopyramide), anticholinergics (atropine, probanthine, benztropine), toxins (botulinum)
Increasing parasympathetic activity	Cholinomimetics (carbachol, bethanechol, pilocarpine, mushroom poisoning), anticholinesterases, reversible carbamate inhibitors (pyridostigmine, neostigmine), organophosphorous inhibitors (parathion, sarin)
Miscellaneous	Alcohol, thiamine (Vitamin B1) deficiency Vincristine, perhexiline maleate Thallium, mercury, arsenic

Table 4.3. Drugs affecting autonomic function screening tests. Adapted from Mathias CJ. Disorders of the autonomic nervous system. In: Bradley WG, Daroff RB, Fenichel GM, Marsden CD. *Neurology in clinical practice*, 3rd ed. Boston: Butterworth-Heinemann, 2000:2131-65.

Subjects were monitored with continuous electrocardiogram (ECG), blood pressure (BP) and heart rate (HR) measurements with an automated sphygmomanometer and finometer (used to measure beat to beat finger arterial pressure). ECG monitoring provided continuous HR monitoring, the finometer a continuous beat to beat variation in BP and the automated sphygmomanometer HR and BP measurements at regular intervals. These measurements were carried out with the following sequence of positions or activities:

1. The subject was supine for 15 minutes with BP measurements every 3 minutes.
2. The subject was then tilted to 60° for 10 minutes (using an automated tilt table) with BP measurements every 3 minutes.
3. At the end of initial supine and end of tilt positions plasma samples for adrenaline and noradrenaline were taken via venepuncture.
4. The subject was then returned to a supine position. In the supine position the following tests were carried out.

- Isometric exercise:

  The subject was asked to squeeze the pump of a manual sphygmomanometer and sustain this at 30-40% of maximum hand grip for 3 minutes. The BP was measured with the sustained hand grip after 2.5 to 3 minutes from the start of the exercise. This exercise was stopped after the BP was measured.

- Mental arithmetic exercise:

  The subject was asked to subtract 7 from 400 continuously. The BP was measured 1.5 to 2 minutes after the start of the mental exercise. The exercise was stopped after the BP had been measured.

- Cold pressor exercise:

  An ice pack was placed on the subject's hand for 90 seconds, and the BP measured at 1 minute from the start of the exercise.

- Deep breathing exercise/ respiratory sinus arrhythmia:

  The subject was asked to do deep breathing at a rate of 6 breaths per minute (5 seconds inspiration and 5 seconds expiration), for a total of 1 minute, with continuous ECG monitor. The mean of difference between maximum and minimum HR for 6 measured cycles were calculated in beats per minute (bpm).

- Hyperventilation exercise:

  The subject was asked to perform rapid shallow breathing for 1 minute with the BP and HR measured 30 seconds after the start of the exercise.

- Valsalva manoeuvre and HR response:

  The subject was asked to blow into a mouthpiece connected to modified sphygomanometer and hold the expiratory pressure of 40mmHg for 10 seconds with continuous ECG monitoring. This was repeated three times with 1 minute intervals. The average of 3 valsalva ratios (ratio of the maximum rise in HR to the maximum fall in HR) was calculated.

Venous blood samples that were collected into heparinised tubes for plasma adrenaline and noradrenaline levels, were added with 1,2-di (2-aminoethoxy) ethan-NNN'n'-tetraacetic acid (EGTA) and glutathione to prevent oxidation. Samples were kept on ice until centrifuged and the plasma then kept at -20 °C until assayed. Plasma concentrations of plasma noradrenaline (norepinephrine), and adrenaline (epinephrine)

were measured by high performance liquid chromatography with an electrochemical detector[349]. The intra-assay and interassay coefficients of variation were 3.1% and 4.6% (respectively) for noradrenaline and 4.6% and 5.1% for adrenaline.

Normal autonomic screening tests responses are summarised in Table 4.4.

Test	System tested	Normal Response
Head Tilt (BP)	Sympathetic	< 20mmHg systolic drop at 3 minutes < 10mmHg diastolic drop at 3 minutes
Head Tilt (HR)	Sympathetic	HR rise of $\geq$ 5 bpm
Isometric, Mental Arithmetic, Cold Pressor	Parasympathetic and sympathetic	$\geq$ 10mmHg systolic rise AND $\geq$ 5 mmHg diastolic rise AND $\geq$ 3bpm HR rise
Respiratory sinus Arrhythmia	Parasympathetic	> 10 bpm HR rise – modest response 6-10 bpm HR rise and minimal response 1-5 bpm HR rise
Hyperventilation	Parasympathetic	$\geq$ 10 bpm HR rise
Valsalva	Parasympathetic	Valsalva ratio > 1.0

Table 4.4. Summary of normal cardiac autonomic responses.

4.5.2   Quantitative sudomotor axon-reflex test (QSART)[350, 351].

The QSART involved using a Q-SWEAT™ machine from WR Medical Electronics Co., Stilwater, Minnesota (Fig 4.1). The Q-SWEAT device accurately measures sweat production from a small area of skin, by evaporating sweat into dry air and measuring the increase in the fractional relative humidity of the air returning from the skin. The device was switched on for 15 – 20 minutes to warm up prior to use. Subjects were seated quietly for 15 – 20 minutes prior to test to acclimatise to the room temperature and humidity. The left medial forearm (75% of the distance from the ulnar epicondyle to the piciform bone), was used as the skin surface area for the test. The medial forearm was chosen as sweat production here is not affected by age[352]. An alcoholic wipe was used to clean the skin and dried for 1 minute. The recording capsule was attached to the skin area that had been cleaned. The recording capsule is made up of a multicompartmental sweat cell. The outer compartment is loaded with 10% acetylcholine solution. The Q-SWEAT device uses a desiccant pack (#5190; WR Medical Electronics Co., Stillwater, Minnesota) as its dry air source. Room air is drawn

in through an intake pump and channelled through a serpentine of drierite (W.A. Hammond Co., Xenia, Ohio). This air is then passed through a set of sensors (Honeywell International, Inc., Morristown, New Jersey), which controls the flow rate. The sensors evaluate the temperature and percent relative humidity. Finally the dried air is delivered to the multicompartmental sweat cell. The test was started when a steady baseline sweat production rate had been achieved. A constant current of 2mA is applied for 5 minutes through the outer compartment containing the 10% acetylcholine solution. The sweat produced in the inner compartment evaporates and was measured by the Q-SWEAT device. The sweat response was recorded during the electrical stimulus and for 5 minutes after. The production of sweat in the inner compartment is based on the neural pathway consisting of an axon reflex mediated by the post-ganglionic sympathetic sudomotor axon (Fig 4.2). Baseline sweat rate, latency (time from electrical stimulus till noticeable sweat rate change occurred) and total sweat volume (in 10 minutes from start of electrical stimulus) was calculated by Test-works software (WRE Medical electronics co., Stilwater, Minnesota). Total sweat volume was corrected for surface area of multicompartmental sweat cell.

Fig 4.1. Q-SWEAT ^{TM} machine from WR Medical Electronics Co., Stilwater, Minnesota.
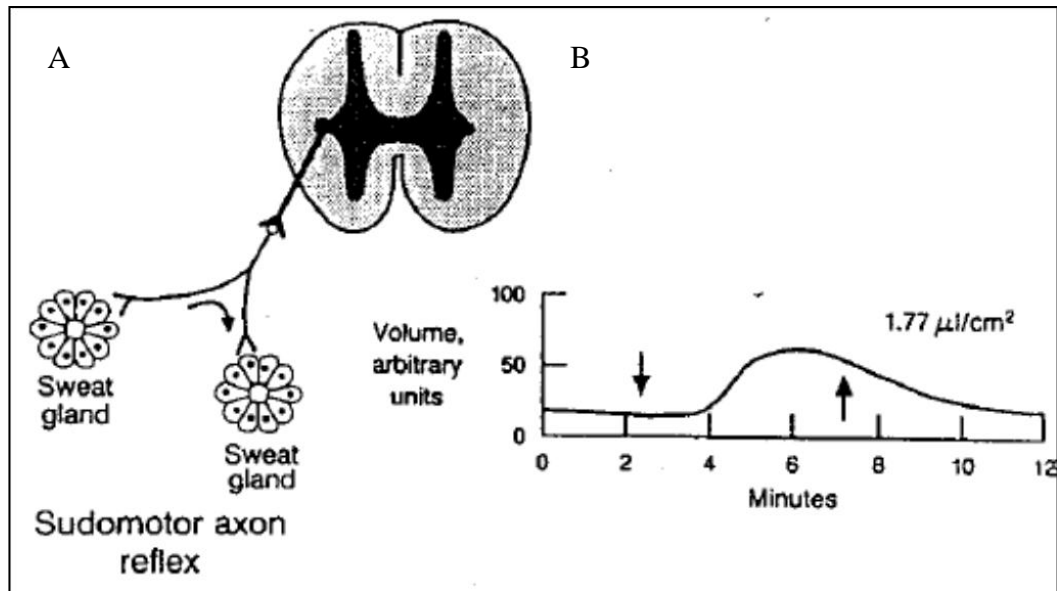
Fig 4.2. (A) Diagram of the sudomotor axon reflex and (B) schematic picture of a normal sweat response. From Clinical autonomic disorders: Evaluation and management; Edited by Philip A.Low, Chapter 14, Laboratory evaluation of autonomic failure, p172.

4.5.3   Questionnaires

Subjects were asked to complete the following 2 questionnaires to assess autonomic symptoms and neuropathic pain.

1. Composite autonomic symptom scale.
2. Leeds assessment of neuropathic symptoms and signs.

4.5.3.1 Composite autonomic symptom scale (COMPASS)[353], (Fig 4.3).

This questionnaire has 73 items subdivided into 9 domains, concerning different aspects of autonomic symptoms and is item-weighted, with higher scores indicating more or worse symptoms. Three different population groups; 41 healthy controls (mean age 46.6 years), 33 patients with non-autonomic peripheral neuropathy (mean age 59.5 years) and 39 patients with autonomic failure (mean age 61.1 years) were tested in developing this questionnaire. Mean scores were 9.8 ($\pm$9), 25.9 ($\pm$17.9) and 52.3 ($\pm$24.2) respectively. This questionnaire was validated by correlating COMPASS scores with the scores of the composite autonomic scoring scale[354] derived from the autonomic reflex screen.

18. In the past year, have you ever felt faint, dizzy, or "goofy" or had difficulty thinking soon after standing up from a sitting or lying down position?   1. Yes   2. No

If you marked YES go to question 19.   *If you marked No go to question 37.*

19. When standing up, how frequently do you get these feelings or symptoms?

      1. Rarely

      2. Occasionally

      3. Frequently

      4. Almost always

20. How would you rate the severity of these feelings or symptoms?

      1. Mild

      2. Moderate

      3. Severe

21. For how long have you been experiencing these feelings or symptoms?

      1. Less than 3 months

      2. 3 to 6 months

      3. 7 to 12 months

      4. 13 months to 5 years

      5. More than 5 years

      6. As long as I can remember

22. In the past year, how often have you ended up fainting soon after standing up from a sitting or lying down?

      0. Never

      1. Once

      2. Twice

      3. Three times

      4. Four times

      5. Five or more times

23. How cautious are you about standing up from a sitting or lying down position?

      1. Not cautious at all

      2. Somewhat cautious

      3. Extremely cautious

24. What part of the day are these feelings worse? (Check only one)

      1. Early morning

      2. Rest of morning

      3. Afternoon

      4. Evening

      5. At night, when I get up after I've been asleep

      6. No particular time is worse

      7. Other time, please specify_____

25. In the past year, have these feelings or symptoms that you have experienced:

      1. Gotten much worse

      2. Gotten somewhat worse

      3. Stayed about the same

      4. Gotten somewhat better

      5. Gotten much better

      6. Completely gone

Please rate the average severity you have experienced in the past year for each of the following symptoms.

	None	Mild	Moderate	Severe
26. Rapid or increased heart rate? (palpitations)				
27. Sickness to your stomach (nausea) or vomiting?				
28. A spinning or swimming sensation?				
30. Blurred vision?				
29. Dizziness?				
31. Feeling of weakness?				
32. Feeling shaky or shaking sensation?				
33. Feeling anxious or nervous?				
34. Turning pale?				
35. Clammy feeling to your skin?				

36. Do you have any biological (blood, natural) relatives among your parents, grandparents, brothers, sisters, or children who have frequent dizziness after standing from a sitting or lying down position?

          1. Yes   2. No

If Yes, please list their names and relationship to you.

Name   Relationship

In the past year, have you ever felt faint, dizzy, or "goofy" or had difficulty thinking:

37. soon after a meal?   1. Yes   2. No

38. after standing for a long time?   1. Yes   2. No

39. during or soon after physical activity or exercise? 1. Yes   2. No

40. during or soon after being in a hot bath, shower, tub, or sauna? 1. Yes   2. No

41. Have you ever felt dizzy or faint or actually fainted when you saw blood or had a blood sample taken?

          1. Yes   2. No

In the past year, have you fainted:

42. while passing urine?   1. Yes   2. No

43. while coughing?   1. Yes   2. No

44. while pressing on side of neck?   1. Yes   2. No

45. before a public speech?   1. Yes   2. No

46. any other time?   1. Yes   2. No

If you checked "Yes" to any of these questions on fainting, please describe circumstances.

47. In the past year, have you ever completely lost consciousness after a spell of dizziness?

          1. Yes   2. No

48. In the past year, have you had any seizures or convulsions? 1. Yes   2. No

If Yes please describe circumstances below

In the past 5 years how would rate the amount of trouble, if any, you have had:

	None	Some	A lot	Constant
49. with paralysis in parts of your face?				
50. with feelings of complete weakness all over your body?				
51. with attacks of uncontrollable movements of your arms or legs?				
52. with attacks in which you couldn't control your speech?				

53. Have you ever in your adult life had a spell of dizziness?   1. Yes   2. No

54. In the past year, have you ever noticed colour changes in your skin, such as red, white, or purple?

1. Yes   2. No

If Yes, go to question 55.   *If No, go to question 65.*

What colour changes have occurred?

55. My skin turns red   1. Yes   2. No

56. My skin turns white   1. Yes   2. No

57. My skin turns purple   1. Yes   2. No

58. Other, please Specify_____

What parts of your body are affected by these colour changes?

59. My hands   1. Yes   2. No

60. My feet   1. Yes   2. No

61. Other parts, please specify_____

62. Entire body   1. Yes   2. No

63. For how long have you been experiencing these changes in skin colour?

     1. Less than 3 months

     2. 3 to 6 months

     3. 7 to 12 months

     4. 13 months to 5 years

     5. More than 5 years

     6. As long as I can remember

64. Are these changes in your skin colour:

     1. Getting much worse

     2. Getting somewhat worse

     3. Staying about the same

     4. Getting somewhat better

     5. Getting much better

     6. Completely gone

65. In the past year, after a long hot bath or shower, have you ever noticed the pads on the ends of your fingers wrinkle up?   1. Yes   2. No

66. In the past 5 years, what changes, if any, have occurred in your general body sweating?

     1. I sweat much more than I used to

     2. I sweat somewhat more than I used to

     3. I haven't noticed any changes in my sweating

     4. I sweat somewhat less than I used to

     5. I sweat much less than I used to

67. In the past 5 years, what changes, if any, have occurred in the amount your feet sweat?

     1. They sweat much more than they used to

     2. They sweat somewhat more than they used to

     3. I haven't noticed any changes

     4. They sweat somewhat less than they used to

     5. They sweat much less than they used to

68. In the past 5 years, what changes, if any, have occurred in facial sweating after eating spicy foods?

    1. I sweat much more than I used to

    2. I sweat somewhat more than I used to

    3. I haven't noticed any changes in my sweating

    4. I sweat somewhat less than I used to

    5. I sweat much less than I used to

    6. I avoid eating spicy foods because I sweat so much

    7. I avoid eating spicy foods for other reasons

In the past 5 years, what changes, if any, have occurred in your ability to tolerate heat during a hot day, strenuous work or exercise, hot bath or shower, hot tub, or sauna?

69. I now get more overheated	1. Yes	2. No
70. I now get dizzy	1. Yes	2. No
71. I now get short of breath	1. Yes	2. No
72. Other changes, please specify_____		
73. No change	1. Yes	2. No
74. Do your eyes feel excessively dry?	1. Yes	2. No
75. Does your mouth feel excessively dry?	1. Yes	2. No
76. Do you have excessive amounts of saliva formation?	1. Yes	2. No

77. What is the longest period of time that you have had any one of these symptoms: dry eyes, dry mouth, or increased saliva production?

    0. I have not had any of these symptoms

    1. Less than 3 months

    2. 3 to 6 months

    3. 7 to 12 months

    4. 13 months to 5 years

    5. More than 5 years

    6. As long as I can remember

78. For the symptom of dry eyes, dry mouth, or increased saliva production that you have had for the longest period of time, is this symptom:

    0. I have not had any of these symptoms

    1. Getting much worse

    2. Getting somewhat worse

    3. Staying about the same

    4. Getting somewhat better

    5. Getting much better

    6. Completely gone

79. What weight changes, if any, have you had over the past year?

    1. I have lost about _____ pounds

    2. My weight has not changed

    3. I have gained about _____ pounds

80. In the past year, have you noticed any changes in how quickly you get full when eating a meal?

    1. I get full a lot more quickly now than I used to

    2. I get full more quickly now than I used to

    3. I haven't noticed any change

    4. I get full less quickly now than I used to

    5. I get full a lot less quickly now than I used to

81. In the past year, have you felt excessively full or persistently full (bloated feeling) after a meal?

   1. Never   2. Sometimes   3. A lot of the time

82. In the past year, have you felt like you had a persistent upset stomach (nausea)?

   1. Never   2. Sometimes   3. A lot of the time

83. In the past year, have you vomited after a meal?

   1. Never   2. Sometimes   3. A lot of the time

84. In the past year, have you had a cramping or colicky abdominal pain?

   1. Never   2. Sometimes   3. A lot of the time

If Never, go to the question 87. *Else, go to question 85.*

85. Are these pains usually after a meal?   1. Yes   2. No

86. How long have you had these cramping or colicky abdominal pains?

   1. Less than 3 months

   2. 3 to 6 months

   3. 7 to 12 months

   4. 13 months to 5 years

   5. More than 5 years

   6. As long as I can remember

87. In the past year, have you had any bouts of diarrhea?   1. Yes   2. No

If Yes, go to question 88.   *If No, go to question 94.*

88. How frequently does this occur?

   1. Rarely

   2. Occasionally

   3. Frequently, _____times per month

   4. Constantly

89. How severe are these bouts of diarrhea?

   1. Mild   2. Moderate   3. Severe

90. What part of the day do they seem to be worse?

   1. First thing in the morning

   2. Rest of the morning

   3. Afternoon

   4. Evening

   5. During the night

   6. No particular time

91. Do these bouts of diarrhea usually occur after a meal?   1. Yes   2. No

92. Are these bouts of diarrhea accompanied by a lot of rectal gas (flatus)?

   1. Never 2. Occasionally 3. Frequently   4. Always

93. Are your bouts with diarrhea getting:

   1. Much worse

   2. Somewhat worse

   3. Staying the same

   4. Somewhat better

   5. Much better

   6. Completely gone

94. In the past year, have you been constipated?   1. Yes   2. No

If Yes, go to question 95.   *If No, go to question 98.*

95. How frequently are you constipated?

    1. Rarely

    2. Occasionally

    3. Frequently, _____times per month

    4. Constantly

96. How severe are these episodes of constipation?

    1. Mild   2. Moderate   3. Severe

97. Is your constipation getting:

    1. Much worse

    2. Somewhat worse

    3. Staying the same

    4. Somewhat better

    5. Much better

    6. Completely gone

98. Overall, are your abdominal symptoms of vomiting, diarrhea, constipation, or weight loss getting:

    0. I have not had these symptoms

    1. Much worse

    2. Somewhat worse

    3. Staying the same

    4. Somewhat better

    5. Much better

    6. Completely gone

99. Which one of the following symptoms have been most troublesome for you? (Check only one.)

    0. None

    1. Vomiting

    2. Diarrhea

    3. Constipation

    4. Weight loss

100. How long have you had this most troublesome symptom?

    0. I do not have any of these symptoms

    1. Less than 3 months

    2. 3 to 6 months

    3. 7 to 12 months

    4. 13 months to 5 years

    5. More than 5 years

    6. As long as I can remember

101. Is this most troublesome symptom getting:

    0. I do not have any of these symptoms

    1. Much worse

    2. Somewhat worse

    3. Staying the same

    4. Somewhat better

    5. Much better

    6. Completely gone

102. In the past 5 years, how would you rate the amount of trouble, if any, you have had with difficulty in swallowing?

    1. No trouble

    2. Some trouble

    3. A lot of trouble

    4. Constant trouble

103. In the past 5 years, how would you rate the amount of trouble, if any, you have had with everything you eat tasting the same?

    1. No trouble

    2. Some trouble

    3. A lot of trouble

    4. Constant trouble

Have you ever in your adult life:

104. been nauseated or vomited?	1. Yes	2. No
105. had a bout of diarrhea?	1. Yes	2. No
106. lost your appetite for at least part of a day?	1. Yes	2. No
107. felt discomfort or pain in the pit of your stomach?	1. Yes	2. No

108. In the past year, have you ever leaked urine or lost control of your bladder function?

    1. Never

    2. Occasionally

    3. Frequently, _____times per month

    4. Constantly

109. In the past year, have you had difficulty passing urine?

    1. Never

    2. Occasionally

    3. Frequently, _____times per month

    4. Constantly

110. In the past year, have you had trouble completely emptying your bladder?

    1. Never

    2. Occasionally

    3. Frequently, _____times per month

    4. Constantly

111. How would you describe your current sexual desire?

    1. Completely absent

    2. Greatly reduced

    3. Somewhat reduced

    4. About the same or more than in the past

If Male, go to question 112   If Female, go to question 124

112. Are you able to have a full erection?

    1. Never, under any circumstances

    2. Much less frequently than in past

    3. Somewhat less frequently than in past

    4. The same, or more frequently, than in past

Which of the following statements apply to your situation?

113. My ability to have intercourse has not changed	1. Yes	2. No

114. I have erections but am unable to have intercourse   1. Yes   2. No

115. I can have intercourse only some of the time   1. Yes   2. No

116. My erections are definitely impaired   1. Yes   2. No

117. I am able to have intercourse, but am unable to ejaculate   1. Yes   2. No

118. I have "dry orgasms" and afterward my urine looks milky   1. Yes   2. No

119. I have been unable to have erections or they have been impaired since I started taking a

medication_____

120. Other situation, please describe_____

121. None of the above apply

122. How long have you had difficulty with erectile function?   0. I do not have this difficulty

1. Less than 3 months

2. 3 to 6 months

3. 7 to 12 months

4. 13 months to 5 years

5. More than 5 years

6. As long as I can remember

123. Is this difficulty getting: 0. I have not had difficulty

1. Much worse

2. Somewhat worse

3. Staying the same

4. Somewhat better

5. Much better

6. Completely gone

124. In the past year, without sunglasses or tinted glasses, has bright light bothered your eyes?

    1. Never 2. Occasionally   3. Frequently   4. Constantly

125. How severe is this sensitivity to bright light?

    1. Mild   2. Moderate   3. Severe

126 In the past year, have you had trouble focusing your eyes?

    1. Never 2. Occasionally   3. Frequently   4. Constantly

127 How severe is this focusing problem?

    1. Mild   2. Moderate   3. Severe

128 In the past year, have you had blurred vision?

    1. Never 2. Occasionally   3. Frequently   4. Constantly

129 How severe is this blurred vision?

    1. Mild   2. Moderate   3. Severe

130 In the past year, have you had difficulty seeing at night?

    1. Never 2. Occasionally   3. Frequently   4. Constantly

131 How severe is this night vision problem?

    1. Mild   2. Moderate   3. Severe

132 In the past year, has the same degree of light seemed:

    1. Excessively dimmer   2. Much dimmer   3. About the same   4. Much brighter   5. Excessively

brighter

133. Which one of the following eye symptoms is the most troublesome for You? (Check only one)

    0. None   1. Trouble Focusing   2. Blurred Vision   3. Difficulty seeing at night

134 How long have your had this most troublesome eye symptom?

    0.I don't have any of these symptoms

    1. Less than 3 months

    2. 3 to 6 months

    3. 7 to 12 months

    4. 13 months to 5 years

    5. More than 5 years

    6. As long as I can remember

135 Is this most troublesome symptom with your eyes getting:

    0. I don't have any of these symptoms

    1. Much worse

    2. Somewhat worse

    3. Staying the same

    4. Somewhat better

    5. Much better

    6. Completely gone

136. In the past year, have you ever noticed or been told that while sleeping you stop breathing for several seconds?

          1. Yes   2. No

137. In the past year, have you ever noticed or been told that while sleeping you snore loudly?

          1. Yes   2. No

Have you ever been told you have or been diagnosed as having:

138. Narcolepsy?	1. Yes	2. No	3. Don't know
139. Obstructive sleep apnoea?	1. Yes	2. No	3. Don't know
140. Abnormal or disordered sleep patterns?	1. Yes	2. No	3. Don't know

141 Currently, how refreshing and restorative is your sleep?

    1. Not at all restorative - derive no benefit

    2. Some slight restorative value

    3. Restorative, but not adequate

    4. Relatively satisfactory

    5. Very satisfactory - feel completely refreshed

142 Compared with a year ago, how would you rate your own sleep over the last month?

    1. Last month was much worse than a year ago

    2. Last month was slightly worse than a year ago

    3. Last month was about the same as a year ago

    4. Last month was slightly better than a year ago

    5. Last month was much better than a year ago

143. Have you ever in your adult life had difficulty getting to sleep or staying asleep once you were asleep?

          1. Yes   2. No

144. In the past year, have you ever noticed or been told that during the day you sometimes breathe very loudly(e.g., croup)?   1. Yes   2. No

Fig 4.3 Composite Autonomic symptom Scale

4.5.3.2 Leeds assessment of neuropathic symptoms and signs (LANSS)[355], (Table 4.5).

This is a pain scale used to identify patients in whom neuropathic mechanisms dominate their pain experience. Developed based on 60 patients with nociceptive and neuropathic pain and validated in a further 40 patients. It is a seven-item instrument which includes 5 self report questions and two sensory tests (with the physician present). A cut-off point of 12 is sensitive (83%) and specific (87%) for differentiating between neuropathic and non-neuropathic pain. It assesses five types of pain (thermal, dysesthesia, paroxysmal, evoked and autonomic dysfunction).

Question	Answer	Score
1) In the area where you have pain, do you also have 'pins and needles', tingling or prickling sensations?	Yes	5
	No	0
2) Does the painful area change colour (look mottled or more red) when the pain is particularly bad?	Yes	5
	No	0
3) Does your pain make the affected skin abnormally sensitive to touch? (Getting unpleasant sensations or pain when lightly stroking the skin might describe this.)	Yes	3
	No	0
4) Does your pain come on suddenly and in bursts for no apparent reason when you are completely still? (Words like 'electric shocks', jumping and bursting might describe this.)	Yes	2
	No	0
5) In the area where you have pain, does your skin feel unusually hot like a burning pain?	Yes	1
	No	0
6) Gently <u>rub</u> the painful area with your index finger and then rub a non-painful area (for example, an area of skin further away or on the opposite side from the painful area). How does this rubbing feel in the painful area?	I feel discomfort (like pins and needles, tingling or burning) different from the normal area.	5
	No difference	0
7) Gently press on the painful area with your finger tip then gently press in the same way onto a non-painful area (the same non-painful area that you chose in the last question). How does this feel in the painful area?	I feel numbness or tenderness in the painful area different from the normal area.	3
	No difference	0
	Total	

Table 4.5 Leeds assessment of neuropathic symptoms and signs questionnaire.

4.5.4 Analysing results

All demographic data were expressed as mean and standard deviation. Statistical analysis used was Wilcoxon matched-pairs rank test unless otherwise stated. Cardiac autonomic tests were expressed as mean and standard deviation in tables; mean, minimum and maximum values graphically and/or expressed as percentage of the AFD subjects with abnormal tests over total AFD subjects tested. Plasma catecholamines were expressed as mean and standard deviation and subdivided based on supine or tilted position, sex or type of mutation. QSART data was compared to normative data from Sletten et al 2010[356] and COMPASS scores were compared to normative data from Suarez et al[353] as no controls were recruited for this study. Statistical analysis used to compare study population with normative data was the 1 sample t-test.

4.6 Results

4.6.1 Cardiac autonomic function tests

Demographics (Table 4.6).
No control group was recruited. 24 AFD subjects were recruited; 9 males and 15 females with a mean age of 42.8 ± 15.9 years, majority were on ERT (79.2%) and had a missense mutation (75.0%).

	n = 24
Age (years)	42.8 ± 15.9
Sex	9 males, 15 females
ERT, n (%)	19 (79.2%)
Missense mutations, n (%)	18 (75.0%)
MSSI	16.5 ± 8.8
iGFR (ml/min/1.73m^2)	82.7 ± 24.9
UACR (mg/mmol Cr)	12.6 ± 23.4
UPCR (mg/mmol Cr)	26.6 ± 36.1
LVMI (g/m^2)	97.4 ± 34.4

Table 4.6. Demographic data for cardiac autonomic tests.

Head tilt (Fig 4.4 – 4.6, Table 4.7)

Mean systolic, diastolic and mean arterial pressure (MAP) rose in AFD subjects from 0 to 9 minutes with an appropriate rise in HR. Systolic BP drop of $\geq$ 20mmHg was present in 1 male and 1 female at 1 minute, 1 female at 3 minutes and 1 female at 6 minutes. Diastolic BP drop of $\geq$ 10mmHg was present in 1 female at 1 minute. HR fall or a rise of $<$ 5 bpm was present in 1 male and 5 females at 1 minute, 1 male and 4 females at 3 minutes, 1 male and 2 females at 6 minutes and 2 females at 9 minutes.

Isometric, mental arithmetic and cold pressor test (Fig 4.7, Table 4.8)

Mean increase in systolic and diastolic BP in all AFD subjects was present in all 3 pressor tests but HR only rose for isometric exercise and not the mental arithmetic or cold pressor tests (mean change in HR was 6.2 ± 11, 0.2 ± 6.7 and 1.4 ± 9.7 bpm respectively). When analysed according to sex of subjects, there was no significant rise in HR in males and females for the mental arithmetic and cold pressor tests. Reviewing each subject individually, in the isometric exercise trial patient 23 had poor response in systolic BP, diastolic BP and HR response, in the mental arithmetic exercise, trial patients 15, 18 and 35 had a poor responses, and in the cold pressor exercise trial patient 17 had a poor response.

Respiratory sinus arrhythmia (Fig 4.8, Table 4.9)

The mean change in HR for all subjects, males and females were 20.8 ± 8.7, 26.1 ± 7.3 and 17.2 ± 7.9 bpm respectively. Of the 22 AFD subjects who completed this test, only 2 females had an abnormal HR response to deep breathing; trial number 14 had a minimal HR rise of 4bpm and trial number 1 had a modest HR rise of 7bpm.

Hyperventilation (Fig 4.9,Table 4.10)

The mean change in HR for all subjects, male and females were 14.2 ± 12.1, 19.8 ± 13.8 and 10.1 ± 9.2 bpm respectively. Of the 21 AFD subjects who completed this test, 2 males and 8 females had HR rises of $<$ 10 bpm (range from -1 to 6 bpm change in HR).

Valsalva manoeuvre (Fig 4.10, Table 4.11)

The mean HR at rest was 66.5 ± 10.7 bpm rising to 88.9 ± 14.1 bpm in phase II with an appropriate fall to 57.0 ± 9.7 bpm in phase IV. The mean Valsalva ratio was 3.4 ± 2.9. Individually reviewing results showed that 1 male and 2 females had valsalva ratios of < 1.0 (abnormal).

Summary of cardiac autonomic tests (Table 4.12)

0 to 15% of AFD patients had an abnormality in 1 of the sympathetic cardiac autonomic tests, 9.1 to 47.6% of AFD patients had an abnormality in of the parasympathetic cardiac autonomic tests and 16.7 to 23.8% of AFD patients had an abnormality in 1 of the sympathetic and parasympathetic cardiac autonomic tests.

	0 min	1 min	3 min	6 min	9 min	0 vs 3 min
All (n=21)						
Systolic (mmHg)	106.8 ± 11.9	108.4 ± 16.1	109.6 ± 11.4	112.8 ± 14.1	111.8 ± 14.5	ns
Diastolic (mmHg)	58.8 ± 8.1	64.0 ± 8.5	66.1 ± 7.3	66.5 ± 11.7	65.5 ±8.8	p = 0.0005
MAP (mmHg)	76.2 ± 8.9	82.2 ± 10.8	85.0 ± 8.6	84.8 ± 12.6	85.5 ± 9.2	p = 0.0004
HR (bpm)	66.9 ±10.5	76.3 ± 11.9	76.7 ± 10.4	77.7 ± 10.8	77.5 ± 9.5	P = 0.0001
Males (n=9)						
Systolic (mmHg)	113.3 ± 10.3	111.9 ± 18.6	114.1 ± 14.4	116.3 ± 18.2	116.4 ± 16.9	ns
Diastolic (mmHg)	60.3 ± 9.1	66.4 ± 10.5	68.9 ± 9.0	69.0 ± 12.3	67.9 ± 11.4	p = 0.0091
MAP (mmHg)	79.7 ± 9.6	85.6 ± 14.4	90.7 ± 9.6	88.2 ± 13.2	89.2 ± 9.6	p = 0.0091
HR (bpm)	64.8 ± 8.2	76.3 ± 9.3	77.9 ± 9.0	77.9 ± 7.9	77.7 ± 8.7	p = 0.0039
Females (n=12)						
Systolic (mmHg)	101.8 ± 10.8	105.8 ± 14.2	106.3 ± 7.5	110.1 ± 10.1	108.3 ±12.0	ns
Diastolic (mmHg)	57.8 ± 7.6	62.2 ± 6.6	64.1 ± 5.3	64.7 ± 11.3	63.8 ± 6.2	p = 0.0262
MAP (mmHg)	73.6 ± 7.7	79.9 ± 6.7	80.7 ± 4.7	82.2 ± 12.1	82.8 ± 8.1	p = 0.0161
HR (bpm)	68.4 ± 12.1	76.4 ± 13.9	75.8 ± 11.7	77.5 ± 12.9	77.3 ± 10.5	p = 0.0058

Table 4.7. Mean and SD of systolic, diastolic, MAP and HR of AFD patients for head tilt test. Statistical analysis used is Wilcoxon matched-pairs rank test.

Blood Pressure and Heart Rate Change Tilt Table test (All)

% of polpulation (ALL) with Blood Pressure or Heart Rate Changes in Head Tilt

Fig 4.4. Head tilt test in all AFD patients; (A) Mean, minimum and maximum of the systolic, diastolic and mean arterial BP, and HR at 0, 1, 3, 6 and 9 minutes, (B) Percentage of AFD patients with abnormal responses at 1, 3, 6 and 9 minutes.
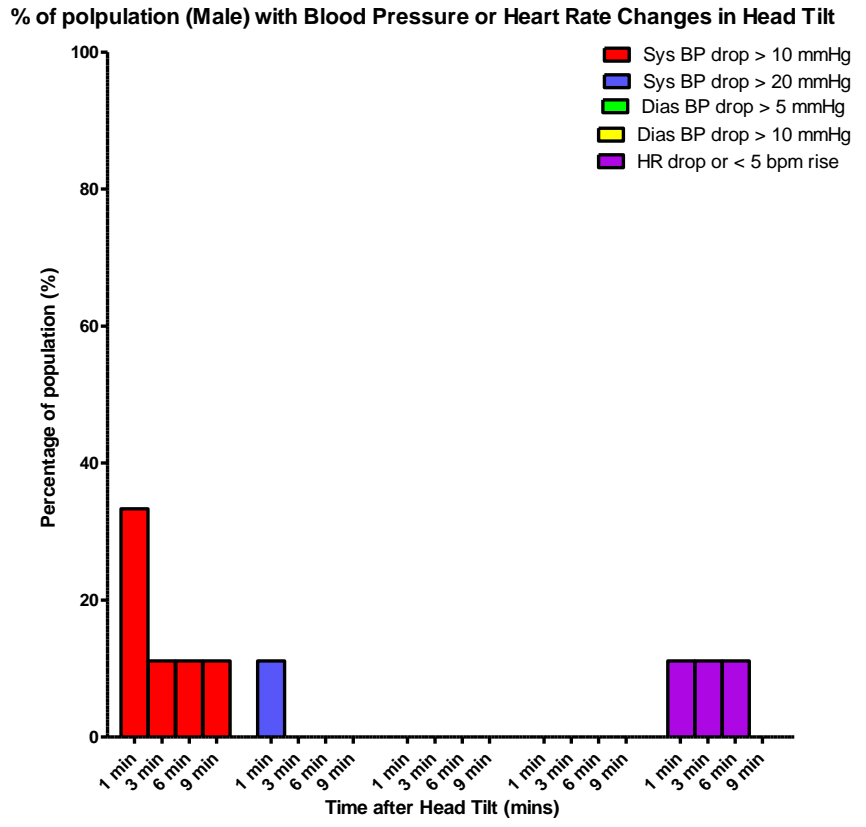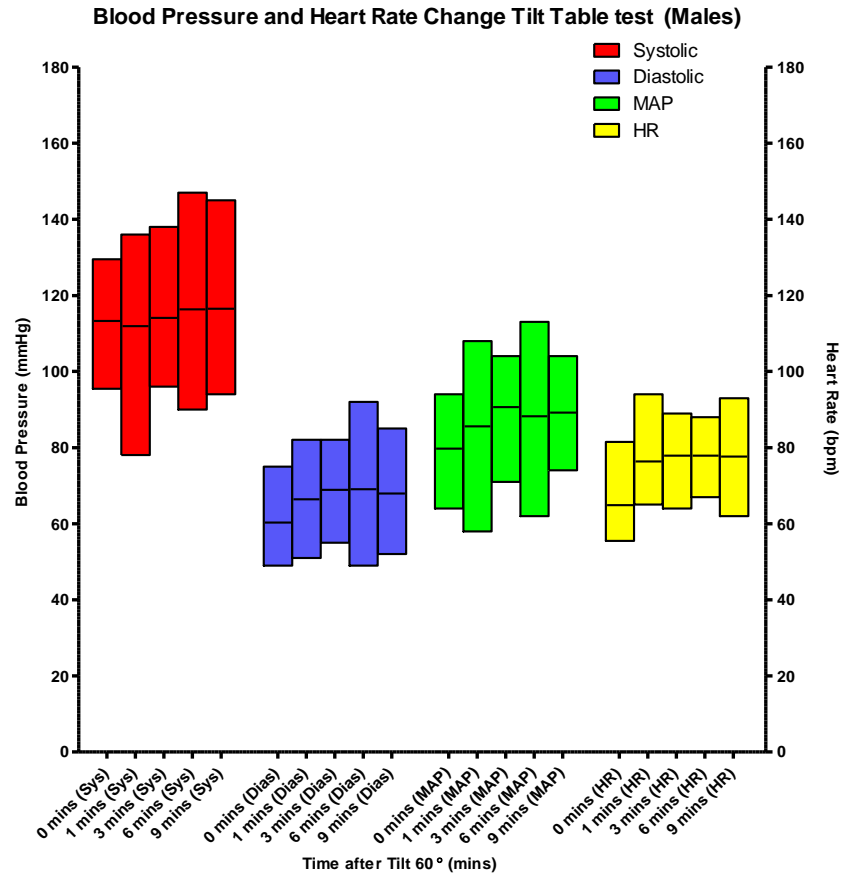
Fig 4.5. Head tilt test in male AFD patients; (A) Mean, minimum and maximum of the systolic, diastolic and mean arterial BP, and HR at 0, 1, 3, 6 and 9 minutes, (B) Percentage of AFD patients with abnormal responses at 1, 3, 6 and 9 minutes.
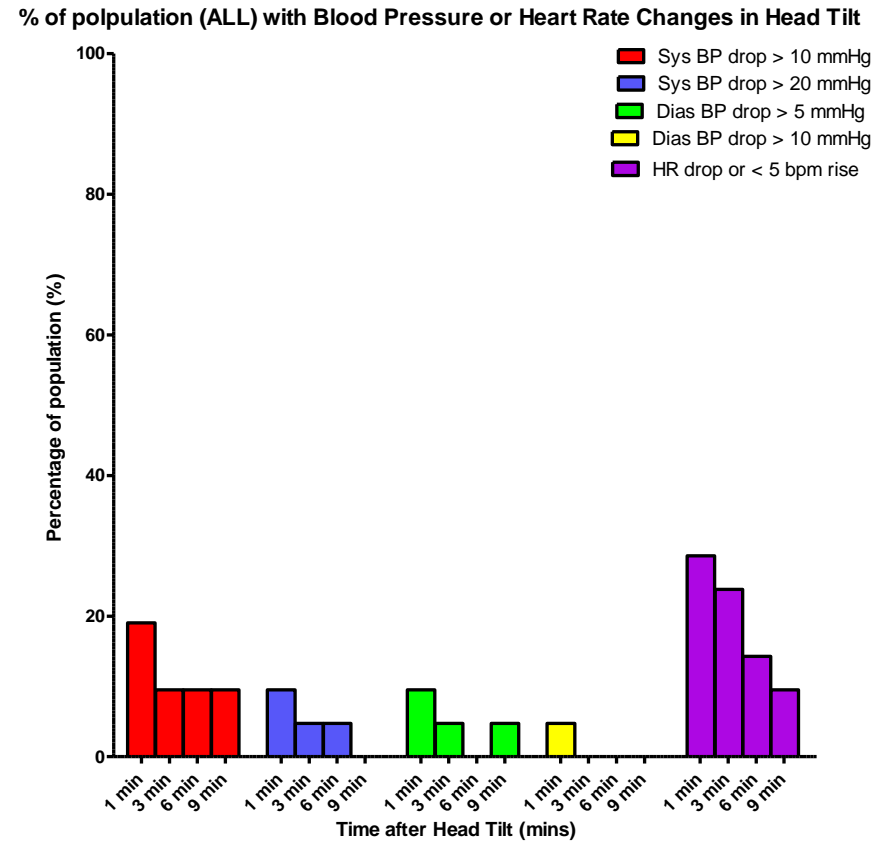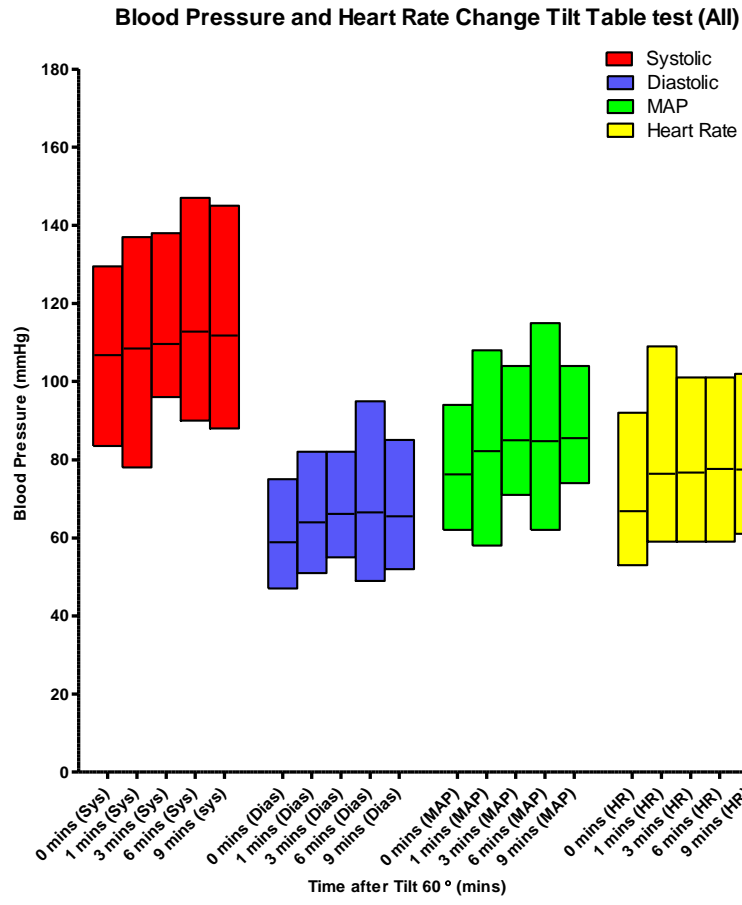
Fig 4.6. Head tilt test in female AFD patients; (A) Mean, minimum and maximum of the systolic, diastolic and mean arterial BP, and HR at 0, 1, 3, 6 and 9 minutes, (B) Percentage of AFD patients with abnormal responses at 1, 3, 6 and 9 minutes.
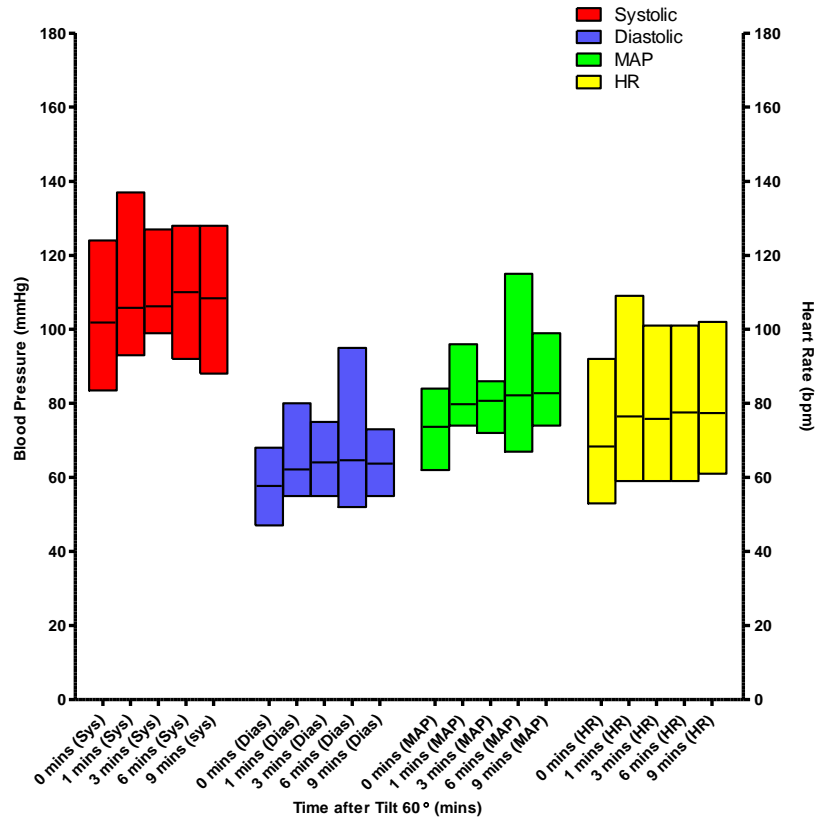
	Isometric Exercise				Mental Arithmetic				Cold Pressor			
	Pre test	Post test	Change	Pre vs Post test	Pre test	Post test	Change	Pre vs Post test	Pre test	Post test	Change	Pre vs Post test
All	**N = 20**				**N = 20**				**N = 17**			
Systolic (mmHg)	108.2 ± 12.1	133.9 ±23.8	25.8 ± 15.6	p < 0.0001	110.6 ± 15.2	122.7 ±18.9	12.1 ± 10.5	p = 0.0003	112.5 ± 17.9	136.3 ± 22.5	23.8 ± 14.0	p = 0.0003
Diastolic (mmHg)	59.0 ± 9.0	75.5 ± 12.6	16.5 ± 10.5	p = 0.0001	63.0 ± 8.6	68.0 ± 11.0	5.0 ± 4.6	p = 0.0012	60.2 ± 9.9	75.9 ± 13.7	15.8 ± 9.9	p = 0.0005
MAP (mmHg)	78.3 ± 9.6	101.1 ± 17.8	22.8 ± 12.5	p < 0.0001	82.6 ± 9.6	89.0 ± 14.3	6.5 ± 7.4	p = 0.0017	79.5 ± 11.2	100.8 ± 16.3	21.3 ± 12.7	p = 0.0005
HR (bpm)	64.6 ±10.9	70.9 ± 13.7	6.2 ± 11.1	p = 0.0083	70.0 ± 10.7	70.2 ± 8.9	0.2 ± 6.7	ns	67.7 ±10.9	69.1 ± 13.7	1.4 ± 9.7	ns
Males	**N = 9**				**N = 9**				**N= 7**			
Systolic (mmHg)	112.0 ± 13.44	150.0 ± 22.1	38.0 ± 13.5	p = 0.0039	118.6 ± 12.9	132.1 ± 16.6	13.6 ±15.2	ns	126.0 ± 13.9	148.4 ± 21.7	22.4 ± 18.3	p = 0.0223
Diastolic (mmHg)	60.9 ± 10.0	84.7 ± 10.1	23.8 ± 8.9	p = 0.0039	67.1 ± 7.9	74.3 ± 9.8	7.2 ± 4.4	p = 0.0138	64.9 ± 11.7	78.9 ± 16.5	14.0 ± 11.0	p = 0.0313
MAP (mmHg)	81.6 ± 7.6	113.9 ± 15.2	32.2 ± 10.8	p = 0.0090	87.7 ± 6.6	95.8 ± 12.1	8.1 ± 6.9	p = 0.0091	88.7 ± 8.0	106.6 ± 18.3	17.9 ± 14.5	p = 0.0343
HR (bpm)	65.3 ± 7.8	74.8 ±13.9	9.4 ± 16.1	Ns	70.8 ± 7.9	71.3 ± 6.3	0.6 ± 9.1	ns	71.1 ± 7.6	66.9 ± 12.5	-4.3 ± 11.38	ns
Females	**N = 11**				**N = 11**				**N = 10**			
Systolic (mmHg)	105.0 ± 10.5	120.7 ± 16.3	15.7± 8.3	p = 0.0038	104.0 ± 14.2	114.9 ±17.6	10.9 ± 4.7	p = 0.0038	103.0 ± 14.1	127.8 ± 19.8	24.8 ± 11.2	p = 0.0059
Diastolic (mmHg)	57.5 ± 8.4	68.0 ± 9.1	10.6 ± 7.6	p = 0.0058	59.6 ±7.9	62.7 ± 9.2	3.1 ± 4.1	p = 0.0492	56.9 ± 7.3	73.9 ± 11.9	17.0 ± 9.5	p = 0.0020
MAP (mmHg)	75.6 ± 10.6	90.6 ± 12.1	14.9 ±7.4	p = 0.0038	78.3 ± 9.9	83.5 ± 14.1	5.1 ±7.8	ns	73.1 ± 8.2	96.8 ± 14.3	23.7 ± 11.3	p = 0.0059
HR (bpm)	64.1 ± 13.3	67.6 ± 13.3	3.5 ± 3.1	P = 0.0122	69.3 ± 13.0	69.2 ± 10.9	-0.1 ± 4.5	ns	65.3 ± 12.5	70.6 ± 15.0	5.3 ± 6.4	ns

Table 4.8. Summary of AFD patients systolic, diastolic, mean arterial BP and HR for isometric, mental arithmetic and cold pressor tests. Results are mean and SD. Statistical analysis used is the Wilcoxon matched-rank pairs test.
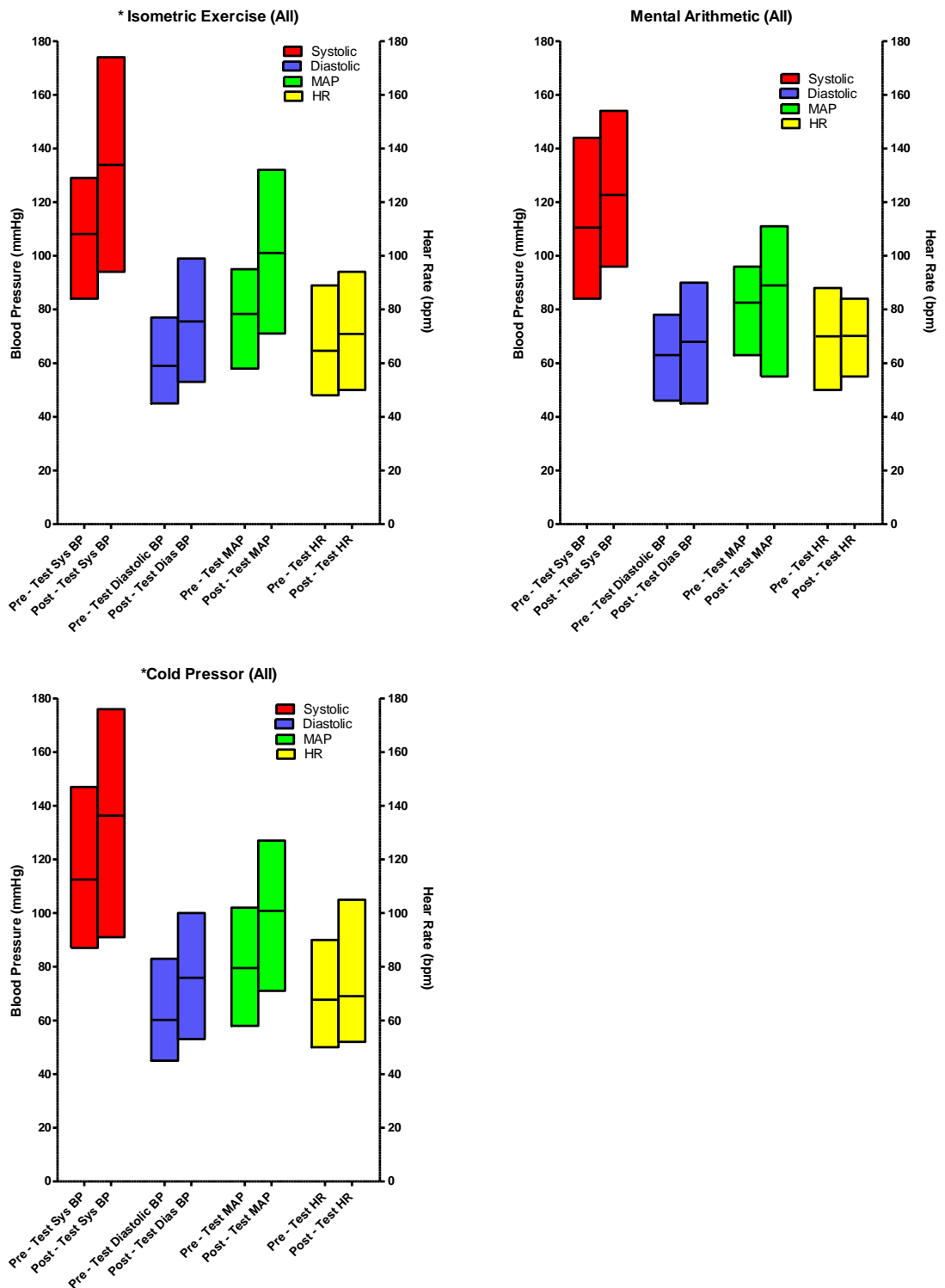
Fig 4.7. Isometric exercise, mental arithmetic and cold pressor tests in AFD patients. Mean, minimum and maximum of the systolic, diastolic and mean arterial BP, and HR; pre and post test.

Respiratory sinus arrhytmia
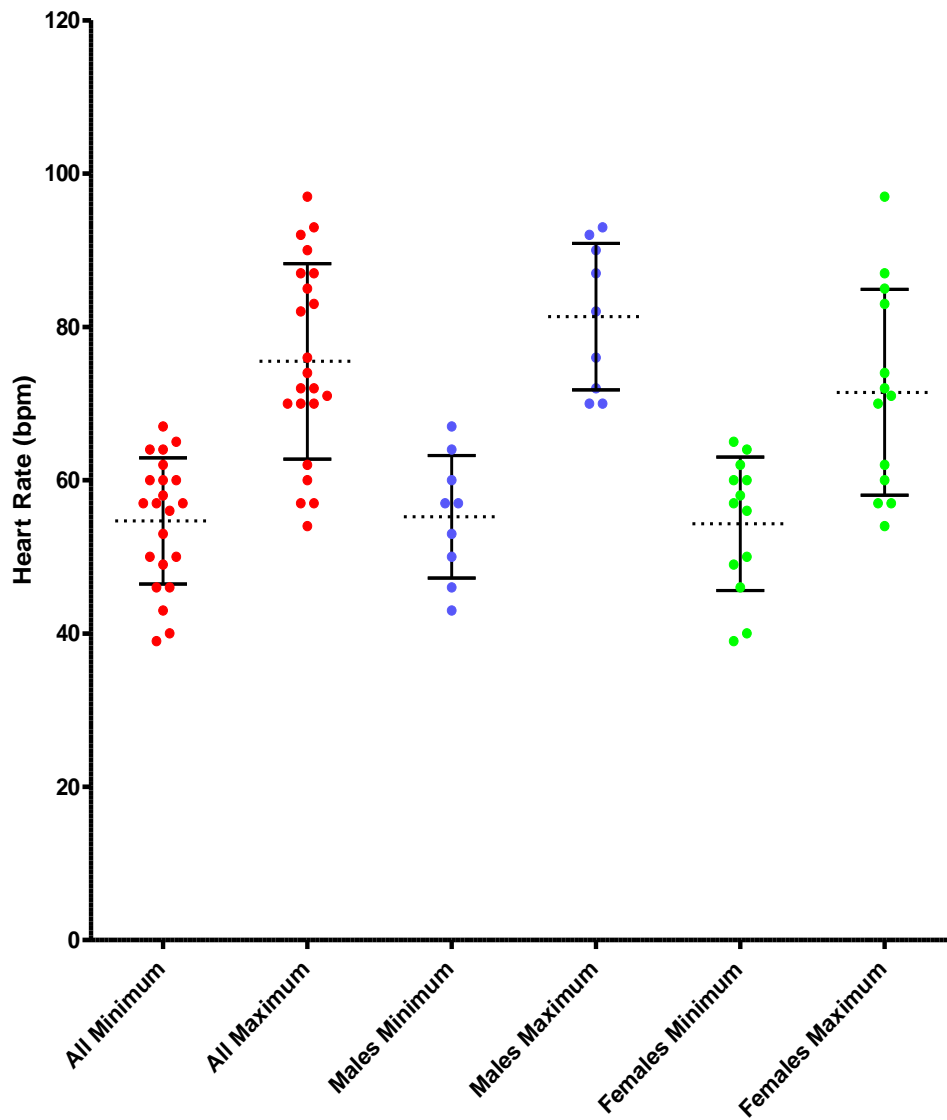
Fig 4.8. Respiratory sinus arrhythmia test with mean and SD of minimum and maximum HR in all male and female AFD patients.

	Minimum HR	Maximum HR	Difference	Pre vs Post test
All (n = 22)	54.7 ± 8.2	75.5 ± 12.8	20.8 ± 8.7	p < 0.0001
Male (n = 9)	55.2 ± 8.0	81.3 ± 9.6	26.1 ± 7.3	p = 0.0091
Female (n=13)	54.3 ± 8.7	71.5 ± 13.4	17.2 ± 7.9	p = 0.0017

Table 4.9. Summary of respiratory sinus arrhythmia data showing mean and SD. Statistical analysis used is the Wilcoxon matched-pairs rank test.
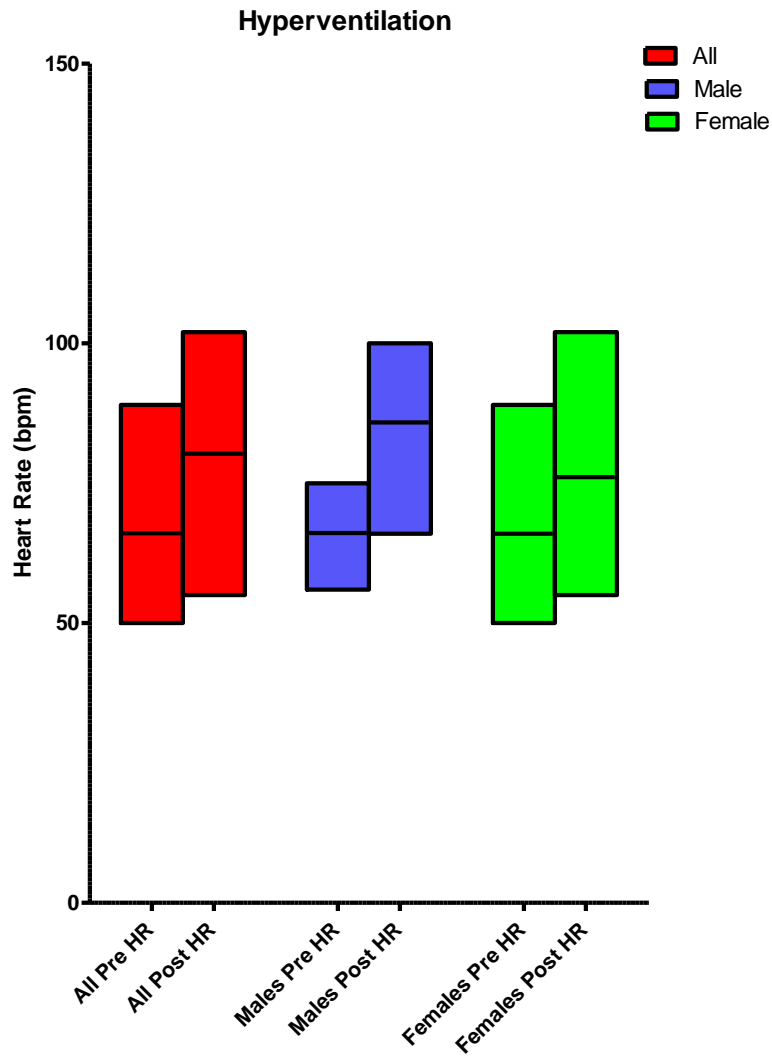
Fig 4.9. Hyperventilation test with mean, minimum and maximum HR pre and post test in all, male and female AFD patients.

	Pre test	Post test	Change	Pre vs Post test
All HR (n = 21)	66.1 ± 10.3	80.3 ± 15.7	14.2 ± 12.1	$p < 0.0001$
Male HR (n = 9)	66.1 ± 7.7	85.9 ± 12.0	19.8 ± 13.8	$p = 0.0078$
Female HR (n = 12)	66.0 ± 12.2	76.1 ± 17.3	10.1 ± 9.2	$p = 0.0025$

Table 4.10. Summary of data from hyperventilation test showing mean and SD of HR pre and 30 seconds after start of the test. Statistical analysis used is the Wilcoxon matched-pairs rank test.
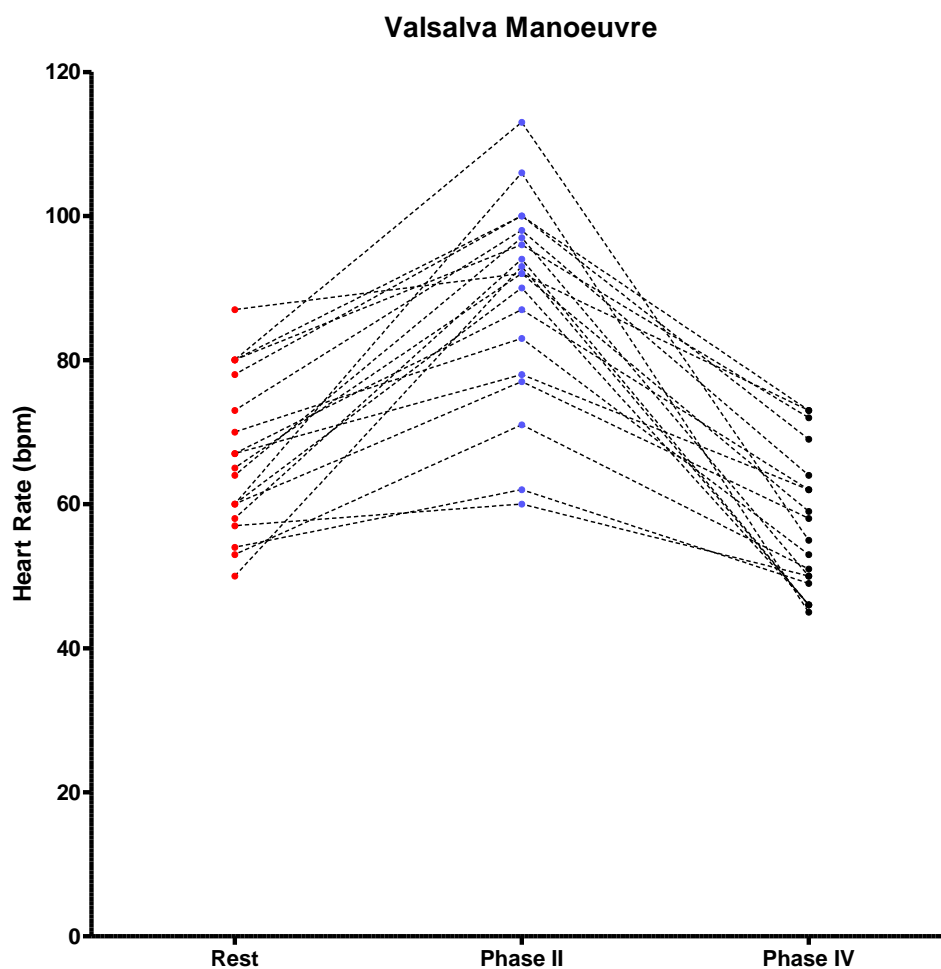
Fig 4.10. Graph of all AFD patients HR during Valsalva manoeuvre.

	Rest	Phase II	Phase IV	Valsalva Ratio
All HR (n = 19)	66.5 ± 10.7	88.9 ± 14.1	57.0 ± 9.7	3.4 ± 2.9
Male HR (n = 9)	68.1 ± 9.3	94.4 ± 11.5	56.3 ± 10.2	3.1 ± 2.2
Female HR (n = 10)	65.0 ± 12.2	83.9 ± 14.8	57.6 ± 9.9	3.6 ± 3.5

Table 4.11. Summary of data from valsalva manoeuvre test showing mean and SD of HR during the rest phase, phase II and phase IV and the valsalva ratio.

Type of Test	Name	Number	Definition of abnormal test	Abnormal test	Trial number	% abnormal
Sympathetic	Head Tilt	N = 21	Systolic BP drop > 20mmHg at 3 minutes	1 Female	11	4.8
Sympathetic	Head Tilt	N = 21	Diastolic BP drop > 10mmHg at 3 minutes	None	none	0
Parasympathetic and sympathetic	Head Tilt	N = 21	HR drop or < 5 bpm rise at 3 minutes	1 Male 4 Females	13, 14, 19, 22, 23	23.8
Sympathetic	Isometric Exercise	N = 20	< 10 mmHg rise in sys, < 5mmHg rise in dias and <3bpm rise in HR	1 Female	23	5
Sympathetic	Mental Arithmetic	N = 20	< 10 mmHg rise in sys, < 5mmHg rise in dias and <3bpm rise in HR	1 Male 2 Females	15, 18, 35	15
Sympathetic	Cold Pressor	N = 17	< 10 mmHg rise in sys, < 5mmHg rise in dias and <3bpm rise in HR	1 Male	17	5.9
Parasympathetic	Respiratory Sinus Arrhythmia	N = 22	Minimal < 5 bpm HR rise Modest 6-10 bpm HR rise	2 Females	1, 14	9.1
Parasympathetic	Hyperventilation	N = 21	< 10 bpm rise in HR	2 Males 8 Females	1, 4, 8, 11, 13, 14, 19, 22, 23, 49	47.6
Parasympathetic and sympathetic	Valsalva Manoeuvre	N = 18	Valsalva ratio < 1.0	1 Male 2 Females	9, 22, 23	16.7

Table 4.12. Summary of cardiac autonomic tests abnormalities in AFD patients.

4.6.2 Plasma catecholamines (Fig 4.11 - 4.12, Table 4.13)

No control group was recruited. 20 AFD subjects were recruited; 9 males and 11 females with a mean age of 40.9 ± 15.4 years, majority were on ERT (81.0%) and had a missense mutation (66.7%). Mean plasma noradrenaline level was 236.6pg/ml in a supine position with a significant rise to 306.9pg/ml in a tilted position. Mean plasma adrenaline level rose from 39.9pg/ml in a supine position to 54.2pg/ml in a tilted position. There was no difference in plasma noradrenaline or adrenaline levels in AFD subjects when subdivided by sex or type of mutation (Fig   4.12).

	n = 20
Age (years)	40.9 ± 15.4
Sex	9 males, 11 females
ERT, n (%)	17 (81.0%)
Missense mutation, n (%)	14 (66.7%)
Plasma Noradrenaline [supine] (pg/ml)	236.6 ± 58.1
Plasma Noradrenaline [tilted], (pg/ml)	306.9 ± 71.1
Plasma Adrenaline [supine], (pg/ml)	39.9 ± 20.5
Plasma Adrenaline [tilted], (pg/ml)	54.2 ± 31.6
MSSI	17.5 ± 9.0
iGFR (ml/min/1.73m^2)	80.5 ± 25.4
UACR (mg/mmol Cr)	14.2 ± 25.3
UPCR (mg/mmol Cr)	28.9 ± 38.2
LVMI (g/m^2)	99.4 ± 35.1

Table 4.13. Demographics of AFD patients tested for plasma catecholamine levels. Mean and SD of results are shown.
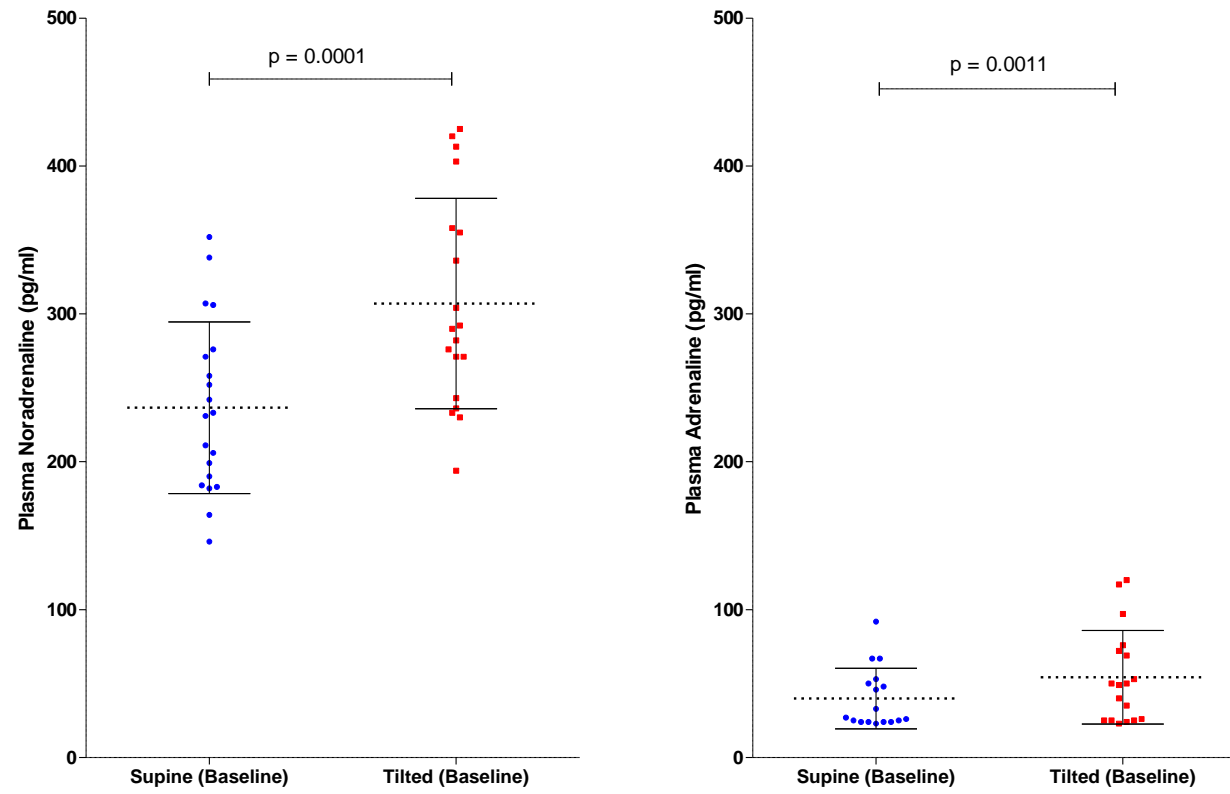
Fig 4.11. Plasma noradrenaline and adrenaline levels in supine and tilted positions for AFD patients. Statistical analysis used was Wilcoxon matched-pairs rank test.
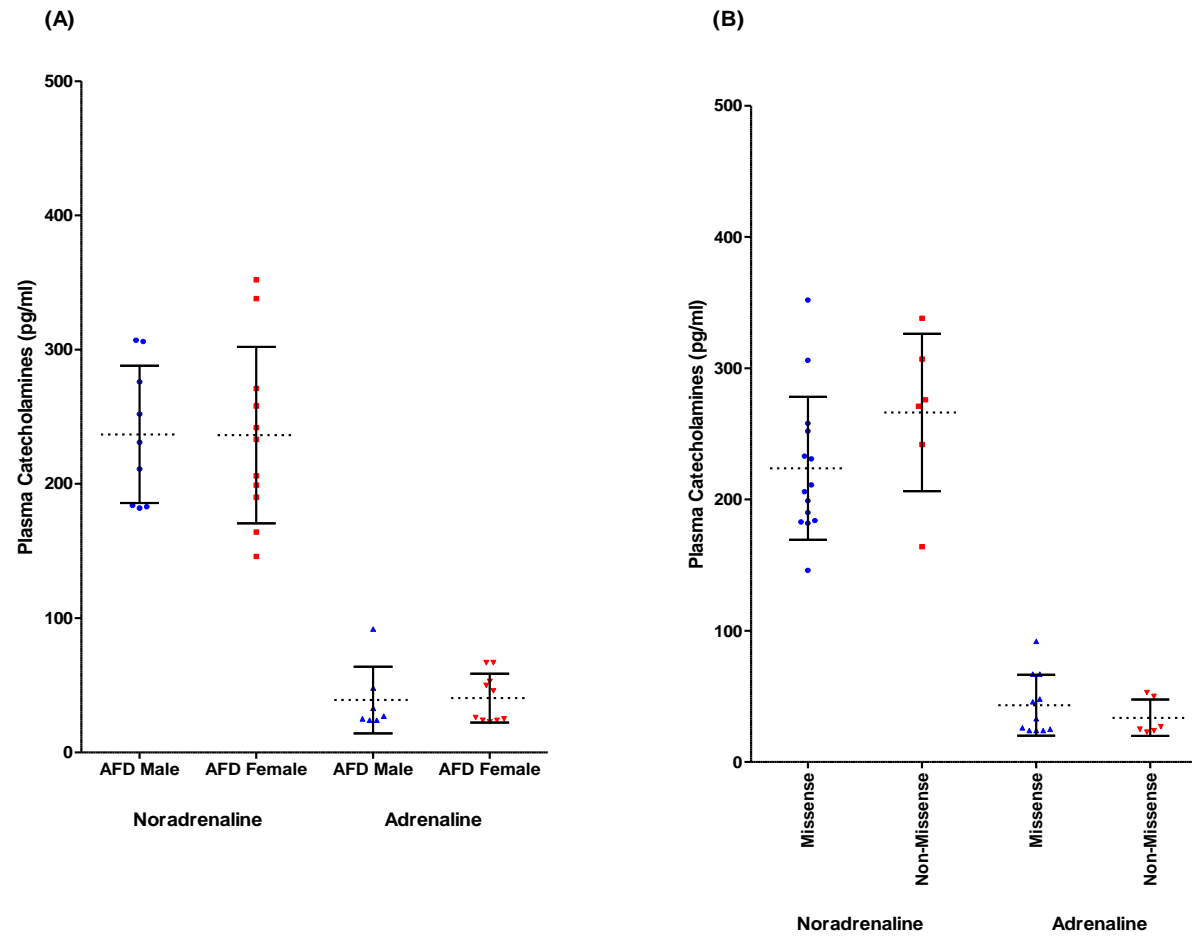
Fig 4.12. Plasma catecholamine levels in AFD categorised by (A) sex and (B) mutation type.

4.6.3 QSART (Fig 4.13, Table 4.14 – 4.15)

No control group was recruited. 20 AFD subjects were recruited; 8 males and 12 females with a mean age of 41.7 ± 15.9 years, majority were on ERT (75.0%) and had a missense mutation (80.0%). Mean baseline sweat rate was 54.8 ± 12.8 nL/min and mean total sweat volume was 0.69 ± 0.53 $\mu L/cm^2$. Comparing our AFD population with controls from Sletten et al[356] there was no significant difference in median total sweat volume in males and females or median latency in males (Table 4.15). Comparing total sweat volumes in AFD subjects showed no significant difference when subdivided for age, sex of type of mutation (Fig 4.13).

	n = 20
Age (years)	41.7 ± 15.9
Sex	8 males, 12 females
ERT, n (%)	15 (75.0%)
Missense mutation, n (%)	16 (80.0%)
Baseline Sweat (nL/min)	54.8 ± 12.8
Latency (min)	1.7 ± 0.6
Total Sweat volume ($\mu L/cm^2$)	0.69 ± 0.53
MSSI	15.2 ± 8.7
iGFR (ml/min/1.73m^2)	84.6 ± 24.2
UACR (mg/mmol Cr)	12.0 ± 24.8
UPCR (mg/mmol Cr)	25.1 ± 38.0
LVMI (g/m^2)	93.2 ± 30.9

Table 4.14. Showing demographic data for AFD population tested with Q Sweat machine.

		Control M (n=44)	Control F (n=50)	AFD M (n=8)	AFD F (n=12)
Total sweat volume ($\mu l/cm^2$)	Median	1.3	0.5	0.90	0.47
	Min	0.3	0.1	0.11	0.18
	Max	3.9	2.4	1.70	1.27
Latency (mins)	Median	1.7	2.0*	1.8	1.7*
	Min	1.0	0.2	0.6	1.1
	Max	3.0	3.5	2.8	2.6

Table 4.15. Summary of Q-Sweat data in AFD patients comparing with normative data from Sletten et al 2010[356]. *No significant difference using 1 sample t-test except for median latency between AFD females compared with control females.
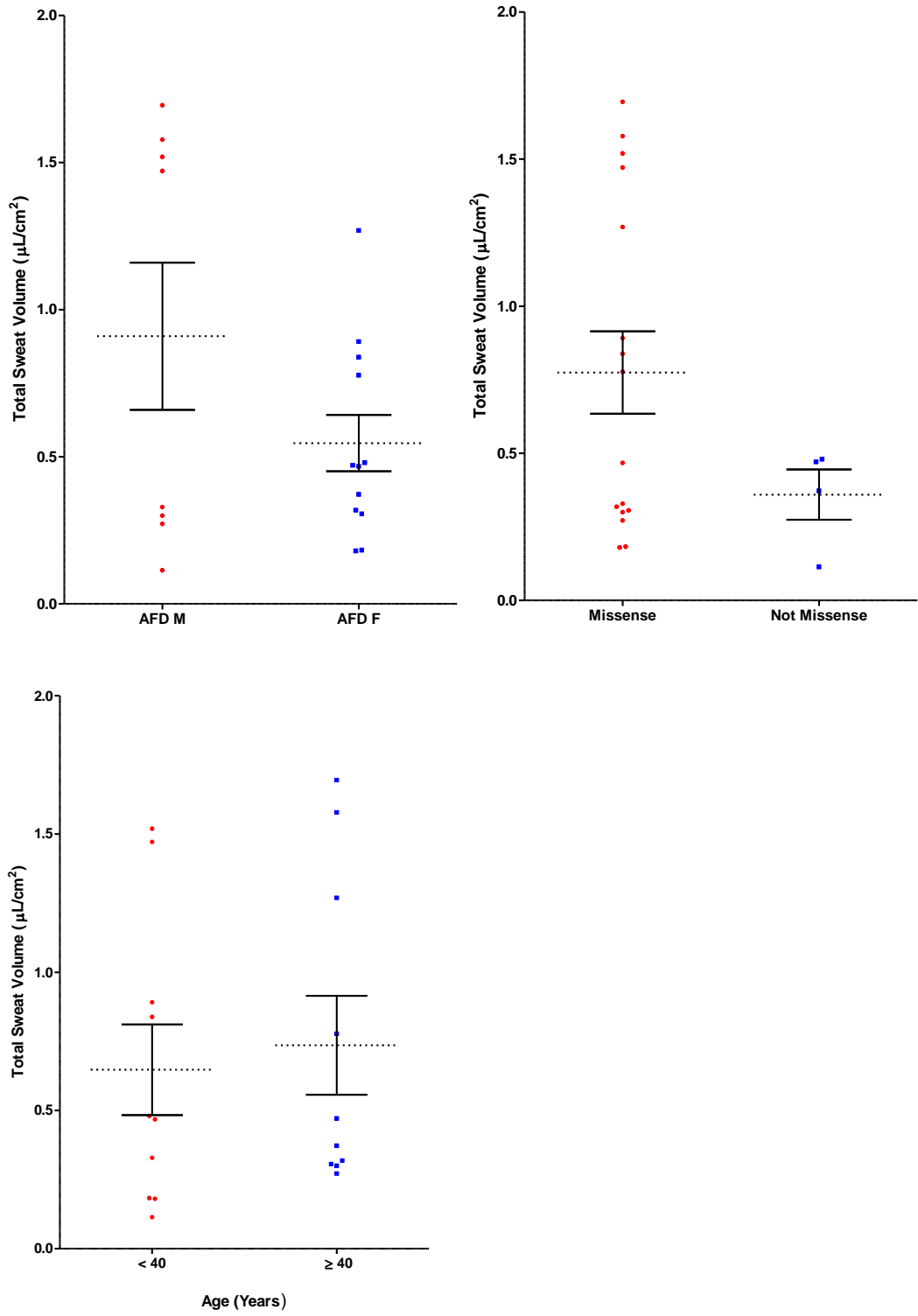
Fig 4.13. Total sweat volume in AFD patients comparing sex, type of mutation and age. Statistical analysis used was the Mann Whitney U-test.

4.6.4 LANSS and COMPASS scores (Fig 4.14 – 4.16, Table 4.16 – 4.17)

No control group was recruited. 37 AFD subjects were recruited; 16 males and 21 females with a mean age of 47.3 ± 15.0 years, majority were on ERT (65.7%) and had a missense mutation (78.4%).

Mean LANSS score was 6.4 ± 8.0 and 11 out of 37 (29.7%) has LANSS score ≥ 12 indicating significant neuropathic pain[355]. No significant difference in LANSS scores in AFD patients differentiated for age, sex, type of AFD mutation or treated with ERT. Multiple regression analysis for LANSS scores showed no significant correlation (Data not shown).

Mean COMPASS score was 34.5 ± 25.3 for AFD subjects which is significantly higher than controls (NML) and peripheral neuropathy (PN) groups and significantly lower than neurogenic autonomic failure (NAF) groups from Suarez et al[353] who had mean levels of 9.8 ± 9, 25.9 ± 17.9 and 52.3 ± 24.2 respectively (Fig 4.15). The most common COMPASS subscores for AFD subjects was pupillomotor, gastroparesis and sleep subscores and least common was reflex syncope. In AFD subjects all COMPASS subscores were higher than controls except for the reflex syncope subscore. When AFD subjects were compared to PN and NAF groups there was no difference in subscores for urinary and vasomotor. In the pupillomotor subscore AFD subjects had a significantly higher mean score than PN and NAF groups. Also AFD subjects had a significantly lower mean score for male sexual dysfunction when compared to PN and NAF groups. For orthostatic intolerance and secretomotor subscores AFD subjects had similar mean scores to PN group but lower than NAF group but for gastrointestinal symptoms (gastroparesis, diarrhoea and constipation) and sleep subscores had significantly higher means to PN group but similar to the NAF group (Table 4.17).

	n = 37
Age (years)	47.3 ± 15.0
Sex	16 males
	21 females
ERT, n (%)	23 (65.7%)
Missense mutation, n(%)	29 (78.4%)
COMPASS score	34.5 ± 25.3
LANSS score	6.4 ± 8.0
LANSS score ≥ 12	6 males, 5 females (no significant difference, Fisher's exact test)
	4 not on ERT, 7 on ERT (no significant difference, Fisher's exact test)
MSSI	16.6 ± 9.8
iGFR (ml/min/1.73m^2)	84.0 ± 25.4
UACR (mg/mmol Cr)	11.2 ± 19.2
UPCR (mg/mmol Cr)	26.1 ± 28.7
LVMI (g/m^2)	101.3 ± 45.9

Table 4.16 Demographic data on AFD population studied for COMPASS and LANSS questionnaires, means ± SD unless otherwise stated.
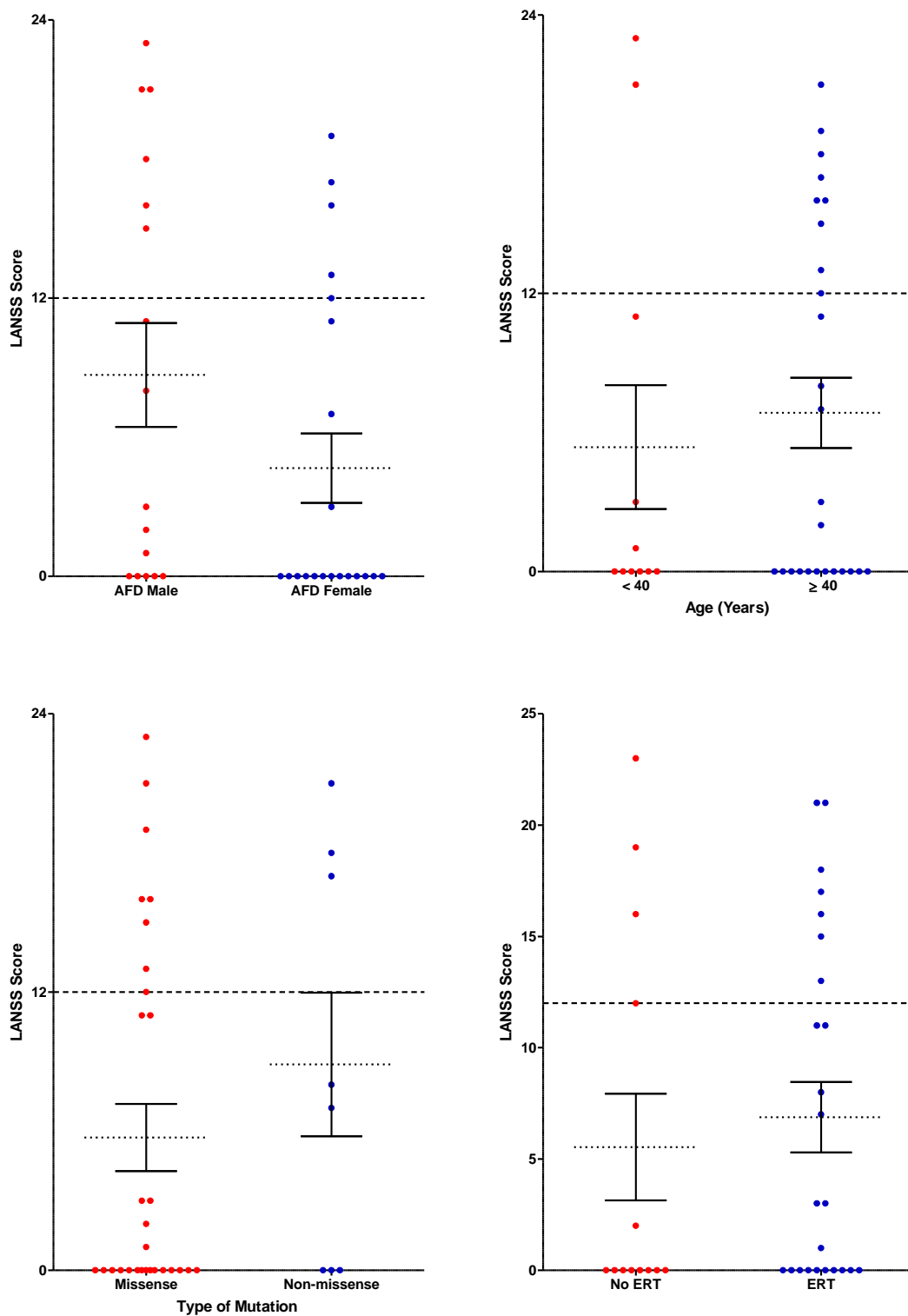
Fig 4.14. LANSS scores of AFD patients categorised according to sex, age, type of mutation and whether on ERT.
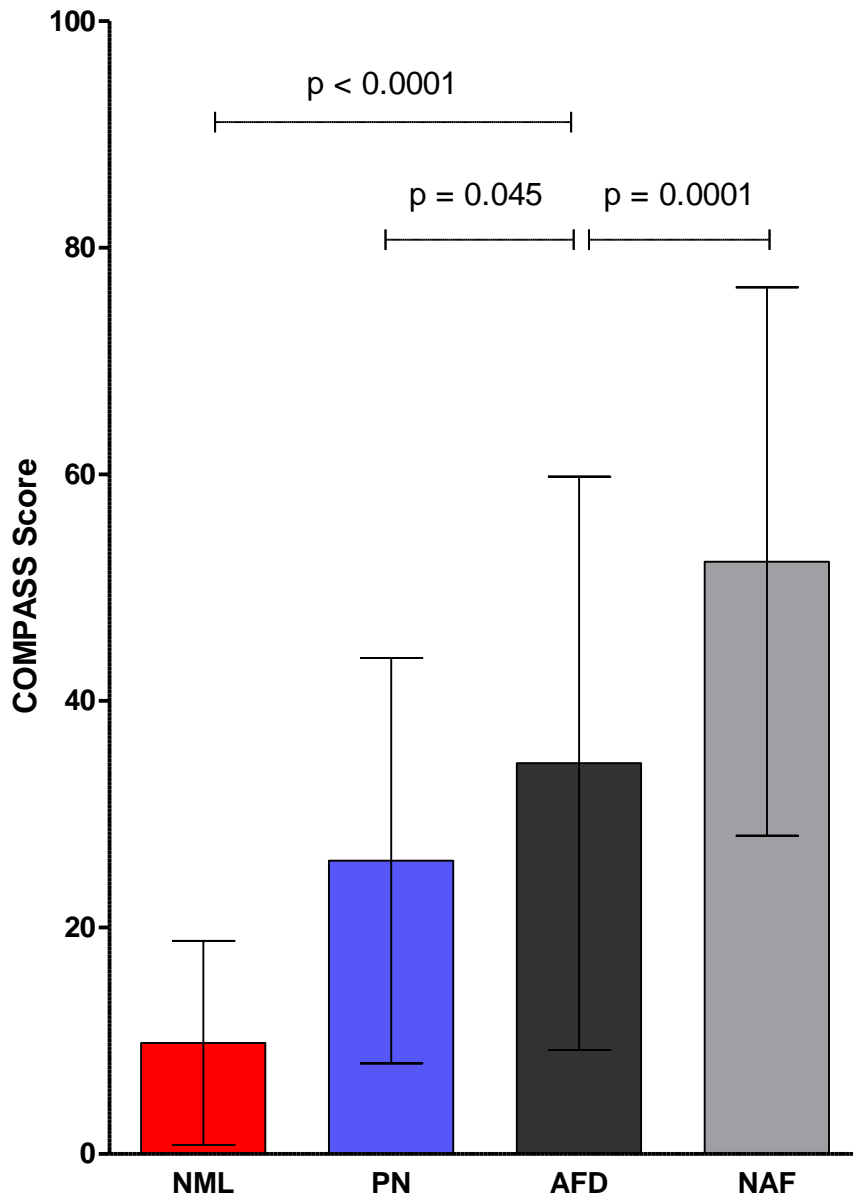
Fig 4.15. Comparison of mean total COMPASS scores of AFD patients in current study to population from Suarez et al 1999[353]. Statistical analysis used was the 1 sample t-test.
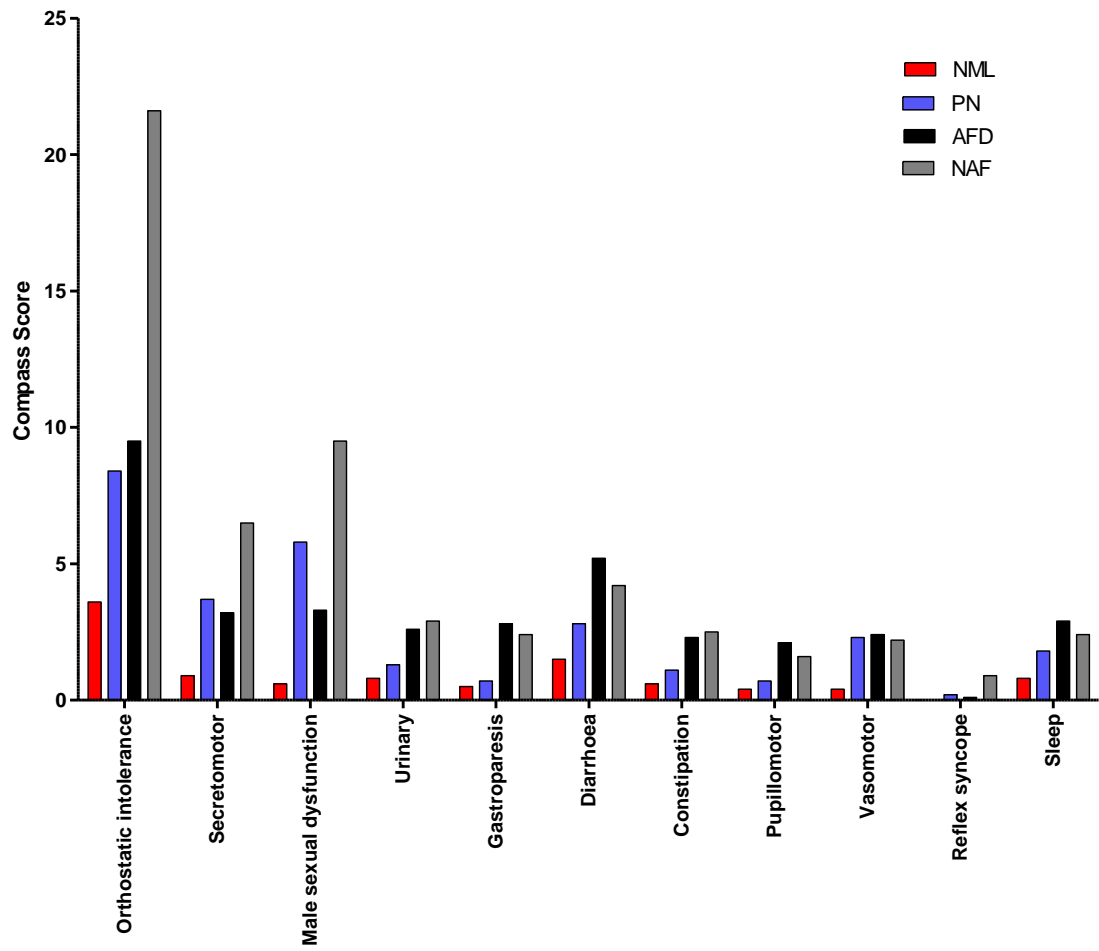
Fig 4.16. Comparison of mean COMPASS subscores of AFD patients in current study to population from Suarez et al 1999[353].

Domain	Maximum Score	Control (C), n=41			Peripheral neuropathy (PN), n = 33			Neurogenic autonomic failure (NAF), n=39			AFD, n=37			AFD vs C	AFD vs PN	AFD vs NAF
		Mean	% > 0	Range	Mean	% > 0	Range	Mean	% > 0	Range	Mean (Median)	% > 0	Range			
Orthostatic intolerance	40	3.6	34.2	0–17.5	8.4	51.5	0-32.5	21.6	89.7	0–37.5	9.5 (10.0)	59.5	0-33	p = 0.0008	ns	p < 0.0001
Secretomotor	20	0.9	31.7	0-6.2	3.7	75.8	0-12.3	6.5	94.9	0-16.9	3.2 (2.0)	64.9	0-12	p = 0.0003	ns	p < 0.0001
Male sexual dysfunction	30	0.6	21.7	0-8.6	5.8	75.0	0-12.9	9.5	71.4	0-19.3	3.3 (0.0)	37.5	0-12	P = 0.0343	p = 0.0407	p < 0.0001
Urinary	20	0.8	31.7	0-4	1.3	45.5	0-8	2.9	69.2	0-14	2.6 (0.0)	48.6	0-18	p = 0.0130	ns	ns
Gastroparesis	10	0.5	22.0	0-5.7	0.7	36.4	0-4.3	2.4	59.0	0-10	2.8 (2.0)	81.1	0-10	p < 0.0001	p < 0.001	ns
Diarrhoea	20	1.5	29.3	0-8	2.8	33.3	0-16	4.2	46.2	0-16	5.2 (6.0)	62.2	0-17	p < 0.0001	p = 0.0049	ns
Constipation	10	0.6	34.2	0-7.1	1.1	42.4	0-7.1	2.5	66.7	0-10	2.3 (0.0)	48.6	0-8.0	p = 0.0006	p = 0.0113	ns
Pupillomotor	5	0.4	36.6	0-1.8	0.7	60.6	0-4.1	1.6	82.1	0-4.5	2.1 (2.0)	86.5	0-5.0	p < 0.0001	p < 0.0001	p = 0.0237
Vasomotor	10	0.4	7.3	0-6.3	2.3	39.4	0-7.5	2.2	38.5	0-8.1	2.4 (2.0)	75.7	0-11	p < 0.0001	ns	ns
Reflex syncope	20	0	0	0-0	0.2	6.1	0-4.0	0.9	23.1	0-4.0	0.1 (0.0)	2.7	0-4.0	ns	ns	p < 0.0001
Sleep	15	0.8	34.2	0-6	1.8	57.6	0-8.3	2.4	56.4	0-12	2.9 (2.0)	81.1	0-8.0	p < 0.0001	p = 0.0032	ns
Male **Female**	200 170										32.9 35.8	93.8 95.2	0-83 0-105			

Table 4.17. Summary of Compass total and subscores in AFD patients compared with Suarez et al 1999 study population[353]. Statistical analysis used was the 1 sample t-test.

4.7   Discussion

The result of this study has demonstrated there is some evidence of cardiac autonomic dysfunction in AFD patients on formal cardiac autonomic screening tests, but screening tests for sympathetic or parasympathetic function are not consistently abnormal in the individual patients. No AFD patients in this study consistently had abnormal sympathetic nervous system tests (head tilt, isometric exercise, mental arithmetic and cold pressor tests). Only 2 female patients, both already on ERT (9.5% of test group) had abnormalities in parasympathetic screening tests (respiratory sinus arrhythmia and hyperventilation tests). This supports the studies by Morgan et al[347] and Biegstraaten et al[346] who concluded that autonomic control of the cardiovascular system is normal. More recently Hilz et al assessed reduced baroreflex sensitivity in response in sympathetic[357] and parasympathetic[358] challenge in untreated AFD males which normalised with ERT (18 to 23 months)[357, 358]. This suggested possible subclinical autonomic dysfunction in AFD males (asymptomatic from autonomic symptoms).

Postganglionic nerve fibres of the sympathetic reflex arc mostly secrete adrenaline and noradrenaline as its neurotransmitters. Plasma noradrenaline and adrenaline concentrations maybe a reflection of sympathetic vasoconstrictor activity and be an indirect measure of activation of sympathetic efferents however, many factors influence noradrenaline and adrenaline concentrations in plasma. In the head up tilt the plasma noradrenaline and adrenaline concentrations increased which may reflect the ability in AFD patients to increase sympathetic nerve activity suggesting normal sympathetic neuroendocrine function.

Nearly a third (29.7%) of our cohort of AFD patients had significant neuropathic pain, reflected by a LANSS score of ≥ 12. Eventhough there was no significant difference in LANSS scores when analysed based on sex, age, type of mutation and treated with ERT, there was a tendency for the mean LANSS scores to be higher in males, older AFD patients, AFD patients with a non-missense mutation and AFD patients on ERT. To delineate this possible difference a much larger cohort of AFD patients would be needed. COMPASS scores in AFD were significantly higher than COMPASS scores from the control cohort described by Suarez et al[353] and significantly higher than the cohort with peripheral neuropathy but lower than the cohort with neurogenic autonomic failure. Higher mean COMPASS scores in AFD patients compared to controls were also shown by Biegstraaten et al[346] especially in the orthostatic intolerance, vasomotor

impairment and gastroparesis subgroups. In our cohort all subgroups had a significantly higher COMPASS score, except for the reflex syncope subgroup, than controls. Also COMPASS subscores in AFD patients for orthostatic intolerance and secretomotor subscores were similar to peripheral neuropathy patients and gastroparesis, diarrhoea and constipation subscores were similar to the neurogenic autonomic failure patients. This reflects a varied distribution of autonomic symptoms in AFD (COMPASS score range of 0 to 150).

In this cohort of AFD patients there was no significant difference in total sweat volumes measured with QSART. Characteristic cytoplasmic inclusions have been observed in the eccrine sweat glands which may be responsible for reduced sweating[359, 360]. Previously one symptomatic AFD female had been shown to have anhidrosis but preserved basal activity and responsiveness of skin sympathetic activity[361]. This suggests that sweat function maybe affected due to sweat gland involvement rather than autonomic nervous system involvement. Schiffmann et al showed there was an acute improvement in sweat function 24-72 hours after ERT infusion[20], demonstrating abnormal sweat function of AFD patients was partly due to a functional defect at the sweat gland level rather than a gross structural abnormality or autonomic neuropathy. In our study, we were unable to demonstrate abnormal sweat function in our cohort as majority (75%) of our cohort were already on treatment with ERT. We can say that the prevalence of abnormal total sweat volumes in this cohort of AFD patients was not significantly different to previous documented control population. Our results of no conclusive autonomic dysfunction in AFD patients, fits with Moller et al's conclusions that reduced sweat output is not because of dysfunction of the autonomic innervations (preserved sympathetic responses) but in addition they showed a reduced skin flare response[362] and together with loss of C-fibers in skin biopsies[340], suggested that C-fiber dysfunction may contribute to sweat dysfunction in AFD patients.

Overall AFD patients have significant symptoms of neuropathic pain and symptoms of autonomic dysfunction but not at the same level as patients with autonomic failure. Clinical testing does not show consistent abnormality in the sympathetic or parasympathetic systems in males or females but this disease is heterogeneous with significant variability that mild symptoms or signs may not be clinically evident on clinical testing. This is further compounded by small study populations due to this disease being rare. Also symptoms of autonomic dysfunction could be related to the end

organ damaged, rather than the autonomic reflex itself as suggested by sweat function in AFD patients.

4.8 Limitations

This study did not have a control group though data was compared to control data from other large published series. Again AFD numbers were small (n=20 for cardiac autonomic screening tests), but this would be difficult to circumvent in view of low prevalence of AFD.

4.9 Conclusions

This cohort of AFD patients had minimal parasympathetic autonomic cardiac abnormalities, normal adrenaline and noradrenaline production with head tilt, normal sweat production but significant symptoms of autonomic dysfunction (based on COMPASS) and neuropathic pain (LANSS). There is currently no definite clinical evidence of autonomic dysfunction but subclinical autonomic dysfunction would need further investigation.

Chapter 5. Final Discussion and Conclusion

AFD is a multisystem genetic disorder with varied phenotypic manifestations. Being an orphan disease with large heterogeneity, recognition and diagnosis of AFD has been difficult. An increasing awareness of AFD and the disease burden imposed on patients together with the availability of therapeutic interventions to prevent or reduce disease progression and improve symptoms, has led to increased availability of therapy to AFD sufferers.

Due to the slow progression of end stage cardiac or renal disease, usually more apparent from the third decade of life onwards, timing of the initiation of expensive therapies and efficacies of these available therapies need to be justified. Progression of the disease process has been shown to continue even with ERT, once significant damage has occurred to end organs. There may be a point of no return when disease burden or damage is irreversible. ERT is expensive and invasive therapy and in the current economic climate, there needs to be evidence the use of ERT would benefit the patient and be cost effective. This thesis examined some possible early markers of organ dysfunction in AFD.

Renal involvement in the form of proteinuria was one of the first symptoms described by William Anderson. Our first study has shown that standard clinical practices of measuring or monitoring renal function in AFD may result in over estimation of GFR. The most commonly used measure of GFR has been the use of serum Cr. It is well known that serum Cr has large variability due to sex, age, muscle mass, ethnicity and laboratory techniques, to name a few. Over the years formulae have been developed to better estimate GFR still using serum Cr, but most of these formulae have usually been validated in Caucasian diabetic populations with CKD stage 3 to 5. Our aims were to document the most appropriate method of measuring GFR in AFD in a clinical setting, and in one of the largest cohorts of AFD, we have shown that the MDRD or CKD-EPI equations had the best eGFR compared to a clinical gold standard method. These equations gave the least bias and had the least number of "missed" or "over-treated" patients when looking at CKD Stage 1 to 3. In terms of decision on initiating ERT may have to be decided on a more invasive or expensive clinical test but regular monitoring of renal function can be done via these equations. Initiating ERT in a patient should be

decided with the best information available as these would mean the patient would be committed to a lifelong expensive and invasive therapy. We also were able demonstrate that a significant proportion of eGFR investigations over or underestimated GFR especially related to sex of the patient. CG, the Mayo quadratic equation and 24 hour urine Cr clearances significantly overestimated GFR but not the MDRD or CKD-EPI when looking at all AFD patients with CKD stages 1 to 3, but further analysis shows that the MDRD and CKD-EPI overestimates GFR in AFD males with CKD 1 to 3 and the MDRD underestimated GFR in AFD females with CKD stages 1 to 3. Based on these findings we conclude that the CKD-EPI is the best method for estimating GFR and AFD clinicians should employ this method in monitoring renal function in AFD patients.

In the second part of our study we aimed to investigate urine α-Gal activity in AFD patients and healthy controls and we clearly showed that AFD males and females had significantly lower α-Gal activity in urine compared to controls. Next we showed that only the lysosomal enzyme β-hex and not chitotriosidase was raised in urine of AFD patients. Despite measures of glomerular function being the common assessment for renal involvement in AFD, histological studies have shown evidence of tubular atrophy and scarring in the kidney. We have shown in our study that urine β-hex is significantly elevated in AFD patients suggesting proximal tubular dysfunction. As urine RBP levels were not raised but urine β-hex activity was, this suggested a normal reabsorptive proximal tubular function. Increased urine β-hex activity is a reflection of renal tubular damage, and we recommend that urine β-hex be incorporated as part of the renal assessment in AFD. The reason for this recommendation is because we know that patients with greater proteinuria or poorer renal function despite ERT, their GFR deteriorates at a increased rate compared to patients with lower proteinuria or better renal function at baseline[10, 11, 363]. To determine earlier renal dysfunction, be it glomerular or tubular dysfunction we need a better array of available investigations, to detect earlier end organ damage. We have shown urine β-hex is increased in AFD patients and associated with end organ damage or disease burden. On the other hand we have not shown longitudinal data on whether urine β-hex activity would improve or normalise on ERT, and whether this would reflect clinical improvement and improvement in mortality and morbidity. Analysing urine β-hex activity is a fairly simple fluoremetric method similar to the analysis of α-Gal activity. This method

should already be available in most units managing AFD patients. Urine of AFD patients are already analysed as part of their routine investigations. Availability of equipment, method of analysis and urine samples would mean it could be fairly straight forward for most units managing AFD patients to analyse urine β-hex in all their patients retrospectively and prospectively. This longitudinal data may help determine the efficacy of managing AFD patients with ERT, or future therapies such as substrate reduction therapy or chaperone therapy. Urine MCP-1 a marker of renal inflammation is also raised in AFD but we would suggest further investigation in its use in AFD patients, in view of small number of urine samples we analysed.

Finally the third part of our study was to investigate autonomic dysfunction, plasma catecholamine levels and symptoms of autonomic dysfunction and neuropathic pain in AFD patients. We have shown that there is no significant evidence of cardiac autonomic system dysfunction on screening tests but AFD patients do suffer from significant neuropathic pain based on the LANSS questionnaire and have significant autonomic symptoms reflected by high COMPASS scores.

In summary we have shown that the CKD-EPI equation is the best method currently for estimating GFR with serum Cr, urine β-hexosaminidase shows evidence of renal tubular dysfunction in AFD patients and should be used to determine renal dysfunction in AFD patients and eventhough AFD patients have symptoms of autonomic dysfunction, this is not evident of screening tests.

This study did have its limitations and the most striking is the small number of patients recruited with great phenotypic heterogeneity. This resulted in a difficult analysis, in trying to tease out correlations with end organ damage with genotype and phenotype of AFD patients. Another problem encountered was the wide spread of AFD patients attending our unit from the United Kingdom. Recruiting for autonomic function screening tests, which could take an extra half a day as patients had to be taken to a different site (St Mary's Hospital, Paddington, London) resulted in a poor uptake for recruitment and follow up data. Another difficulty was the long term follow up of patients. Many of the patients recruited in the study were on ERT or were to be started on ERT. We do not know if disease markers assessed would change with time as part of the natural history or improve/stabilise with the use of ERT. To improve on these

limitations, collaborations with other AFD units to obtain urine samples and analyse a cross-section and with follow up samples would improve the significance of these tests as markers of disease severity and progression. Also follow up samples on a 6 monthly basis over a 10 year period would probably give us a more robust analysis.

Looking back at the study there are a few ways to take these experiments and research forward. More novel biomarkers in CKD have been proposed and these in the future should be investigated in AFD to help us in the decision making of initiating and monitoring efficacy of therapy. Two examples are Cystatin C and Neutrophil gelatinase associated lipocalin (NGAL). Cystatin C is a sensitive biomarker of kidney function in mild to moderate kidney disease[364] and can predict progression of CKD[365]. In AFD, it has been shown that Cystatin C is a more reliable and sensitive marker of renal function than serum Cr or the MDRD equation[105, 366, 367]. NGAL was initially identified as an early biomarker of acute kidney injury[368-370] but recent evidence suggests it may be involved as a mediator of CKD and urinary NGAL has been shown to be higher in patients with adult polycystic kidney disease who progressed more rapidly to end stage renal failure[371]. Also cross sectional studies have shown higher urine and serum NGAL in CKD due to a variety of primary causes[372-375].

Another suggestion for the future is based on a more international level of cooperation. Currently there are two large international databases in AFD, the Fabry Outcome Survey and the Fabry Registry. These international databases hope to show longitudinal data with respect to natural history and outcomes in AFD patients with or without treatment. Similar to this an international collaboration of stored samples (e.g. serum, urine) in AFD patients could potentially be a large research source as more novel markers on disease or organ progression are developed in the future. This may have more ethical and financial considerations but is not unfeasible.

The most robust level of evidence for disease progression and effect of therapies in this orphan disease would be to have large prospective randomised trials looking at early biomarkers of end organ damage with the end point being the emergence of conventional features of AFD. Early biomarkers could be in the form a specific biochemical assays such as urine β-hexosaminidase as demonstrated in this thesis.

We analysed a variety of urine proteins in this thesis. A further potential tool or assessment in AFD patient is the use of mass-spectrometry based profiling of urinary proteins. Increased levels or activity of urine proteins in differing combination related to type of genetic mutations, sex or the use of ERT could help determine the pathophysiology in relation to the AFD kidney or other end organ damage, and may be able to distinguish whether treatment strategies are beneficial or not and even possibly when to initiate therapy.

The link between the genetic abnormality in AFD to organ damage, which then manifests as patient symptoms and signs, is still not clearly understood. Hopefully the identification of measurable biomarkers can help to determine the best way in managing the disease burden for these patients.

Publications and Presentations

Poster presentations:

1. Increased urine β-hexosaminidase is associated with end organ damage in Anderson Fabry disease. European round table on Fabry disease, Berlin, May 2012.
   Jeevaratnam P, Baker R, Reed M, Mehta A, Unwin R, Hughes DA.

2. Isotopic glomerular filtration rate is a more suitable method to monitor renal function in Anderson Fabry disease patients. 8th International symposium on Lysosomal storage disorders, Paris, April 2008.
   Jeevaratnam P, Burns A, Holmes A, Reed M, Baker R, Ayto R, Mehta AB, Hughes DA.

Publications:

In process of submitting a manuscript titled "Increased urine β-hexosaminidase is associated with end organ damage in Anderson Fabry disease".

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