Pulmonary Involvement in Anderson Fabry Disease

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I, Nadia Tazin Shafi, 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.

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Abbreviations

AFD	Anderson Fabry Disease
ATS	American Thoracic Society
BAL	Bronchoalveolar lavage
BD	Beckton Dickinson
BSA	bovine serum albumin
CD	Cluster of differentiation
COPD	Chronic obstructive pulmonary disease
Cr51 EDTA	51-chromium ethylenediamine tetraacetic acid
CXR	chest radiograph
DGJ	1-deoxygalactonojirimycin
DNA	deoxyribonucleic acid
DTT	ditriothreitol
ECG	electrocardiogram
ELISA	enzyme linked immunosorbent assay
EMG	electromeylography
ERS	European Respiratory Society
ERT	enzyme replacement therapy
FBS	fetal bovine serum
FEV$_1$	forced expiratory volume in 1 second

FOS	Fabry Outcome Survey
FRC	functional residual capacity
FVC	forced vital capacity
Gb3	globotriaosylceramide
GFR	glomerular filtration rate
GLA	α-galactosidase A
GOLD	Global Initiative for Obstructive Lung Disease
He	Helium
HPLC	high performance liquid chromotography
HRCT	high resolution computed tomography
IL-10	interleukin-10
IL1-α	interleukin 1-α
IL1-β	interleukin 1-β
IL-6	interleukin-6
IL-8	interleukin-8
iNKT	invariant natural killer T cell
KCO	diffusion capacity corrected for alveolar volume
LCI	Lung Clearnance Index
lyso-Gb3	globotriaosylsphingosine
MEF$_{25-75}$	maximal expiratory flow rate between 25% and 75% of vital capacity
MEF$_{50}$	maximal expiratory flow rate at 50% of vital capacity
MPS	mucopolysaccharidoses

MRI	magnetic resonance imaging
MSSI	Mainz Severity Score Index
NHYA	New York Heart Association
NKT	natural killer T cell
NSCAG	National Specialist Commisioning Advisory Group
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFT	pulmonary function test
pky	pack year
QSART	quantitative sudomotor axon reflex thermoregulatory testing
RFH	Royal Free Hospital, London
RV/TLC	residual volume/total lung capacity
SOP	standard operating procedure
TIA	transient ischaemic attack
TLCO	diffusion capacity
TNF-α	tumour necrosis factor α
VEGF	vascular endothelial growth factor

Abstract

Aim: To investigate the clinical, physiological, radiological and pathological changes which occur in the lungs in Anderson Fabry Disease (AFD)

Methods: In this study we have used lung function testing, high resolution CT scanning and induced sputum examination to investigate the lung. We have measured sputum enzyme activity using fluorometric assays, cell populations using flow cytometry and cytokines using enzyme linked immunosorbent assays. We have compared investigation findings from AFD subjects with those from patient's with airways disease in the form of chronic obstructive pulmonary disease (COPD) and healthy controls

Results: We have shown that respiratory symptoms are common, and airway involvement is widespread though mild in AFD. Pulmonary involvement is more common in males, in subjects with worse overall disease as measured by Mainz Severity Score Index, and is independent of smoking. No significant radiological changes were evident on CT chest imaging in AFD. We have presented novel data on α-galactosidase A activity from lung derived samples, which demonstrate low sputum enzyme activity in AFD males compared to controls and AFD females, and consistently higher enzyme activity in sputum derived leucocytes compared to those derived from peripheral blood. We did not find any detectable differences in blood or sputum α-galactosidase A activity in subjects on enzyme replacement therapy. Cell populations from induced sputum in AFD subjects demonstrated a predominance of monocytes/macrophages, similar to the COPD subjects, and there was the suggestion of an increased T cell population in AFD subjects with airway obstruction compared to those without. Elevated concentrations of sputum IL-8 were seen in the sputum of AFD subjects compared to controls.

Conclusion: There is demonstrable and clinically relevant involvement of the lungs in AFD, which appears to occur as a result of deficient α-galactosidase A in the lungs and subsequent inflammatory processes.

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Chapter 1 – Introduction

1.1 The Nature of Lysosomal Storage Disorders

1.1.1 Lysosomes

Lysosomes are essential cellular organelles found in the cells of all human tissues. They contain a number of hydrolytic enzymes such as lipases, proteases, amylase, nucleases and phosphoric acid monoesters (1). They are responsible for the catabolism of peptides and carbohydrates, and for digestion of effete organelles and mitochondria. In addition they are involved in degradation of phagocytosed pathogens (2).

Lysosomal enzymes are formed from the rough endoplasmic reticulum (3) and modified in the Golgi apparatus where mannose-6-phosphate residues are added to allow targeting to lysosomes via the mannose-6-phosphate receptor. However the mannose-6-phophate receptor is recycled from the late endosomes and therefore not found in the lysosome itself (4). Lysosomes are usually spherical, EM dense, and granular (5). Each lysosome has a plasma membrane which protects the cell from hydrolytic enzymes, and can assist in repairing cell membrane damage if required. The interior of the lysosome is acidic compared to the cell cytosol, and this acidic environment is necessary for the optimal functioning of the hydrolytic enzymes. The pH difference also provides some protection to the cell should there be any leak of hydrolases into the cytosol, though despite this lysosomes do have a role in cell autolysis (6). Lysosomes perform their function by fusing with other cellular vacuoles forming endo-lysosomes and phago-lysosomes and allowing the hydrolases to contact the vacuole contents and digest them (7) .

1.1.2 Lysosomal storage disorders (LSD)

There are over 50 lysosomal storage disorders arising from dysfunction of lysosomal proteins. They are a diverse group of disorders occurring largely as a result of inherited deficiencies of lysosomal enzymes, although may also result from defects in protein cofactors, membrane proteins or proteins involved in post translational modification or enzyme transport. As a result of the enzyme

deficiency there is an accumulation of substrate within various tissues, resulting in multi-organ pathology, which is progressive over time.

Broadly LSDs may be divided into the Mucopolysaccharidoses (MPS), Sphingolipidoses and Mucolipidoses, in addition to type II glycogen storage disease Pompe disease, where there is a defect in the lysosomal enzyme acid maltase. Figure 1.0 demonstrates the pathways involved in glycosphingolipid metabolism, highlighting the lipids and intermediates involved, and the enzymes which have been shown to cause disease as a result of their absence or deficient activity.

LSDs may present in childhood with conditions such as the mucopolysaccharidoses, GM2 gangliosidosis (Tay-Sachs disease) and infantile Pompe disease presenting overtly with developmental delay, neurological abnormalities and cardio-respiratory disease or more subtly with failure to thrive or musculoskeletal abnormalities. However a number of diseases such as Anderson Fabry Disease (AFD), Gaucher disease, the milder forms of MPS and adult onset Pompe may not become apparent until adolescence or adulthood. Presentation in adulthood is heterogenous such that many subjects will have seen multiple physicians of differing specialities before having an LSD diagnosis confirmed including cardiologists, neurologists, renal physicians, haematologists and dermatologists. As a result, diagnosis is often delayed and on occasion missed altogether. Given the recent significant advances in treatment in the form of enzyme replacement therapy (ERT) and specialist centre multidisciplinary care this could significantly impact on clinical outcome.

The collective incidence of LSDs has been estimated at 1 in 8000 live births (8-10). The most common of these are the lipidoses, mainly sphingolipidoses, of which Anderson Fabry Disease and Gaucher disease have the highest prevalence.

Figure 1.0 Pathways and intermediates involved in lysosomal storage disorders with sphingolipids (black), deficient enzyme (red) and resulting disorder (blue)

SAP = sphingolipid activator protein (diagram modified from Sandhoff et al (11))

GM_1 ganglioside

GM_1 - β-galactosidase

GM_1 gangliosidosis

GM_2 ganglioside   Globoside

B - hexosaminidase A,   β – hexosaminidases

GM_2 activator   A and B

Tay-Sachs, Sandhoff

Tay-Sachs AB variant   Sandhoff

GM_3 ganglioside   Globotriaosylceramide

neuraminidase,   α-galactosidase A,

SAP-B   SAP-B

Sialidosis   **Anderson Fabry**

Lactosylceramide   Digalactosylceramide

GalCer-β-galactosidase   α-galactosidase A,

GM_1 - β-galactosidase   SAP-B

SAP-B and SAP-C   **Anderson Fabry**

Glucosylceramide

Galactosylceramide

Glucocerebrosidase

SAP-C   β-galactosylceramidase,

Gaucher   SAP-A and SAP-C

Sphingomyelin   Ceramide   Krabbe

sphingomyelinase   Arylsulphatase A

Niemann-Pick   Acid ceramide   SAP-B

types A and B   SAP-D   Metachromatic

Farber   leucodystrophy

Sphingosine   Sulphatide

1.2 Anderson Fabry Disease (AFD)

1.2.1 Defect

Anderson Fabry Disease (AFD) (OMIM #301500) is an X-linked condition caused by mutations in the gene which encodes the enzyme α-galactosidase A (GLA), resulting in an absolute or relative deficiency of this enzyme (12). This deficiency results in an accumulation of the glycosphingolipid globotriaosylceramide (Gb3) in plasma and in the lysosomes of cells (13-15) resulting in a multi-system disorder most detrimentally affecting the cardiovascular, renal and neurological system, but also affecting the skin, blood vessels and auditory systems and lungs.

The nature of AFD as an X-linked disease results in disease manifestations that are more severe and occur earlier in males, though with further investigation it has been demonstrated that females are also significantly affected by the condition (16;17). Manifestations of the disease have been documented in childhood, largely comprising skin, gastrointestinal, neurological, auditory and ophthalmologic involvement (18). The clinical symptoms and signs worsen with age, with significant renal and cardiac involvement being major causes of morbidity and mortality of these patients in adulthood.

1.2.2 Epidemiology

The prevalence of AFD in the general population has been estimated to be 1 in 117,000 in one Australian study (19) and 1 in 476,000 in another from the Netherlands (20), though it is thought likely that these underestimate true prevalence. Other studies have produced contrasting results such as that based on newborn screening in Italy where the condition was present at a frequency of 1 in 3,100 males with mutations associated with later onset phenotypes (21). A similar study in Taiwan suggested 1 in 1,500 males were affected, also with later onset phenotypes, and a high frequency of a recognised splicing mutation (22). In part this finding may reflect some geographical variation but AFD occurs in all racial groups and is geographically widespread. There is however a discrepancy

between the findings from newborn screening studies and the prevalence of diagnosed AFD patients in the general population, implying that not all subjects who have the disease will necessarily manifest significant clinical symptoms.

1.2.3 Genetics

Over 300 mutations of the GLA gene which is located on chromosome Xq22 have been demonstrated. These include point mutations as well as deletions, substitutions, premature stop codons, missense and nonsense mutations. The majority of these have been shown to be private mutations (23;24). This, along with the large phenotypic differences evident between subjects with the same mutation, has made assessments of correlation between genotype and phenotype demanding (25). In addition the influence of modifiers on the penetration of symptoms leads to differences in disease manifestations and severity between individuals.

However, it is likely that one of the factors influencing clinical presentation is the amount of residual enzyme activity associated with a mutation. This has been shown to affect the prevalence of neuropathic pain, the onset to significant renal disease (26), and to correlate with hearing loss (27). Broadly speaking AFD patients have been divided into those with a classic phenotype who have <1% residual enzyme activity, such as base deletions which are associated with little or no residual enzyme activity and produce more severe phenotypes (28), and those with variant phenotypes who have >1% residual enzyme activity and later onset disease. Mis-sense mutations, such as N215S have been associated with higher levels of residual enzyme activity, and a later onset AFD phenotype (29). Whilst disease may be of later onset it is possible that over time ultimately the manifestations would be as severe as that of classic AFD. It has been suggested that mutations which result in a non-conservative amino acid chain have the lowest residual enzyme activity, and that the exon in which the mutation is present may be pertinent to this and therefore determine disease severity.

The phenomenon of X-inactivation also has a role in the heterogenous presentation of AFD phenotypes and severity in females (30). Historically it was thought that females were carriers of the disease with no clinical manifestations. Further investigation has shown this not to be the case, with evidence that some may be as severely affected as males (31;32).

1.2.4 Diagnosis and screening

Once suspected, the diagnosis of Anderson Fabry Disease in males is confirmed by activity of α-galactosidase A enzyme in plasma, peripheral blood-derived leucocytes, or in cultured fibroblasts, using either a fluorometric assay or tandem mass spectrometry. The fluorometric assay uses substrates such as 4-methylumbelliferyl-α-D-galactopyranoside and measures enzyme activity by differences in the fluorescent properties of this substrate and its products of hydrolysis.

In AFD females lyonisation results in potentially normal enzyme activity in both plasma and peripheral blood leucocytes despite subjects having clinical manifestations of the disease. It is therefore essential for all females, to undergo mutational analysis. This is also advised in males to allow genetic counselling and may be used for rapid family screening. Genotyping is performed by polymerase chain reaction (PCR) sequencing. Genetic counselling is offered to all AFD patients who are considering starting a family.

Further supporting evidence for the diagnosis of AFD may come from clinical features such as corneal verticillata and angiokeratomata though these are not condition-specific having been detected in patients receiving lysosomal toxic drugs such as chloroquine and amiodarone. In addition Gb3 may be quantified in both plasma and urine, where it has been shown to be significantly elevated in the majority of AFD patients (33). However it has been demonstrated that not all subjects have elevated Gb3 in the urine, with some subjects with the N251S mutation having been shown to have normal Gb3 levels in the urine (34). When the kidneys of such subjects have been examined histologically there has

been evidence of Gb3 deposition in podocytes, but not in other renal cell types or in the vascular endothelium (35). Demonstration of storage material on histological examination of biopsies from the kidney, skin or myocardium may provide conclusive evidence for AFD, though is clearly a more invasive way of making the diagnosis (36).

More recently there has been focus on non-invasive, quicker and more practical methods of diagnosing LSDs including AFD for the purposes of screening. Several studies have examined the utility of newborn screening using dried blood spots on filter paper with either fluorometric assays or tandem mass spectrometry (37-39). As enzyme activity may be normal in affected AFD females, this test is less sensitive in female neonates (40).

The aim of newborn screening for any disease is to achieve earlier detection of the disease with a view to preventing the development of irreversible pathology. As a concept this is somewhat less straight forward in AFD, particularly with the detection of mutations that have been associated with later onset disease, as it calls into question whether these subjects would definitely develop significant morbidity in the future. The fact that such studies have demonstrated a much higher incidence of α-galactosidase A deficiency than the current estimated prevalence of the disease does suggest that a significant proportion of subjects may not ever manifest significant symptoms, especially females (41;42). In addition it has not been established as yet that treatments available such as enzyme replacement therapy would be effective in entirely preventing the sequelae of the disease if commenced prior to the clinical manifestations of the disease being evident.

Screening for AFD in populations considered to be high risk may however be of benefit, such as those with renal failure, cardiomyopathy and strokes prior to the age of 55. There is a detectable yield, with one review suggesting the prevalence of AFD in males on dialysis to be 0.33%, and in subjects with left ventricular hypertrophy 1% (43). A recent European survey amongst subjects

with unexplained hypertrophic cardiomyopathy reported a prevalence of 0.5% AFD in this group (44). An international multicentre study assessing the incidence of AFD in young stroke patients found that 0.5% of 18-55 year old subjects with stroke had a diagnosis of AFD and a further 0.4% were likely to have AFD though did not meet all of the diagnostic criteria (45). Screening using dried blood spot testing in such high risk populations has been performed, and suggests a similar prevalence (46-48), giving the advantage of examining samples on a much larger scale, with the additional ease of storage and transport of samples.

1.2.5 Clinical Manifestations

The combination of AFD's relative rarity and its insidious nature results in average delays from onset of symptoms to correct diagnosis of 13.7 and 16.3 years in males and females respectively (49). The spectrum of clinical symptoms and signs is similar in males and females, though the latter generally present with milder disease at an older age of onset and with slower progression (50).

Findings from a UK-based survey demonstrated that the main causes of mortality amongst AFD subjects were renal failure, heart disease and stroke, with life expectancy of 50 years in males, and around 70 years in heterozygous females at a time prior to the introduction of enzyme replacement therapy (15). Data from the Fabry Registry reported in 2008 also showed significantly reduced life expectancy compared to the general US population at 58.2 and 75.4 years in males and females respectively - with the most common cause of death being cardiac disease (51), followed by renal and neurological disease.

The main clinical manifestations of AFD are summarised in Table 1.2.5 along with the common investigation findings and the findings from studies examining the histological changes present in the organs involved.

Table 1.2.5 Summary of clinical manifestations, investigation findings and histological changes in AFD

Organ system	Clinical manifestations	Investigation findings	Histological findings
Cardiac	SOB, chest pain, reduced exercise tolerance; conduction abnormalities; sudden cardiac death (52); "cardiac variant" or atypical AFD (53;54)	ECG abnormalities On echocardiography: concentric, non-obstructive ventricular hypertrophy, changes in septal thickness (55); heart failure (56)	Gb3 within cardiac myocytes and vascular smooth muscle and endothelial cells have abnormal coronary vascular flow (57); myocardial fibrosis (58)
Renal	renal impairment; progressive renal failure requiring renal replacement therapy and transplantation(59-61)	microalbuminuria ; proteinuria; reduced glomerular filtration rate	widespread Gb3 in endothelial, parenchymal and epithelial cells (62); fibrosis, sclerosis, tubular atrophy (63;64)
Neurological	neuropathic pain, acroparaesthesia (65;66); transient ischaemic attacks; stroke (67); headaches, vertigo,dizziness and dementia (68;69)	abnormal nerve conduction studies (70); periventricular white matter lesions on MRI brain (71;72); pulvinar sign (73)	inclusions in peripheral nerves (74); widespread cerebral vasculopathy (75); cortical and brain stem lipid accumulation (76)
Skin	angiokeratomas in "bathing trunk" area and mucosa hypo/hyper/anhidrosis (77)	abnormal quantitative sudomotor axon reflex and thermoregulatory sweat testing parameters (78)	superficial angiomas (79) inclusions in sweat gland cells and axon of nerve (80)
Gastrointestinal	abdominal pain and bloating, nausea, vomiting, diarrhea on eating	abdominal symptom scores	lipid deposition on duodenal, jejunal and rectal biopsy (81); depositon in muscularis mucosa and vasculature (82)
Eyes and ears	tinnitus; hearing loss	corneal verticillata on slit lamp conjunctival and retinal vessel tortuosity on retinal imaging (83); high frequency sensorineural hearing loss(35)	lipid deposition in subepithelial layer of cornea (84); cochlea Gb3 in AFD mice (85)

1.2.6 Initial investigation, follow up and monitoring

At diagnosis a full and comprehensive assessment of all aspects of AFD is made and documented as a baseline, prior to the commencement of any treatments. This includes a full medical history and clinical examination, along with a family pedigree. Patients are asked to complete questionnaires to assess pain scores and quality of life. Further investigations into underlying organ involvement are undertaken. As summarised in Table 1.2.6. Patients are followed up at six-monthly intervals, with clinical assessment and examination, and investigations assessing disease progression and effectiveness of therapy. Those who are on ERT require annual measurement of renal and cardiac investigations, with repeat blood tests at six monthly intervals. Magnetic resonance imaging (MRI) brain scans are performed every 2 years, unless clinical indications suggest otherwise.

Table 1.2.6 Summary of initial investigations on diagnosis of AFD

Blood and urine	Eyes and ear	Renal	Cardiac	Neurology
- full blood count - urea & electrolytes - Liver function - Fasting Lipid profile - Plasma and urine Gb3 - urine albumin/ creatinine	-Slit-lamp examination -retro-illumination -retinal examination and photography - pure tone audiogram	- Glomerular Filtration Rate by Cr51 EDTA - 24 hour urine Creatinine Clearance if this is not available - 24 hour urine protein - renal biopsy	- ECG - 24 hour ECG - echocardiogram - exercise testing	- MRI brain - QSART assessment of sweating - EMG

QSART – quantitative sudomotor axon reflex and thermoregulatory testing

EMG – electromyelography

1.2.7 Treatment

1.2.7.1 Supportive

The clinical manifestations of AFD are managed with attention to symptom control, lifestyle advice, risk factor control and regular assessments of organ involvement. Patients are seen regularly by an appropriate specialist according to their pattern of organ involvement. Since the introduction of newer therapies, patients must be treated in specialist centres by a multidisciplinary team, who co-ordinate their care with the involvement of the appropriate specialist services.

1.2.7.2 Enzyme Replacement Therapy (ERT)

Following the example of successful therapy for Gaucher disease, enzyme replacement therapy for AFD was developed and subsequently introduced in 2001 following double blind placebo controlled clinical trials (86;87). In Europe, two such products are in regular use. These are agalsidase alfa (Replagal®, Shire Pharmaceuticals, UK) produced using a human fibroblast cell line, and administered at a dose of 0.2mg/kg intravenously fortnightly, and agalsidase beta (Fabrazyme®, Genzyme Corporation, UK) produced using a Chinese hamster ovary cell line, and administered at a dose of 1mg/kg intravenously fortnightly.

There has been much interest in the efficacy of both enzyme preparations which have now been in use for over 10 years. Both the products are licensed for clinical use in AFD in Europe and the UK, and Fabrazyme® in the US. As is shown in Table 1.2.7.3 there have been numerous studies demonstrating good clinical outcomes in the different organs affected by AFD. This includes data from the original double blind placebo controlled clinical trials (original trials marked with *). However there have also been meta-analyses and systematic reviews of the Cochrane database of randomised controlled clinical trials in this area – and these do not indicate there to be robust evidence for efficacy with either product (88;89). The difference between the Cochrane review and the separate trials is likely due to the limited numbers of subjects in the trials, the heterogeneity of the condition and the differences in the end points between individual studies.

Data from the Fabry Outcome Survey (FOS) and the Fabry Registry have been much more compelling with regards to the efficacy of ERT. They have the advantage of data that arises from larger numbers of subjects followed up over longer periods of time (90).

The two preparations of the enzyme have been shown to have similar biochemical properties and their activity per milligram, with only minor differences in glycosylation (91;92). The difference in dose between the two preparations has been the subject of much discussion. Whilst both preparations improve clearance of Gb3 from the plasma as well as renal and cardiac cells, data from randomised controlled clinical trials and open label extension studies suggest that algalsidase-β (Fabrazyme®) is of greater efficacy due to its use at a higher dosing regimen of 1mg/kg fornightly (93). ERT literature reviews which include more studies with broader criteria and greater heterogeneity in outcome measures show more similar efficacy between the two preparations (94;95).

The demonstration of α-galactosidse A antibodies with both preparations of the enzymes has generated interest in the immunogenicity of the preparations. In one study 56% of males on algalsidase-α were shown to have antibodies (96); and 88% of males on agalsidase-β in another (97). Adverse events such as fever and chills appear to be more common in such individuals. There appeared to be no difference in the proportion of subjects developing antibodes when agalsidase-α and agalsidase β were given at the same dose of 0.2mg/kg fortnightly (98). However, subjects receiving the recommended dose of algasidase-β at 1mg/kg fortnightly were much more likely to develop antibodies.

It has been proposed that the presence of antibodies may affect clinical efficacy of the enzyme replacement therapy. The higher dose of agalsidase-β appears to overcome the effect of neutralising antibodies by significantly reducing urinary Gb3 - implying that in antibody positive subjects higher doses of enzyme may be beneficial (99). There is also some evidence to suggest that the presence of agalsidase-β antibodies reduces clearance of tissue substrate in mice and cultured fibroblasts (100). Overall, however, clinical impact of antibodies

requires further work, as studies to date have used different methodologies, which complicates the interpretation of their findings (101).

Criteria for the commencement of ERT have been set out based on clinical measures of disease severity as summarised in Table 1.2.7.2. On the basis of initial investigations, validated severity scores including the Mainz Severity Score Index (MSSI) (102) and an age adjusted severity score (103) are calculated, which can then be used to follow the effect of treatment.

Table 1.2.7.2 Clinical indications for the commencement of enzyme replacement therapy in AFD

General and GI symptoms	Renal disease	Cardiac disease	Neurovascular disease
Abdominal pain, vomiting, altered bowel habit causing significant impact on quality of life	GFR <80 Proteinuria >300mgs/24 hrs Microalbuimnuria with renal biopsy evidence of endothelial deposits	ECG: Left ventricular hypertrophy or Isolated repolarisation abnormalities or Conduction abnormalities	Stroke/TIA in the absence of other risk factors Abnormal MRI scans with progressive change
Uncontrolled pain		Echocardiography: Increased left ventricular mass	
		Arrhythmia on 24 hr ECG	
		Ischaemia in the absence of coronary artery disease	

TIA – transient ischaemic attack

GFR – glomerular filtration rate

Table 1.2.7.3 Summary of clinical manifestation of AFD shown to improve with enzyme replacement therapy

Clinical manifestation	Demonstrated benefit of ERT	Enzyme preparation	Duration	Study
Acroparaesthesia	Improved Brief Pain Inventory Scores Improve C-, A-delta and A-beta nerve fibre function	Replagal® Fabrazyme®	3 years 18 months	Schiffman et al 2003* Hilz et al 2004
Hypo/anhydrosis	Improvement in quantitative sudomotor axon reflex and thermoregulatory sweat testing parameters	Replagal®	3 years	Schiffman et al 2003
Hearing loss	Improvement in sensorineural hearing loss from baseline testing	Replagal®	12 months	Hajioff et al 2007
Abdominal pain	Improvement in visual analogue score assisted assessments in paediatric subjects	Fabrazyme®	1 – 8 years	Bogwardt et al 2012
Renal involvement	Increased creatinine clearance, reduced urinary Gb3, improved clearance of Gb3 on renal biopsy, reduced rate of decline in GFR Clearance of Gb3 on renal biopsy Stabilisation of serum creatinine, eGFR and clearance of Gb3 from renal cells on biopsy	Replagal® Fabrazyme® Fabrazyme®	12 weeks 20 weeks 54 months	Schiffman et al 2001* Eng et al 2001* Germain et al 2007
Cardiac involvement	Reduced QRS complex duration Reduction in LV mass on MRI and Gb3 deposition on endomyocardial biopsy Reduction on LV mass on echocardiography Sustained reductions in LV mass and midwall shortening	Replagal® Replagal® Fabrazyme® Replagal®	12 weeks 6 months 12 months 5 years	Schiffman et al 2001* Hughes et al 2008 Spinelli et al 2004 Mehta et al 2009
Neurological Involvement	Possible improvement in cerebral blood flow	Replagal®	6 months	Moore et al 2002

1.2.8 Pathophysiology

The underlying pathological processes that result in the changes seen in different organs in AFD remain unclear. It is likely that factors beyond the effect of substrate accumulation contribute to end-organ damage. A disease model was described by Wanner et al (104) involving three overlapping stages in a cascade, starting with Gb3 storage in the cells as the initial event. Over time this results in tissue damage due to ischaemia, inflammation, hypertrophy and fibrosis, ultimately leading to the third stage of gross organ damage and dysfunction.

Supporting evidence for this comes from autopsy findings of AFD patients with significant cardiac involvement, who had received ERT, though late in their disease (105). Pathological examination of three cases with cardiac changes revealed that whilst cytoplasmic inclusion bodies were present, the extent of cardiac muscle thickening could not be accounted for by Gb3 deposition alone. Cardiac myocytes throughout the heart were hypertrophied and extensively vacuolated. There was evidence of focal myocyte apoptosis and necrosis with an associated accumulation of macrophages and a T cell infiltrate. In addition, there was significant myocyte disarray and extensive areas of fibrosis. The trigger for apoptosis under these conditions is not clear, but this may be key in understanding the changes seen in AFD.

Whilst numerous studies have shown that ERT reduces plasma, urinary and tissue Gb3 (106), it has been shown that Gb3 does not appear to correlate well with disease severity, and therefore cannot be used as a biomarker for the disease (107;108). Factors such as deacylated Gb3, globotriosylsphingosine (lyso-Gb3) have been proposed as having a causative role in the pathogenesis of AFD. This is because significantly elevated levels of lyso-Gb3 are present in AFD plasma, and its presence results in smooth muscle proliferation and the production of factors that lead to kidney injury (109;110). Levels of lyso-Gb3 have also been shown to fall with ERT, though not to normalise entirely (111).

AFD-associated vasculopathy appears to be key to the pathogenesis of the organ dysfunction seen in AFD, with widespread vascular changes seen in the

vessels of the brain, kidneys and heart (112), these being the major sites involved in morbidity and mortality (113-115). Although changes in the vessels involve both the vascular endothelium and the smooth muscle layers, it has been suggested that Gb3 deposition in the smooth muscle layer is of more clinical significance (116). It has also been shown that the use of ERT leads to clearance of substrate more readily from the vascular endothelial cells than the smooth muscle cells (117).

Changes in the vascular smooth muscle affect arterial structure and function, with measurable increases of intima-medial thickness (118). This does not appear to result in much atherosclerosis, though may cause arterial stenosis (119). Evidence from studies assessing the effect of AFD subjects' plasma on rat vascular smooth muscle cells suggests that hypertrophy of the smooth muscle cells occurs as a result of circulating growth factors (120). This may include factors such as sphinogosine-1-phosphate as well as lyso-Gb3 (121;122) . It has been proposed that this smooth muscle hypertrophy and increase in intima-media thickness is then responsible for triggering an inflammatory cascade through upregulation of angiotensin II, resulting in a pro-inflammatory state with increased local cytokines and chemokines such as vascular endothelial growth factor (VEGF) (123;124).

Oxidative stress may be relevant as the presence of Gb3 in the vascular endothelium increases the production of reactive oxygen species and adhesion molecules (125). Lower concentrations of antioxidants and higher levels of the pro-inflammatory cytokines interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) have been demonstrated in AFD subjects compared to controls (126). This adds further support to the concept of an inflammatory component being present. Safyan et al demonstrated a predilection for certain TNF-α and IL-10 gene polymorphisms in patients with AFD when compared to controls, though could not show an association between cytokine and enzyme levels (127). Equally, markers of inflammation such as C-reactive protein are not elevated in individuals with AFD, either (128).

Apoptosis could be an additional contributing factor to the changes in AFD, with sphingolipids having been shown to have a role in apoptosis in the heart (129). There have also been studies demonstrating higher levels of apoptotic markers in peripheral blood mononuclear cells, correlating with Gb3 concentrations in AFD patients, and of cell death when normal cells were treated with similar concentrations of Gb3 to that seen in patients (130).

It has been postulated that differences in cell populations in the peripheral blood of AFD patients may contribute to the reported pro-inflammatory state. In the context of lipid antigen there has been some research focussed on iNKT cells and the pathophysiology of AFD. Gadola et al examined mouse models of lysosomal storage disorders and found reduced numbers of iNKT cells and iNKT cell-mediated lysis of antigen presenting cells carrying lipid antigen (131). They observed reduced numbers of iNKT cells in all stages of thymic development, and that the effects on thymic selection of iNKT cells resulted in the reduced presentation and processing of lipid antigen in mouse models of LSDs. This idea was also supported by work from Schuman et al (132) who demonstrated that lipid accumulation resulted in impaired lipid antigen presentation and processing in β-galactosidase and Niemann Pick C protein deficient mice. It was also suggested that the iNKT cells present may not be fully functional, given that it was not possible to detect the predicted cytokine response (133).

Further studies in this area have demonstrated changes in T cell populations as well as NKT cells, where reduced numbers of CD4+ T cells and increased numbers of CD8+ T cells result in a two-fold reduction in the CD4+/CD8+ ratio in the liver of Fabry mice compared to controls (134). A similar imbalance of T cells was evident in the spleen of the mice, though less marked. In the same study, no such difference was present in the peripheral blood of AFD patients.

However, elsewhere AFD patients' peripheral blood contained a higher percentage of lymphocytes than controls (135). And whilst no difference was seen in CD4+ T cell and NKT cell numbers, the proportion of CD8+ T cells was

lower in AFD subjects naïve to ERT compared to those on ERT and controls; plus the percentage of B cells, monocytes and dendritic cells were higher in AFD subjects compared to controls.

A more recent study in both AFD mice and AFD subjects showed a reduction in percentages of CD4+ iNKT cells and a corresponding reduction in anti-inflammatory cytokine IL-4, and percentage increases in dendritic iNKT cells (136), further suggesting a pro-inflammatory state in AFD. This study also observed a reduction in CD8+ iNKT cells in the first month after the commencement of ERT, implying that iNKT cells may have a role in the pathophysiology of AFD.

1.3 Rationale for a pulmonary study in AFD

1.3.1 Existing evidence

Lung involvement by AFD was initially regarded as non-existent. Bartimmo et al in 1972 (137) reported that AFD had little or no impact on the lung, and that any changes seen were likely to be secondary to other factors such as smoking or the cardiac components of AFD. However, Kariman et al in 1978 (138) documented a 32 year old male presenting with AFD and significant airway obstruction and bullous disease out of keeping with his smoking history, and with no evidence of another cause for his respiratory morbidity.

Further evidence for lung involvement was subsequently presented by Rosenberg et al (139) who in 1980 reported significant chronic airflow obstruction in 7 AFD subjects and demonstrated the presence of inclusion bodies in bronchial brushings and biopsy samples of airway epithelium. In addition the possibility was raised that smoking might worsen the severity of the airflow limitation in AFD.

Sometime after this in 1997, Brown et al undertook a larger study on lung involvement in AFD (140). They examined 25 male subjects, 72% of whom had never smoked, and found 36% had obstructive spirometry, all of them aged 26 or over. They also noted only a weak association between smoking and worsening

of airflow, with a similar frequency of reported symptoms (24%) including shortness of breath, cough and wheeze in smoking and non-smoking subjects. A number of the obstructed subjects demonstrated a degree of apparent reversibility to bronchodilators though on methacholine challenge testing, no positive responses were found. Radio-nuclide scanning was also performed on 18 of the subjects, and no abnormalities demonstrated. Overall the findings of this study were highly suggestive of an effect of AFD on the lungs, restricted to the airways and not directly related to smoking.

The largest study to date on the lung in AFD provides further evidence of airway obstruction. Here, Magage et al (141) examined 50 AFD subjects, 39 of whom were naïve to enzyme replacement therapy (ERT). This study found mostly mild to moderate airway obstruction was present in men (assessed by all spirometric parameters compared to controls), which progressed with age. In women with AFD, significant reductions in FEV_1 and FEF_{25-75} were evident compared to controls. Small airway obstruction, as measured by FEF_{25-75}, was widespread in both sexes and all age groups. This parameter was also shown to progress in women over a median duration of 24 months.

Further supporting evidence for lung involvement in AFD has come from two separate case reports of individual female AFD subjects presenting with primarily pulmonary symptoms and signs and significant dyspnoea and hypoxia (142;143). In both cases the subjects demonstrated lung function abnormalities with obstructive and restrictive components and a significantly reduced diffusion capacity. Computerised tomography (CT) scanning demonstrated pulmonary infiltrates and air trapping, suggestive of both parenchymal lung involvement and small airways disease. In one case there was also evidence of significant pulmonary hypertension on CT and echocardiography, though no evidence of thromboembolic disease. In the other subject, an open lung biopsy revealed peribronchial and peribronchiolar fibrosis, with the demonstration of lamellar inclusion bodies, indicative of Gb3 deposition, within lung smooth muscle cells and vascular endothelium. In both of these cases there was a marked improvement in pulmonary symptoms and signs, as well as pulmonary function

tests and radiological appearance, on commencing enzyme replacement therapy. This was sustained for up to 2 years in one case.

Whilst it is difficult to draw any firm conclusions from such cases, they suggest that enzyme replacement therapy may positively influence pulmonary dysfunction in AFD. However the effect of ERT on lung changes in AFD requires further exploration, given that previous studies have largely involved enzyme naïve subjects (144). It is noteworthy that in both case reports the subjects were female. However due to the small numbers, possible gender differences in AFD lung involvement remains an area that requires further investigation.

Given the high frequency and severity of cardiac involvement in AFD, symptoms such as shortness of breath and reduced exercise tolerance are likely to be attributed to cardiovascular dysfunction. However, it is possible that these symptoms may reflect respiratory involvement. One cardiac study in AFD patients that included cardiopulmonary exercise testing also reported a high incidence of respiratory symptoms, obstructive spirometry and two patients with abnormalities on CT (145), though overall it was judged that the reporting of pulmonary symptoms correlated best with the degree of left ventricular dysfunction. Elsewhere, cardiopulmonary exercise testing parameters in AFD subjects were reported to improve with enzyme replacement therapy (146). Here too it was proposed that the cardiovascular, as opposed to pulmonary, changes in AFD were the main contributor to reduced exercise tolerance in AFD.

1.3.2 Other relevant factors

1.3.2.1 α-galactosidase A activity

The exact nature of the underlying changes in the lung in AFD is unclear. Whilst α-galactosidase A activity is most often measured in plasma and from peripheral blood leucocytes, it can also be detected in cultured fibroblasts, tissue homogenate and tears (147;148). However, little is known about α-galactosidase A activity in the lungs, as thus far this has not been quantified. Hence the relationship between enzyme activity in blood and lung is unknown. Given the phenotypic variations in AFD presentation that occur with differing levels of

residual enzyme activity, this may be relevant when exploring the possible mechanisms by which lung involvement occurs and the extent to which the lungs are affected.

1.3.2.2 Airways disease

The finding of airway obstruction in reported pulmonary studies in AFD suggests that patients with similar airway dysfunction may be a useful reference population with which to compare AFD patients. The two commonest obstructive airway conditions are asthma and chronic obstructive pulmonary disease (COPD). Asthma is characterised by symptoms of wheeze, cough and chest tightness. These are features of airway hyper-reactivity, reversibility to bronchodilators and largely eosinophilic inflammation, which may be associated with atopy (149). It has been predominantly thought to be a large airways disease though there is evidence to suggest the small airways may also be involved (150). Chronic inflammation results in structural changes including the hyperplasia of mucous secreting cells and increases in airway smooth muscle cells and airway remodelling (151).

The definition of COPD in the recently updated and published GOLD Report 2013 includes the description of "…persistent airflow limitation, which is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lungs to noxious particles or gases…" (152). It has been shown to occur as a result of chronic inflammation, with a resultant fixed narrowing of the small airways and increased levels of inflammatory mediators including cytokines, growth factors and chemokines, and increased numbers of cytotoxic T cells, neutrophils and macrophages (153). COPD affecting the more peripheral airways in an irreversible manner is more in keeping with what is already known with regards to the nature of the lung changes in AFD.

The relatively fixed airway obstruction reported in AFD suggests that COPD may be a helpful model with which to compare the AFD lung. Also, as the impact of smoking in previous AFD studies is conflicting, examination of COPD subjects

might also help to clarify whether changes seen in AFD are similar or distinct from those found in COPD, which largely arises as a consequence of smoking.

1.3.2.3 The lungs in other LSDs

The lungs have been shown to be involved in other lysosomal storage diseases to different extents and through different mechanisms, though data in this area are very limited. Lung involvement is uncommon in Gaucher disease, and tends to be present in severely affected patients, in whom the outcome is particularly poor. It manifests as interstitial pulmonary infiltrates which can improve on ERT (154). In a recent bronchoalveolar lavage study, lipid laden macrophages were seen, and lung biopsy revealed foamy histiocytes in the alveolar spaces and interstitium, though no fibrosis (155). In some cases of Gaucher disease pulmonary hypertension and hepato-pulmonary syndrome can occur as complications of type I Gaucher disease (156).

In the Mucopolysaccharidoses lung involvement when present tends to occur as a result of glycosaminoglycan deposition in the soft tissues upper and lower airway resulting in airway obstruction, recurrent respiratory tract infections, and in some cases restrictive lung disease secondary to skeletal abnormalities (157). In glycogen storage disease type II, Pompe disease, progressive respiratory muscle weakness results in ventilatory failure that leads to significant morbidity and mortality (158).

Overall there is little known about the effect of LSDs on the lungs. Limited data suggest that other LSDs do not have similar pulmonary findings to those reported to occur in AFD.

1.3.2.4 Induced sputum

Induced sputum sampling is increasingly used as both a clinical and research tool in many different forms of pulmonary disease. This includes COPD (159-161), asthma (162) and pulmonary tuberculosis (163). In 2002 a European Respiratory Society Working Group reviewed studies on induced sputum sampling and reported on safety and methodology with a view to standardisation of induced sputum sampling (164;165). It has the advantage of

being non-invasive, well tolerated, quick and relatively simple to perform when compared to other methods of obtaining lung derived samples, such as bronchoscopy. It is however recognised that cell profiles obtained by induced sputum sampling are not the same as those from broncho-alveolar lavage (166). It is likely that the origin of induced sputum samples is from larger airways, and more in keeping with that seen in bronchial washings.

Case reports in AFD have shown evidence of change at a cellular level through demonstration of storage material in induced sputum from an AFD subject (167). It is therefore possible that induced sputum may be a useful and non-invasive means of evaluating changes in the lung related to AFD, such as α-galactosidase A activity, cytokine concentrations and for determining cell populations that may be of relevance in our understanding of lung involvement of AFD.

1.3.2.5 Flow cytometry

Examination of the cellularity of lung-derived samples and determining differential cell counts has largely been performed by the preparation of cytospins and cell counting with light microscopy (168;169). However, more recently flow cytometry has been used to delineate cell populations in samples such as resected lung parenchyma and bronchial biopsies in COPD where in particular CD8+ T cell populations have been identified (170;171). Subsequent studies have compared samples from COPD subjects obtained by BAL, bronchial washes and induced sputum using flow cytometry (172). Here differences between the sample methods are predominantly due to changes in neutrophils, with the greatest proportion being present in induced sputum.

In interstitial lung diseases, flow cytometry of bronchoalveolar lavage samples reveals increased CD4+/CD8+ T cell ratios. This can be used as an aid to diagnose pulmonary sarcoidosis (173). It has subsequently been shown to also apply to induced sputum sampling (174). The diagnostic value of induced sputum flow cytometry has been demonstrated for active pulmonary tuberculosis using

recognition of CD4+ T cells specific to Purified Protein Derivative of *Mycobacterium tuberculosis* (175).

1.2.3.6 Other considerations

It is not particularly surprising that there are only a limited number of studies investigating the lungs, as the main causes of significant disease in AFD result from renal, cardiac and neurological damage. However with the advent of enzyme replacement therapy offering the possibility of improved outcomes and an altered disease natural history (including greater longevity for AFD patients who are now at risk of non AFD age-related diseases), it is an opportune time to investigate the lung.

Support for this argument comes from HIV medicine, where the significant advances in treatment and improvements in outcome have enabled there to be more focus on longer term conditions, such as bone disease, nephropathy (176;177) and smoking-related conditions, of importance in a surviving, ageing population. Whilst treatment of AFD is not as effective as that for HIV infection, better AFD therapies will result in longer survival and hence a greater risk of lung related disease.

In the context of AFD one could also propose that further pulmonary investigation is warranted on the basis that for a multisystem disease which causes life threatening morbidity in other organ systems, the lungs appear "protected". Hence there may be additional value in understanding the impact of AFD on the lungs as this may provide insights on the mechanisms of protection, and how pulmonary changes relate to overall disease severity.

1.4 Study Hypothesis, Aims and Objectives

Our aim was to examine the respiratory system of a cohort of AFD subjects with carefully documented disease under the care of the Royal Free Hospital. Such a cohort could be comprehensively investigated and consistently sampled in ways not previously performed. This could be undertaken both longitudinally and also by cross sectional analysis. We aimed to examine the pulmonary

changes in AFD through clinical, physiological, radiological and pathological assessments.

Our overriding hypotheses were that AFD does involve the lung significantly though perhaps not severely, and that changes would be demonstrable on lung function testing, imaging and from sampling the lungs by induced sputum. We hypothesised that this would show similar changes to those seen in the other organs involved in AFD with α-galactosidase A activity lower than in controls, and that we would find differences in the sputum cell populations in AFD subjects, particulary lymphocytes, and that these may be similar to the changes seen in COPD.

The hypotheses tested in each section of the study are summarised as follows:

1.4.1 Lung function and imaging

Hypotheses

The degree of pulmonary involvement in AFD

- is related to

  o Gender

  o Age

  o Overall disease severity

- Is progressive over time and not influenced by ERT

- Is demonstrable on CT scanning

- Is worsened by smoking

Additional aims and objectives

1. To establish the extent and severity of airway obstruction and changes in diffusion capacity in the AFD population

2. To examine the differences in spirometry and diffusion capacity between genders in AFD compared to sex matched controls

3. To determine changes in spirometry and diffusion capacity over time in AFD

4. To determine the impact on the airways of smoking in AFD subjects

5. To examine the severity of lung involvement in AFD as measured by spirometry and diffusion capacity in relation to the duration of enzyme replacement therapy (ERT)

6. To examine the relationship between lung involvement in AFD and overall disease severity and genotype

7. To document pulmonary imaging findings in AFD using frontal chest radiographs and high resolution CT scanning

1.4.2 Pulmonary α-galactosidase A activity

Hypotheses

α-galactosidase A activity:

- is measurable in induced sputum

- is lower in AFD subjects than in controls in sputum

- is lower in AFD males than AFD females

- is consistently related between blood and induced sputum

- is lower in the induced sputum of AFD subjects with pulmonary involvement

- is higher in AFD subjects on enzyme replacement therapy (ERT)

Additional aims and objectives

1. To establish a normal reference range for α-galactosidase A activity in induced sputum

2. To compare α-galactosidase A activity in the induced sputum of AFD subjects and controls

3. To compare α-galactosidase A activity in the induced sputum of AFD males and females

4. To delineate a relationship between peripheral blood and lung derived α-galactosidase A enzyme

5. To examine the effect of enzyme replacement therapy on α-galactosidase A activity in induced sputum

6. To examine the relationship between induced sputum α-galactosidase A enzyme activity and lung involvement in AFD

1.4.3 Induced sputum cell populations

Hypotheses

Induced sputum cell populations in AFD subjects with pulmonary involvement compared to those without would have:

- a greater proportion of CD8+ T cells than in AFD subjects

- a higher proportion of neutrophils than that of AFD subjects without airway obstruction

- a higher concentration of pro-inflammatory cytokines IL-8 and IL-6

Additional aims and objectives

1. To delineate the cell populations present in the induced sputum of AFD subjects

2. To compare cell populations in the induced sputum of AFD subjects to COPD and healthy control subjects

2. To compare the cell populations present in the induced sputum of AFD subjects with airway obstruction and those without

3. To examine the inflammatory processes in the lung in AFD by examining cytokine profiles

4. To examine the impact of smoking on inflammatory cytokines in induced sputum in AFD

Chapter 2 – Methods

2.1 Ethics

The study was performed at the Royal Free Hospital, London, and all patient assessments and investigations performed on site. The study was approved and sponsored by the Royal Free Hospital Research and Development Department, and was ethically approved by the Local Research and Ethics Committee.

All patient data were stored securely within a password protected system, under individual study numbers assigned at the time of recruitment. All potential study subjects were sent written information regarding the study in the form of the Patient Information Sheet (Appendix 1, Sections 1.0-3.0) relevant to the appropriate arm of the study for them, and contacted by telephone to answer any questions raised by the written study information.

2.2 Recruitment and Clinical Assessments

2.2.1 Anderson Fabry Disease (AFD) subjects

Subjects were recruited from the clinical cohort of Anderson Fabry Disease patients under the care of the Lysosomal Storage Disorders Unit at the Royal Free Hospital, London. If willing to participate, arrangements were made for the study investigations to be performed on the day of their next clinic attendance, and at subsequent follow up appointments over the following 12 months.

All AFD subjects were under the care of the Lysosomal Storage Disorders Unit at the Royal Free Hospital, a National Specialist Commissioning Advisory Group (NSCAG) designated centre for the diagnosis and management of lysosomal storage disorders. Their attendance for study participation was timed to coincide with their scheduled medical appointment, in order to minimise any inconvenience or additional expense given that many subjects live some distance away from the hospital. Study data collection and investigations were performed alongside their routine clinical care.

2.2.1.1 Diagnosis

All patients recruited to the AFD arm of the study had a confirmed diagnosis of Anderson Fabry disease by plasma enzyme activity and DNA mutational analysis, with the majority of these performed by the Molecular Diagnostic Laboratory at the Royal Free Hospital. If carried out elsewhere the mutation was confirmed by obtaining source data. In addition over half the subjects also had blood leucocyte enzyme activity from the time of diagnosis.

2.2.1.2 Medical Assessment

In general, AFD patients attend the Lysosomal Storage Disorders Unit for a clinical review on a six-monthly basis. This involves an assessment by a physician, with the completion of a clinical assessment proforma (Appendix 1, Section 8.0). A full set of observations is recorded at each visit, including blood pressure, pulse rate, oxygen saturations, temperature and weight. In addition a full clinical examination of the cardiovascular, respiratory, abdominal, neurological and musculoskeletal systems undertaken and documented. AFD patients also regularly undergo investigations for evidence of disease progression in the different organ systems affected. These include the following:

- Blood tests (every six monthly)

    o Full blood count, clotting screen, iron studies

    o Bone profile, liver function tests

    o Glucose, lipid profile

    o Immunoglobulin levels

- Renal assessments (every six months)

    o Blood test for urea, electrolytes, creatinine

    o Cr-51 EDTA GFR performed by the Nuclear Medicine Department

    o 24 hour urinary protein collection performed by Biochemistry Department

- o Spot urine for albumin/creatinine ratio performed by Biochemistry Department

- Cardiac assessments (six-monthly to annually)

  - o Clinical review at the Heart Hospital, Westmoreland Street, London, by Dr. Perry Elliot (Consultant Cardiologist) and team

  - o echocardiogram performed by Echocardiography Department at the Heart Hospital, with attention to left ventricular mass and septal wall thickness and other changes related to AFD

  - o Cardiopulmonary Exercise Testing as indicated

  - o ECG and 24 hour tape as indicated

- Ophthalmology (six monthly)

  - o clinical review by Dr. Pauline Wilson (Consultant Ophthalmologist, Royal Free Hospital, London)

  - o slit lamp examination

  - o retinal photography

- Dermatology (six monthly)

  - o clinical review by Dr. Cate Orteu (Consultant Dermatologist, Royal Free Hospital, London)

- Audiology (six monthly)

  - o Pure tone audiograms performed by the Royal Free Hospital Audiology Department

- Neurology (annually)

  - o T2 weighted MRI scanning of brain performed by Radiology, MRI Department at Royal Free Hospital, with specialist neuroradiology reporting (two yearly)

- o Clinical review by Professor Lionel Ginsberg (Consultant

    Neurologist, Royal Free Hospital, London) and team as indicated

2.2.1.3 Additional Study Information

For the purpose of the study, additional information was collected specifically related to the respiratory system and enzyme replacement therapy for each individual study subject. This included the following:

- Smoking History

    - o tobacco/pack years

    - o recreational drugs

- Drug History

    - o bronchodilator use

    - o frequency

    - o use of beta blockers

- Enzyme Replacement Therapy (ERT)

    - o duration

    - o dose

    - o date of last infusion

- Family History of respiratory disease

- Social History

- Employment History

    - o asbestos exposure

    - o dust/chemical exposure

- Plasma enzyme activity at diagnosis

- Leukocyte enzyme activity at diagnosis

2.2.1.4 Severity Scores

Mainz Severity Score Index (MSSI)

Using the clinical information and investigation results gained from these reviews at the time of the first study visit, the Mainz Severity Score Index, a validated tool for assessment of the severity and monitoring of treatment response in AFD, was calculated for each AFD subject recruited at the start of the study (178). An overview of the clinical features considered in the calculation of the MSSI is given in Table 1.0.

Age Adjusted Severity Score

In addition, the age adjusted severity score based on the adapted Fabry Outcome Survey (FOS) MSSI was also calculated using the clinical information gathered at the first study visit (179)

Table 2.0 Clinical features taken into consideration for calculation of Mainz Severity Score Index in AFD

General appearance	Neurological	Cardiovascular	Renal
Facial appearance	Tinnitus	Changes in cardiac muscle thickness	Proteinuria
Angiokeratoma	Vertigo		
Oedema			Abnormalities of glomerular filtration
	Acroparaesthesia	Valve insufficiency	
Musculoskeletal abnormality	Fever pain crisis		
		ECG abnormalities	
	Cerebrovascular abnormalities		
Corneal verticillata		Pacemaker	
Diaphoresis	Depression	Hypertension	
	Fatigue		
Abdominal pain	Reduced activity level		
Diarrhoea/constipation			
Haemorrhoids			
Pulmonary			
New York Heart Association (NYHA) Classification			
Maximum score = 18	**Maximum score = 20**	**Maximum score = 20**	**Maximum score = 18**

2.2.2 Chronic Obstructive Pulmonary Disease (COPD) subjects

Patients were recruited from the London COPD cohort based at the Royal Free Hospital, with the kind permission of Professor Wedzicha and the assistance of the Academic Respiratory Department and team. None of the patients recruited were participating in a clinical trial at the time, and care was taken to ensure that all study investigations were performed with the patients at baseline, at least six weeks clear of any pulmonary exacerbations.

Patients were identified based on their close matching using gender, spirometry and age to an AFD subject with airway obstruction. All spirometric data used for matching were from within 6 months of recruitment to the study. Subjects were approached initially by the Academic Respiratory Department to ascertain whether they would be amenable to a discussion regarding the study at their next routine visit, and to receiving written information regarding the study through the post in advance of this visit. If happy to participate they were reviewed, consented provided blood and respiratory samples under the direction of Dr. Nadia Shafi at their subsequent clinic visit, using the same standardised methods as the AFD group. Samples were processed on the same day of collection (within 2 hours for sputum samples).

2.2.3 Healthy Control Subjects

Control subjects were recruited from amongst healthy volunteers within the Royal Free Hospital, and matched as closely as possible to an AFD subject by gender, spiromtery and age. It was ensured that they did not have a diagnosis of a pre-existing respiratory condition, and had no clinical symptoms to suggest an undiagnosed lysosomal storage disorder. A smoking history was documented for each of the control subjects.

2.3 Study Investigations

2.3.1 Lung Function Testing

All lung function tests were performed in the Lung Function Department at the Royal Free Hospital by qualified lung function technicians under the supervision of Ms Tina Adamou (Head, Lung Function Technician).

2.3.1.1 Equipment

The system used was a complete on-line computerised system MasterScreen PFT (Carefusion Vyasys Products) for recording flow-volumes loops, pulmonary diffusion capacity and lung volumes. This system uses a pneumotachograph to record volume as a function of integrated flow and

incorporates fast gas analysers for recording Helium, Oxygen and Carbon monoxide. It has a gas reservoir of up to 8 litres volume displacement.

2.3.1.2 Method

The method used for measuring absolute lung volumes was by the closed circuit helium dilution re-breathing method and gas transfer factor by single breath carbon monoxide. The system meets the technical minimum specification laid out by the European Respiratory Society (ERS) and American Thoracic Society (ATS) guideline standards. A graphical display of real-time gas analysis and tidal volume is available on the screen during the measurements. The flow, volume and gas analysers are calibrated daily for quality assurance according to the manufacturer's instructions. The unit is assembled on a smoothly running trolley and has an adjustable support arm.

2.3.1.3 Measurements

All measurements were performed according to the American Thoracic Society/European Respiratory Society guidelines (180;181). The test gas used for DLCO was in the region of 0.28% carbon monoxide, 9% helium (He) balance nitrogen. The mean of two technically accepted recordings were obtained with a 4 minute rest between measurements (rationale in guidelines). One measurement of functional residual capacity (FRC) was made using 9% He in air with a balance of nitrogen, with subjects asked to take relaxed deep breaths during the He dilution method to ensure mixing, and total lung capacity (TLC) and residual volume (RV) calculated. The system detects end-tidal breath automatically and will correct for any shift in the FRC resting level. The gases were provided from pre-prepared cylinders (BOC special medical gases), with additional Oxygen supplied by a separate cylinder containing 100% medical Oxygen. The CO_2 in the circuit was removed by soda lime and the oxygen supply is controlled by an oxygen sensor.

2.3.1.4 Results interpretation

All lung function tests were interpreted based on the criteria set out by the Global initiative for Chronic Obstructive Lung Disease (182), (Table 2.0).

2.3.1.5 Reversibility testing

All bronchodilator reversibility testing was performed using nebulised salbutamol 2.5mg in 2.5mls via a mouth piece. Post bronchodilator spirometry was performed 15 minutes after bronchodilator administration.

Table 2.1 GOLD staging for spirometry in Chronic Obstructive Pulmonary Disease

GOLD Spirometric Criteria for COPD Severity	
I. Mild COPD	$FEV_1/FVC < 0.7$ $FEV_1 >$ or $= 80\%$ predicted
II. Moderate COPD	$FEV_1/FVC < 0.7$ FEV_1 50% to 79% predicted
III. Severe COPD	$FEV_1 /FVC < 0.7$ FEV_1 30% to 49% predicted
IV. Very Severe COPD	$FEV_1 /FVC < 0.7$ $FEV_1 < 30\%$ predicted or $FEV_1 < 50\%$ predicted with chronic respiratory failure

2.3.2 Blood Sample Preparation

All blood samples obtained for the study were processed by Dr Nadia Shafi on the same day as acquired.

2.3.2.1 Plasma

Whole blood samples from study subjects were collected in 6 ml Lithium heparin tubes. Samples were kept on ice for 25 minutes prior to being centrifuged at 2500*g* for 5 minutes at 4°C. The plasma was carefully aspirated from these samples and stored in microcentrifuge tubes [Fisher Scientific, UK] at - 80°C until further use.

2.3.2.2 Peripheral blood mononuclear cells (PBMC)

Whole blood samples form study subjects were collected in 10ml EDTA tubes. This was layered onto 5mls of Lymphoprep™ [Axis-Shield, Norway] in a 15ml Falcon™ tube and centrifuged at 650g for 25 minutes at 4°C. Following gradient centrifugation, the white cell layer was removed using a sterile pipette, and the recovered cells placed in 3 or 4 1.7ml Eppendorf micro-centrifuge tubes depending on the volume obtained. The cells were then spun down in a micro-centrifuge at 4000g for 2 minutes. The resulting cell pellets were re-suspended in 1ml of red blood cell lysis solution (8.29g NH_4Cl [Sigma Aldrich, UK]; 1g $KHCO_3$ [Sigma Aldrich, UK]; 0.02g EDTA, di-sodium [Sigma Aldrich, UK]; 1 litre distilled water) and left at room temperature for 5 minutes. This step was repeated if red cell lysis were incomplete, given that contaminating red blood cells may lead to artificially high results in enzyme assays. The peripheral blood mononuclear cells were subsequently washed in PBS (5 minutes; 300g) prior to cell lysis using 400µl of ProteoJET Mammalian Cell Lysis solution [Fermentas, Fisher Scientific]. The cell pellet was dispersed by pipetting up and down and vortexing for 30 seconds, and subsequently rocked at room temperature for 10 minutes at 80rpm. Following a further brief vortex, the sample was placed in the micro centrifuge for 15minutes at 10,000g. On removal of the sample the cell lysate was aspirated and stored at -80°C for future processing.

2.3.3 Fluorometric assays for plasma and leucocyte α-galactosidase A

For all measurements of enzyme activity the standardised protocol for the Royal Free Hospital Molecular Diagnostics Laboratory were followed for the sample type obtained. These enzyme assays are based on the use of a substrate, in this case 4-methylumbelliferyl-α--D-galactopyranoside, being hydrolysed at acid pH to 4-methylumbelliferone and galactose by α-galactosidase A. Stopping the enzyme reaction after a controlled time period, by adding an alkaline buffer, enables the differences in the fluorescent properties of the unhydrolysed substrate compared to the products of hydrolysis to be used to give a measure of α-galactosidase A activity.

Enzyme assays were performed using a BMG Fluorstar Galaxy fluorimeter, set at excitation 360nm and emission 460nm. For this study the measurement of sputum supernatant α-galactosidase A activity was performed as per plasma samples, and for sputum leucocytes as that for peripheral blood leucoytes, following sputum processing (subsequently described in this chapter).

2.3.3.1 Plasma and sputum supernatant α-galactosidase A assay

A minimum of 0.6ml plasma or sputum supernatant was used for each assay. For this assay, in order to accurately measure the activity of α- galactosidase A it was necessary to inhibit the activity of α-galactosidase B, as this has significant activity in plasma. This was achieved using an inhibitor of the enzyme, N-acetyl-D-galactosamine [MWt 221.2) Sigma Aldrich, UK]. The substrate used was 4-methylumbelliferyl-α-D-galactopyranoside in solution [250mg in 74ml of 0.5M acetate buffer (MWt 338), Sigma Aldrich, UK]. A substrate/inhibitor solution was made by dissolving 500mg of the inhibitor in 11.3mls of substrate solution.

The reaction mixture of 100µl plasma or sputum supernatant with 100µl of substrate/inhibitor was set up in duplicate in the last 2 wells of the 24 well plate. After 2 hours incubation at 37 $^{\circ}$C, the reaction was stopped using the stopping reagent of 1M glycine buffer with a pH 10.4 [55.7ml of 75g glycine/58g sodium chloride in 1 litre of distilled water with 44.3ml of 1M sodium hydroxide solution]. A standard for comparison was set up in Well 2 using an equal volume (200µl) of a standard solution of 4-methylumbelliferone [(MWt 176), 1nmol per 200µl, Sigma Aldrich, UK], with a standard blank in the neighbouring well using an equal volume of water, adjacent to a substrate blank with 100µl of substrate/inhibitor and 100µl of plasma. These standards allow compensation for changes in fluorescence due to other factors such as haemolysis and drugs. With each step of addition, mixing was performed.

The plate was then inserted into the BMG FLUOstar Galaxy™ plate reader (excitation 365nm, emission 450nm) to be read. For each sample using this method, the reading from the Standard minus the reading from the Standard

blank was equivalent to fluorescence of 1nmol 4-MU. The activity of the test sample was calculated using the formula:

$$\frac{(T-B)}{S} \times \frac{60}{120} \times \frac{1000}{100} = nmol/hr/ml\ plasma$$

where T = average of the test readings for the sample, B = reading for blank sample, S = reading from standard sample

The steps followed for each plasma or sputum supernatant sample in a 24 well plate are summarised in order going down the table in Table 2.3.3.1. Results of the plasma enzyme activity were interpreted based on the reference range used by the Royal Free Molecular Diagnostics laboratory, as outlined below:

Reference Range for plasma α- galactosidase A

Normal   4.0 – 21.9 nmol/hr/ml

Hemizygote Fabry   0 – 0.9 nmol/hr/ml

Heterozygote Fabry   0.22 – 7.4 nmol/hr/ml

Table 2.3.3.1 Summary of procedure for the measurement of α- galactosidase A activity in plasma and sputum supernatant samples

Well 2	Well 3	Well 4	Well 5	Well 6
Standard	Standard Blank	Substrate Blank	Reaction Mixture	Reaction Mixture
		100µl substrate/ inhibitor	100µl plasma + 100ul substrate/ inhibitor	100µl plasma +100ul substrate/ inhibitor
			Mix	Mix
		Incubate at 37°C for 2 hours in cell culture incubator		
		1.0ml stopping reagent	1.0ml stopping reagent	1.0ml stopping reagent
		100ul plasma		
200µl standard solution	200µl H$_2$O			
0.9ml stopping reagent	0.9ml stopping reagent			
100µl plasma	100µl plasma			
Mix	Mix			
Insert plate into plate reader to read fluorescence and calculate activity in nmol/ml/hr				

2.3.3.2 Peripheral blood and sputum leucocyte α-galactosidase A assay

In leucocytes usually over 95% of the total α-galactosidase activity is α-galactosidase A, with a much smaller contribution from α-galactosidase B. Peripheral blood leucoytes were obtained as described in Methods Section 2.3.2.2, and sputum leucocytes as described in Methods Section 2.3.6.2. Leucocyte cell lysate from sputum samples was prepared as described in Methods Section 2.3.2.2.

The protein content of the prepared cell lysate from both blood and sputum samples was measured using the Bicinchoninic Acid Protein Assay Kit [Sigma, product code BCA-1and B 9643] in a 96 well microtitre plate. Protein content was quantified by colorimetric measurement using a BMG FLUOstar Galaxy™ plate reader (absorbance wavelength 562nm; reference wavelength 650nm), using a standard curve generated from dilutions of bovine serum albumin (BSA) between 0-1000 mg/ml to determine the protein content of each lysate sample. This was then used in the calculation of leucocyte α-galactosidase A activity for each sample.

For leucocyte enzyme measurements the reagants used including the substrate/inhibitor, standard solution and stopping reagent were prepared as described in Methods Section 2.3.3.1. For each reaction mixture, which was set up in duplicate, 15μl of leucocyte protein sample was mixed with 150μl of substrate/inhibitor solution in a 24 well plate. This was incubated for 30 minutes at 37 ^{o}C before the reaction was stopped using the stopping reagent. The substrate blank was set up using 150μl of substrate/inhibitor which was incubated with the reaction mixture before 1ml of stopping solution and 15μl of the leucocyte protein sample were mixed in. The standard blank and the standard were set up with 200μl of water 200μl of the standard solution respectively, along with 950μl stopping reagent and 15μl of sample. With this set up, for each sample the difference in fluorescence between the Standard and the Standard blank was equivalent to a fluorescence of 1nmol 4-MU.

The formula used to calculate α-galactosidaseA activity in each sample was:

Fluorescence of sample x   30min x 1000µl   x   1mg

Fluorescence of standard   60min   15µl   Protein conc. (mg/ml)

= leucocyte α-galactosidase A activity (nmol/hr/mg of protein)

The steps followed in the procedure using a 24 well plate are summarised in Table 2.3.3.2. Sample results for peripheral blood leucocyte α-galactosidaseactivity were interpreted based on the reference ranges used by the Molecular Diagnostics laboratory at the Royal Free Hospital as shown below:

Reference Range for peripheral blood leucocyte α-galactosidaseA

Normal   33 - 134 nmol/hr/mg of protein

Hemizygote Fabry   0.4 - 5 nmol/hr/mg of protein

Heterozygote Fabry   4.3 - 73 nmol/hr/mg of protein

Table 2.3.3.2 Summary of the procedure for measurement of α-galactosidase A activity in leucocytes

Well 1	Well 2	Well 3	Well 4	Well 5
Standard	Standard Blank	Substrate Blank	Reaction Mixture	Reaction Mixture
			15µl leucocyte protein sample	15µl leucocyte protein sample
		150µl substrate/ inhibitor	150µl substrate/ inhibitor	150µl substrate/ inhibitor
		Mix	Mix	Mix
		Incubate at 37°C for 30 minutes in cell culture incubator		
		1.0ml stopping reagent	1.0ml stopping reagent	1.0ml stopping reagent
		15ul leucocyte protein sample		
200µl standard solution	200µl H_2O			
950µl stopping reagent	950µl stopping reagent			
15µl leucocyte protein sample	15µl leucocyte protein sample			
Mix	Mix			
Insert plate into plate reader to read fluorescence and calculate activity in nmol/ml/hr				

2.3.4 Radiology

All chest radiographs (CXR) and high resolution computed tomography (HRCT) scans were performed by the Radiology Department at the Royal Free Hospital using standardised protocols. Films were reported by different members of the Radiology Department and then reviewed by a specialist Consultant Thoracic Radiologist (Dr. J. Haddock, Royal Free Hospital, London).

2.3.4.1 CXRs

All chest radiographs were performed using digital radiography at the first study visit. The film taken was in the standard postero-anterior, non-rotated position with scapula rotated away from the lung fields and with full inspiration on exposure. The equipment used 125kVp and automatic exposure chambers (AEC), with a scatter reduction grid delivering an average of 1.8mAs for the average sized adult. The focal film distance (FFD) was 180cm. The approximate average dose per CXR was 0.9dGycm2.

2.3.4.2 HRCT scans

Scans were performed on a Brilliance 64 [Philips Medical Systems] or GE Lightspeed [GE Healthcare] CT scanners using the same lung algorithm. Inspiratory and expiratory images were captured using 1.25mm slices at 10mm increments with 120kVp and 200mA, with a 0.5s rotation time and field of view (FoV) of 350mm. Both scanners possessed 2 x 0.625mm detectors with lung enhanced filters.

2.3.5 Sputum Induction Procedure

2.3.5.1 Equipment and setting

All sputum induction procedures were performed in the isolation system housed in the Out Patient Department of the Royal Free Hospital, London. This isolation system consists of a framework 2.2m high x 1.5m wide, glass clear envelope with a Nyplax reinforced floor fitted with inner polypropylene sheet. It has a single inlet and double outlet filter pack with high efficiency particulate air fan unit with single extraction and fixed negative pressure. Fifteen air exchanges

occur per hour within the chamber. Waste air is double HEPA filtered and then extracted from the chamber via a hose.

An ultrasonic nebuliser [DeVilbiss Ultraneb 3000] was set up inside the chamber for the delivery of the hypertonic saline, with the use of disposable cups and lids, elephant tubing and mouthpieces to ensure no possibility of cross infection between subjects. This nebuliser operates at a nebuliser rate of 3ml/min delivering a maximum flow rate of 20l/min, with a particle size of <4μm. This ensures a good delivery of the hypertonic saline to the lower airways in order to generate a sputum sample representative of this part of the lung.

Experiments were performed on volunteers to determine the optimal concentration of hypertonic saline that would yield sufficient cell numbers per sample obtained, and therefore 4% hypertonic saline was used in all induced sputum sampling.

2.3.5.2 Procedure

All subjects were given written and verbal information with regards to the procedure, and given the opportunity to ask questions prior to the procedure. Subjects were all sampled in their baseline state, and were excluded from induced sputum sampling if they had any symptoms of chest infection within the preceding 6 weeks.

Sputum induction was performed after the subjects had undergone lung function testing to ensure that poor lung function did not preclude them from having the procedure (FEV_1<1litre). They were encouraged to keep well hydrated leading up to the procedure by drinking water, but not to eat in the 2 hours immediately prior to the procedure if at all possible. Baseline oxygen saturations were also taken.

In order to minimise the possibility of bronchospasm, subjects were pre-medicated with Salbutamol either in nebulised form during their lung function tests, or with a 200mcg dose via a metered dose inhaler. At least 10 minutes after this a baseline measurement of FEV_1 was recorded using a MicroPlus hand

held spirometer [MicroMedical, UK] with a bacterial/viral filter. This reading was used for comparison with subsequent readings through the course of the procedure to ensure there was no significant bronchospasm as a result of saline inhalation.

For study subjects up to a total of 40mls of 4% hypertonic saline was used in each sputum induction procedure - each of which was performed under the supervision of the same operator, Dr. Nadia Shafi. Subjects were asked to sit inside the chamber and inhale the nebulised saline via a mouthpiece, breathing in and out normally, with a deep breath every tenth breath. They were encouraged to keep their mouth free of saliva by spitting into a waste pot rather than swallow, and to blow their nose if required to minimise salivary contamination of the sample.

After 5 minutes of inhalation, subjects were asked to rinse their mouth out with water, to take 2 deep breaths and then encouraged to cough and expectorate any sputum produced into a collection pot. They were also taught the technique of huffing to assist expectoration. Following this, a repeat measure of FEV_1 was taken, and as long as there had not been a drop of >15%, the steps were repeated a further 3 times to a total of 20 minutes of saline inhalation. At the end of the whole procedure subjects were kept in the chamber for a further 10 minutes until they had stopped coughing altogether.

The expectorated sample was then taken to be processed within 2 hours of obtaining it to ensure maximal cell viability.

2.3.5.3 Safety

Resuscitation equipment in the form of cannulae, Guedel airways, bag and mask, oxygen cylinder and emergency drugs were also available in the room at all times, including Adrenaline 1:10000, Hydrocortisone 200mg, Piriton 10mg for anaphylaxis, and Salbutamol 2.5mg nebules for bronchospasm. In addition Salbutamol metered dose inhalers were available for pre-medication.

At 5 minute intervals throughout the course of the procedure repeat measures of FEV_1 were taken using a hand held MicroPlus spirometer, as a safety measure, to ensure that no significant bronchospasm was occurring as a result of hypertonic saline inhalation. Should there have been a significant drop in FEV_1 (>15% from baseline), the procedure would have been stopped, and subject given further inhaled Salbutamol if required, and observed until their FEV_1 had returned to close to baseline.

Bacterial/viral filters were used on all equipment to ensure no possibility of cross infection of any respiratory pathogens between subjects. Following each sputum induction procedure, the sputum induction chamber and equipment within were all cleaned using Tristel Sterilising Wipes [Tristel Solutions Ltd, Cambridgeshire, UK] to ensure no possibility of cross infection of any kind between subjects.

Overall, the procedure was very well tolerated by all patients. The procedure did not have to be stopped for any safety reasons during the study.

2.3.6 Preparation of sputum samples, fluid and cellular components

Induced sputum sample processing was commenced with 2 hours of obtaining the samples at all times to ensure accurate measurement of cell counts and viability. The samples were kept at room temperature until processed.

The processing method used was based on the recommendations of the European Respiratory Society Working Group on methods for sputum processing for cell counts (183) with some modification to account for the accurate measurement of α-galactosidase A in both the fluid and cellular phase of the sputum. Experiments were performed to determine the effect of 0.1% Dithiothreitol (DTT) on α-galactosidase A enzyme activity in both fluid and cellular components of the sputum, and the recommended processing method was adjusted for this.

2.3.6.1 Experiments

These were performed on blood from healthy volunteers, given that the method for measurement of enzyme activity in blood components is established using this medium.

2.3.6.1a Effect of 0.1% DTT on α-galactosidase A activity in plasma

This experiment was performed to ascertain the impact of 0.1% DTT on enzyme activity or its measurement in the fluid - which is equivalent to the fluid phase of sputum. A-galactosidase A activity was measured in ratios of between 1:1 and 1:4 of the following:

Plasma alone

Plasma with Phosphate Buffered Saline (PBS)

Plasma with 0.1% Dithiothreitol (DTT)

α-galactosidase A activity was then measured using the standard fluorometric assay for plasma as previously described in Methods Section 2.3.3.1, and corrected for the dilution factor for each sample.

This experiment revealed that 0.1% DTT significantly reduces the α-galactosidase A activity in plasma (Figure 2.3.6.1a).

Figure 2.3.6.1a Effect of 0.1% DTT on α-galactosidase A activity in plasma
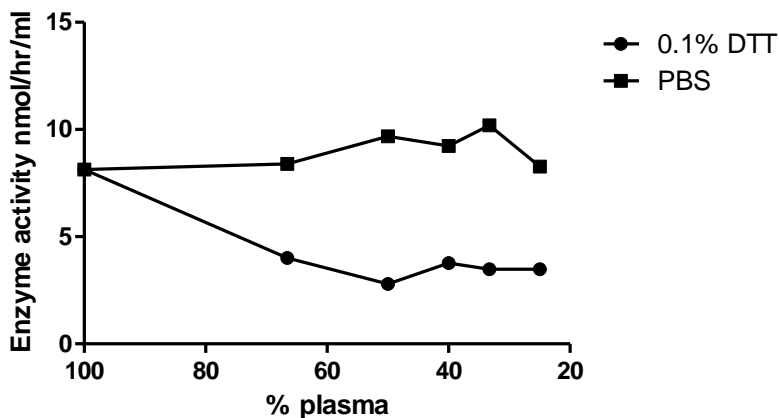
2.3.6.1b Effect of 0.1% DTT on α-galactosidase activity within intact blood leucocytes

This experiment was performed to ascertain the impact of 0.1% DTT on the measurement of α-galactosidase A activity in peripheral blood derived leucocytes from for the purpose of extrapolating the results to apply to sputum derived leucocytes. Five healthy controls donated leucocytes; and these were separated from the peripheral blood as described previously in Methods Section 2.3.2.2. Any contaminating red blood cells were lysed from the cell pellet. The intact leucocytes were then re-suspended in 4 mls PBS and divided into 4 equal portions.

Either PBS or 0.1% DTT was added to the cells in ratios of 1:1 or 1:4 and the samples subsequently processed in the same way as planned for study sputum samples, in that they were placed in a water bath at 37°C and shaken at 100rpm for 20 minutes. The samples were subsequently filtered through a 50µm filter, and centrifuged at 400g for 10 minutes.

The cells from each sample were washed twice using PBS [Sigma Aldrich, UK], then lysed using 200µl of ProteoJET Mammalian Cell Lysis solution [Fermentas, Fisher Scientific, UK] and the lysate collected as previously described. The α-galactosidase A activity was subsequently measured using the fluorometric assay as described in Methods Section 2.3.3.2 in each sample and compared.

This experiment demonstrated no significant difference in the α-galactosidase A activity between intact peripheral blood leucocytes that had been treated with 0.1% DTT and those that had not, demonstrating that 0.1% DTT did not affect α-galactosidase A activity within intact leucocytes (Figure 2.3.6.1b) (One way ANOVA and Friedman's test).

Figure 2.3.6.1b Effect of 0.1% DTT on α-galactosidase A activity from peripheral blood derived leucocytes
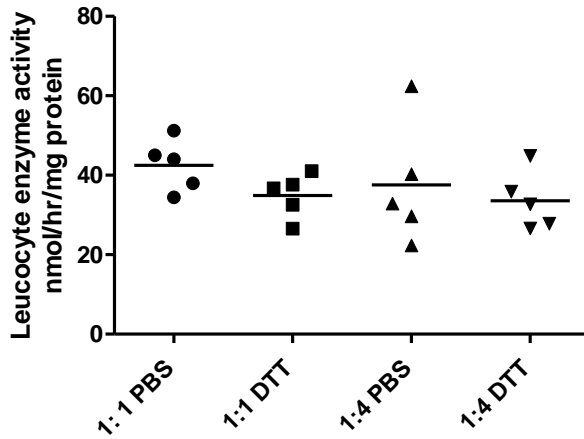
2.3.6.1c Effect of 0.1% DTT on α-galactosidase activity in blood leucocyte lysate

Peripheral blood samples from volunteers were collected and the leucocyte lysate prepared as previously described in Methods Section 2.3.3.2. Varying ratios of the lysate were then made up with PBS and 0.1% DTT, and warmed to 37ºC in the water bath at the same time as being shaken for 20 minutes. α-galactosidase A activity was then measured using the standard fluorometric assay outlined previously and results compared.

α-galactosidase A activity was found to be lower in blood leucocyte lysate when mixed with 0.1% DTT than when mixed with equal volumes of PBS, having corrected for the dilution factor. This suggested that 0.1% DTT may affect the activity of the enzyme from lysed leucocytes (Figure 2.3.6.1c).

Figure 2.3.6.1c Effect of 0.1% DTT on blood leucocyte lysate α-galactosidase A activity compared to PBS

70
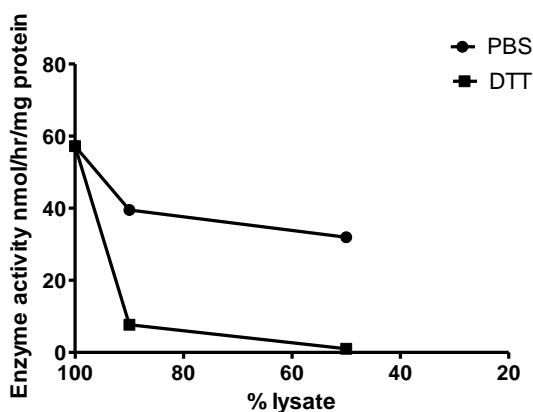
2.3.6.2 Sputum processing method

To maximise yield and viability of the cellular sample, induced sputum processing was commenced within 2 hours of obtaining the sample as recommended in the Report of the ERS Working Group 2002 (184). The whole sample was weighed, and this documented, before being transferred into a petri dish, where the plugs of sputum were picked out from the saliva to minimise the salivary contamination of the sample (185).

The separated sputum sample was then transferred into a 50ml Falcon tube and re-weighed. This weight was used to determine the actual volume of the sample, where 1gm of sputum was equivalent to 1ml. This volume was subsequently used to determine the volumes of the reagents required in the processing method.

The sputum sample was then diluted with an equal volume of PBS [Sigma Aldrich, UK], and the mixture vortexed to homogenise it. On the basis of the results from the experiment performed (Methods Section 2.3.6.1a) prior to the addition of any further reagents, a portion was taken in 1.7ml Eppendorf tubes and centrifuged at 4000g for 2 minutes. The supernatant, being representative of the fluid phase of the sputum, from these samples was then aspirated and stored at -80°C for future enzyme and cytokine assays, and the remainder of each returned to the sputum/PBS mixture.

Following this 0.1% DTT was added to the sputum/PBS mixture, also in an equal volume to that of the sputum, and vortexed for a further 30 seconds, prior to being placed in a water bath warmed to 37°C and shaken at 100rpm for a total of 20 minutes.

On completion of this step the sample was removed from the water bath, and filtered through a 50 micron mesh filter [CellTrics 50µm filters, Partec, UK] to remove any debris without significant loss of cells from the sample.

This filtrate was then centrifuged at 400g for 10 minutes and a cell pellet formed. The cell pellet was dispersed, the cells washed with PBS and re-spun in a

micro centrifuge for 2 minutes at 4000g before re-suspending in R10 growth medium containing RPMI 1640 [Sigma Aldrich, UK], 10% heat-inactivated foetal calf serum [Invitrogen™, Paisley, UK], 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer [Invitrogen, Paisley, UK], 2mM L-glutamine [Sigma Aldrich, UK] and penicillin/streptomycin [>10,000U/ml penicillin, >10mg/ml streptomycin; Sigma Aldrich, UK].

Cell counts were performed by light microscopy using a Neubauer haemocytometer with an etched grid system. 10μl of cell suspension was added to 10μl of 0.2% trypan blue (diluted in filtered phosphate buffered saline, PBS; [Sigma Aldrich, UK]) for 1 minute in a micro-centrifuge tube. Assessment of cell viability was based on cell appearance under light microspcopy, with live cells being colourless and dead cells staining blue due to their inability to exclude dye. A minimum of 200 cells were analysed. Only samples with 80% cell viability, not including squamous cells, were used for further studies. All samples used had a squamous cell count of <20% of the total cell number.

The cells obtained were then divided up for the study investigations required, depending on total cell number.

2.3.7 Sputum α-galactosidase A assays

2.3.7.1 Sputum supernatant

For the measurement of α-galactosidase A in the fluid phase of the sputum, stored samples of sputum supernatant which had been frozen at -80°C were allowed to thaw at room temperature. Once fully thawed, they were vortexed for 30 seconds to ensure full mixing. Measurement of α-galactosidase A activity was then performed as described in Methods Section 2.3.3.1.

2.3.7.2 Sputum leucocytes

A minimum of 1 x10^6 sputum leucocytes from sputum samples processed as per Methods section 2.3.6.2 were used for the measurement of sputum leucocyte α-galactosidase A activity. Cell pellets were lysed using 200μl of the ProteoJET Mammalian Cell Lysis Solution [Fermentas, Fisher Scientific, UK] and α-

galactosidase A activity in the cell lysate measured flourometrically using the procedure described in Methods Section 2.3.3.2.

2.3.8 Preparation and staining of sputum cell cytospins

Of the cells obtained from sputum samples a proportion were used to make cytospin slides for the purpose of examining cell morphology and for correlation with flow cytometry findings if required. For this purpose the concentration of the cell suspension was adjusted to 0.5 x10^6 cells/ml with R10, and 100µl of this cell solution was used for each slide.

Slides were labelled with the study number and date of sample prior to being loaded into the metal holders along with adsorbent filter paper [Thermo Electron Co-operation, East Grinstead, UK]. Cuvettes were secured into place, and 100µl of the cell suspension pippetted into each. The cuvettes with slides were then loaded into place in the centrifuge [Shandon Cytospin© 2] and spun at 450rpm for 2 minutes.

On removing the cuvettes from the centrifuge, slides were allowed to air dry for 2 hours prior to any fixing procedures. For each sample obtained, one cytospin slide was stained with Giemsa. Air dried slides were fixed fully submerging in methanol for 7 minutes before being removed and allowed to air dry once more. Once dry, slides was fully submerged in Giemsa [Sigma Aldrich, UK] having been diluted 1 in 20 with distilled water and filtered, for 25 minutes before being rinsed and air dried. Glass coverslips were mounted on each cytospin slide using DPX mountant [Sigma Aldrich, UK] with care to avoid introduction of air bubbles into the fixative, and allowed to dry for at least 1 hour prior to the slides being viewed under a light microscope.

If cell numbers allowed, additional cytospin slides from the samples were fixed after the initial air drying step by submerging in a 1:1 mixture of methanol and acetone for 20 minutes, and allowed to dry. Following this they were wrapped in aluminium foil and stored in a container with silica gel to remove any moisture, in a fridge at - 20°C for future staining.

2.3.9 Flow Cytometry

To examine the cell populations present, cells obtained from the induced sputum of subjects were stained with fluorescent antibodies. For the majority of samples, a 3 colour Becton Dickinson (BD) FACScan flow cytometer was used to acquire cell data, along with BD antibodies. The antibodies used were direct conjugates with fluorescein isothiocyanate (FITC), R-Phycoerythrin (PE) and peridinin-chlorophyll-protein complex (PerCP) and following fluorochrome excitation, emitted light was detected in the FL1, FL2 and FL3 channels respectively. In addition, on a small number of samples a fourth fluorochrome APC was used and emitted light detected in the FL4 channel of a 4 colour BD FACScan.

Templates for flow cytometry plots and gating were established using blood cells stained with the same antibodies as used for the sputum cells. Sputum samples were processed as described in Methods Section 2.3.6.2.

For each flow cytometry sample a cell suspension of 1x10^6 cells/ml in PBS [Sigma Aldrich, UK] was used, with 100µl of cell suspension incubated in the dark for 15-30 minutes with 5-10µl of antibody in separate tubes. Excess antibody was removed by washing twice in PBS before the cells were re-suspended in 400µl of PBS [Sigma Aldrich, UK] prior to acquisition. Up to 4 tubes were prepared for each sputum sample using the antibodies shown in Table 2.3.9 in the following combinations:

Tube 1 : unstained

Tube 2 : CD3, CD4 and CD8

Tube 3 : CD3, CD16, CD56

Tube 4 : CD45, CD14, CD15 +/- CD19

Flow cytometry data were analysed with CellQuestPro^{TM} (BD) or Flowjo 7.6.1 software ($^{©}$Tree Star, Inc, Oregon, USA). For each flow cytometry sample, the maximum number of events possible was recorded until the sample was

entirely used up, to ensure as many cells as possible were identified with a minimum of 1,000 gated events collected for analysis.

Table 2.3.9 Antibodies used for cell staining of sputum derived cells for flow cytometry analysis

Targets	Species	Ig Class	Product code	Company	Clone	Conjugate	Dilution/ incubation time
Human CD3	Mouse	IgG1k	345766	BD	SK7	PerCP	1:20 15 minutes
Human CD4	Mouse	IgG1k	345768	BD	SK3	FITC	1:10 15 minutes
Human CD8	Mouse	IgG1k	345773	BD	SK1	PE	1:10 15 minutes
Human CD16	Mouse	IgG1k	335035	BD	NKP15	FITC	1:10 15 minutes
Human CD56	Mouse	IgG1k	345810	BD	MY31	PE	1:5 15 minutes
Human CD45	Mouse	IgG1k	345809	BD	2D1	PerCP	1:10 15 minutes
Human CD14	Mouse	IgG2k	345785	BD	MøP9	PE	1:10 15 minutes
Human CD15	Mouse	IgMk	332778	BD	MMA	FITC	1:10 15 minutes
Human CD19	Mouse	IgG1k	345777	BD	4G7	PE	1:10 15 minutes

2.3.10 Cytokine Assays

Concentrations of the cytokines interleukin 6 (IL-6) and interleukin 8 (IL-8) were measured using ELISA kits for the purpose. For IL-6 this was the BD OptEIA™ ELISA Set for human IL-6 [BD Sciences, San Diego, California; Cat. No.

555220], and for IL-8 the Quantikine® Human CXCL8/IL-8 ELISA [R&D systems; Cat. No. D8000C]

2.3.10.1 IL-6 assay

Sputum leucocyte lysate samples were prepared as described in Methods Section 2.3.6.2 and 2.3.7.2, with a minimum of 1×10^6 cells used for cytokine measurements. Frozen samples were thawed at room temperature prior to use.

Assays were performed in the 96 well plates provided, with the microwells pre-coated with 100µl of a solution of capture antibody (80µl anti-human IL-6 monoclonal antibody) diluted in coating buffer buffer (20mls of 0.1M sodium carbonate, pH 9.5 [2.1g $NaHCO_3$ and 0.89g Na_2CO_3 in 250mls deionised water]) sealed and incubated overnight at 4°C. Wells were aspirated and washed thoroughly 3 times with >300µl of freshly made up well wash buffer (50µl of 0.05% Tween-20 in 100mls PBS [Sigma Aldrich, UK]). Plates were then blocked with at least 200µl of freshly prepared assay diluent per well (250mls PBS [Sigma Aldrich, UK] with 2.5mls 10% FBS [Hyclone; Cat. No. SH30088; heat inactivated]), incubated at room temperature for 1 hour, after which the wells were aspirated and washed thoroughly.

A stock standard solution was prepared by reconstituting the lyophilised standard [120ng recombinant human IL-6] warmed to room temperature with 1.0ml of deionised water, vortexing gently to mix and allowed to equilibrate for 15 minutes. A 300pg/ml standard prepared from the stock standard was then used to make standard solutions of 150pg/ml, 75pg/ml, 37.5pg/ml, 18.8pg/ml and 4.7pg/ml by serial dilution, with the assay diluent alone serving as the zero standard. 100µl of each standard, sample and control were pipetted into a 96 well plate in duplicate, and the plate sealed and incubated at room temperature for 2 hours, after which the wells were aspirated and washed thoroughly with wash buffer.

Following this, 100µl of working detector (Detection antibody [Biotinylated anti-human IL-6] and enzyme reagent [Avidin-horseradish peroxidise conjugate]) were added to each well, and the plate sealed and

incubated at room temperature for a further 1 hour prior to aspirating and washing thoroughly again, with soaking of the wells in wash buffer for 30-60 seconds for each wash at least 7 times. Subsequently, 100μl of substrate solution [Tetramethyl benzidine (TMB) and hydrogen peroxide; BD Pharminogen™ TMB Substrate Reagent set; Cat. No. 555214] was added to each well, and the plate incubated without sealing at room temperature in the dark for 30 minutes. At this point, 50μl of stopping reagent [1M H_2SO_4] was added to each well. The plate was then read within 30 minutes by colorimetric measurement using a BMG FLUOstar Galaxy™ plate reader (absorbance wavelength 450nm; reference wavelength 570nm). The IL-6 concentration of the samples was ascertained by their absorbance on the standard curve.

2.3.10.2 IL-8 assay

Sputum leucocyte lysate samples were prepared as previously described in Methods Section 2.3.6.2 and 2.3.7.2. Concentrations of IL-8 were measured in samples from AFD subjects and controls using the Quantikine® Human CXCL8/IL-8 ELISA kit, using the equipment and reagents provided. The IL-8 standard was prepared by reconstituting with 5.0mls of single strength Calibrator Diluent RD5P. Serial dilutions were performed using this 2000pg/ml stock solution to obtain solutions of 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml and 32.5pg/ml with the calibrator diluent alone serving as the zero standard.

100μl of the assay diluent RDI-85 were added to wells in a microstrip plate, followed by 50μl of IL-8 standard, control or sample. Plates were then left to incubate at room temperature for 2 hours.

Wells were then aspirated and washed thoroughly using wash buffer. Following this 100μl of the IL-8 conjugate (polyclonal antibody against IL-8 conjugated to horseradish peroxidise with red dye and preservatives) was added to each well, and the plate sealed and incubated for a further hour at room temperature, after which the wells were again aspirated and washed.

A substrate solution was freshly prepared using equal volumes of stabilised hydrogen peroxide and stabilised tetramethylbenzidine as the chromogen, and 200µl of substrate solution added per well. Plates were then incubated in the dark at room temperature for 30 minutes before adding 50µl of stop solution (2N sulphuric acid) to each well, and thorough mixing was ensured by observation of the colour change within the wells from blue to yellow. The optical density of each well was then measured using a BMG FLUOstar Galaxy™ plate reader (absorbance wavelength 450nm; reference wavelength 570nm), with sample IL-8 concentrations calculated by comparison with the standard.

2.3.11 Transmission Electron Microscopy

A proportion of the cells obtained from the induced sputum were also used for electron microscopy imaging to ascertain the presence of any storage material within the lysosomes. Cell pellets were obtained as previously described in Methods Section 2.3.6.2, and 1ml of EM preservative media refrigerated fixative solution (20mls of 20% parformaldehyde [Sigma Aldrich, UK], 16mls of 25% glutaldehyde [Sigma Aldrich, UK] and 59mls of PBS) was added to the pellet in a 1.7ml Eppendorf micro centrifuge tube and kept refrigerated at 4°C prior overnight until further preparation for EM was performed by Mr. Innes Clatworthy, Department of Electron Microscopy, Royal Free Hospital, London.

Cells were washed twice in PBS and incubated for 10 minutes in staining solution (1% osmium tetroxide [TAAB Laboratories Equipment, Aldermaston, UK], 1.5% potassium ferricyanide [TAAB Laboratories Equipment], PBS). Cells were washed 5 times in distilled water and dehydrated with increasing concentrations of ethanol (30%, 50%, 70%, 90% and 100%). Samples were left in a mixture of 50% alcohol/50% Lemix epoxy resin [TAAB Laboratories Equipment] on a mixer overnight. The following day, resin infiltrated samples were placed in 100% Lemix resin for at least 4 hours before being embedded in 100% Lemix Resin overnight. Semi-thin (1µm) sections were cut, using glass knives, on a Reichert-Jung Ultracut microtome before being stained with 1% Toluidine Blue overnight [TAAB Laboratories Equipment]. Ultra-thin sections were cut using a

diamond knife [Diatome; TAAB Laboratories Equipment] and collected on 300HS 3.05mm copper grids [Gilder Grids, Grantham, UK].

Sections were stained with saturated alcoholic uranyl acetate [TAAB Laboratories Equipment] for 5 minutes and transferred into Reynold's lead citrate [TAAB Laboratories Equipment] for a further 5 minutes. Specimens were viewed and photographed using a Phillips CM120 transmission electron microscope [Philips, Eindhoven, Netherlands].

2.4 Statistical Methods

All experimental data were analysed using GraphPad Prism 5.0™ (GraphPad Software Inc, California, USA) or Microsoft Excel 2003 (Microsoft Co-operation, California, USA). All data were tested using the D'Agostino & Pearson omnibus normality test to identify whether the data had a Gaussian distribution. Standard deviation (SD) and mean were used to express results for parametric data, whilst median and range were used to summarise non-parametric data.

An unpaired t-test was used to compare parametric data, whereas non-parametric data were analysed using a Mann-Whitney U test. One-way ANOVA (analysis of variance) was used to compare the means of 3 or more discrete populations where required. A paired t-test and Wilcoxon signed rank test were used for paired parametric and non-parametric data respectively. A p value <0.05 was considered to be statistically significant.

Data correlations were performed using a Spearman rank test. All statistical tests were two-tailed. Multivariate linear regression analyses were performed on normally distributed data using SPSS version 20 (IBM SPSS Statistics, USA). All statistical analysis was reviewed by a statistician, Dr. Colette Smith (Lecturer in Biostatistics, Royal Free Hospital, London).

Chapter 3 – Demographics, clinical features, pulmonary function and imaging

3.1 Introduction

Overall, studies into the involvement of the lungs in AFD have been limited. Those that exist have established that respiratory symptoms such as wheeze, cough and dyspnoea are frequently reported by AFD patients (186), and that airway obstruction occurs commonly, particularly in men, and worsens with age (187). Whilst this pattern between the genders is in keeping with the pattern of AFD involvement in other organ systems, no links between lung involvement and overall disease severity have been investigated in either sex.

From previous data, there has been the suggestion that the airflow limitation seen may be partially reversible to bronchodilators, though in one of these studies no response was seen on methacholine challenge testing. However, establishing the presence of airway reversibility may be pertinent to symptoms, to the consideration of cardiac comorbidity and the choice of drugs used for this (188).

The suggestion has also been raised that smoking may be particularly detrimental in the context of AFD and the lung, and smoking cessation advice is given to patients though the limited evidence from studies on the lung has been conflicting (189;190). Clearly this advice is also beneficial from a cardiac perspective, and so also relevant in this context.

Case reports have raised the possibility of spirometric and radiological improvement on commencing ERT in cases of AFD where presentation of the disease has been primarily with respiratory symptoms (191). However, there has not as yet been any assessment of the potential impact of ERT and AFD in the lung in the wider scheme, as in these previous studies the majority of subjects had not been commenced on ERT. In addition radiological findings in AFD subjects have varied, with some reports of bullous changes, and some reports of interstitial changes in cases where subjects have been further investigated on the basis of respiratory symptoms (192).

3.2 Hypotheses

This chapter aimed to test the following hypotheses:

Hypothesis 1:

The presence and severity of airway involvement in AFD is related to gender, age and overall disease severity

Rationale:

Aspects of the disease in different organ systems have been shown to be more severe in males, and with increasing age higher disease severity scores

Hypothesis 2:

The severity of airway obstruction in AFD is progressive over time, independent of ERT

Rationale:

On the basis of the accumulation of storage material over time, and the inability of ERT to completely halt this process in other organ systems

Hypothesis 3:

Pulmonary involvement in AFD is demonstrable on HRCT imaging

Rationale:

Air trapping on CT scanning representing small airways disease is recognised in many conditions and this has been reported in AFD

Hypothesis 4:

 A significant smoking history is associated with increased severity of airway obstruction in AFD

Rationale:

The impact of smoking in AFD has previously been suggested to be more harmful in terms of airway changes than in non-AFD subjects, though not proven

3.3 Objectives

The main objectives of this chapter were:

1. To establish the extent and severity of airway obstruction and changes in diffusion capacity in the AFD population

2. To examine the differences in spirometry and diffusion capacity between genders in AFD and compared to sex matched controls

3. To determine changes in spirometry and diffusion capacity over time in AFD

4. To determine the impact on the airways of smoking in AFD subjects

5. To examine the severity of lung involvement in AFD as measured by spirometry and diffusion capacity in relation to the duration of enzyme replacement therapy (ERT)

6. To examine the relationship between lung involvement in AFD and overall disease severity and genotype

7. To document pulmonary imaging findings in AFD using frontal chest radiographs and high resolution CT scanning

3.4 Methods

All recruitment, including contacting and consenting of study subjects, as well as clinical data collection and historical data collection from patient case notes were performed personally by Dr. Nadia Shafi, in addition to all clinical assessments at each study visit. All study investigations were also organised and co-ordinated by Dr. Shafi.

Lung function testing was performed by the Lung Function Department at the Royal Free Hospital using the same equipment as described in Method

Section 2.3.1. This was undertaken and reported according to the European Respiratory Society and American Thoracic Society guidelines for lung function testing (193;194). Analysis and interpretation of the lung function data for individual study subjects were performed by Dr. Nadia Shafi. Individual lung function parameters were compared between AFD subjects and healthy controls to investigate possible differences in both spirometry and diffusion capacity, which were felt to be of most interest.

All recruited subjects were asked to undergo induced sputum sampling on each study visit for the purposes of analysis of enzyme activity, cell populations and cytokine concentrations. Samples were collected from all those who consented and where time allowed around their scheduled clinical investigations, which had been arranged prior to their appointments.

For all study subjects, plain chest radiographs (CXR) were performed at the first study visit. All subjects were also offered high resolution computed tomography scans (HRCT scans) of the chest, and if in agreement these were arranged for their next attendance. All CXRs and HRCT scans were performed by the Radiology Department at the Royal Free Hospital using standard lung algorithms as outlined in Methods Section 2.3.4.1 and 2.3.4.2, with assessment of air-trapping made on inspiratory and expiratory CT images. Images were reviewed by a Consultant Thoracic Radiologist, Dr. Jamanda Haddock, and the data collated by Dr. Nadia Shafi.

3.5 Patient Population

3.5.1 Recruitment and follow up

Forty five study subjects were prospectively recruited to the AFD group over the period of a year from 02/06/2009 to 08/06/2010, including 20 males and 25 females. Of these, follow up lung function data were collected from 30 subjects at 6 months and from 14 subjects at 12 months.

A control cohort of 9 healthy volunteers was also recruited, matched for age and gender only to 9 of the AFD subjects (Appendix 2, Table 1.0). The main

purpose of this matched cohort was for comparison of sputum findings in subsequent chapters. Therefore for the analysis of lung function data these subjects were analysed with the AFD group as a whole.

A COPD cohort of 14 subjects was also recruited from the London COPD cohort based at the Royal Free Hospital. As outlined in Methods Section 2.2.2, these COPD subjects were matched as closely as possible to 14 of the AFD subjects with airway obstruction. Priority for matching was given to gender and spirometric GOLD stage over age. As a consequence, the participants in the COPD group were significantly older than those in the AFD group and the healthy control group (Appendix 2, Table2.0), (p<0.0005). Despite matching for GOLD stage, individual lung function parameters aside from FVC% predicted were significantly lower in the COPD group compared to the matched AFD group (Appendix 2, Table2.0), (p<0.05 for each). The purpose of this COPD cohort was mainly for the comparison of induced sputum samples. The COPD subjects were, therefore, not included in all of the lung function analysis.

3.5.2 Respiratory symptoms in AFD subjects

Clinical information was collected at baseline study visits for all study subjects with AFD. In total 28 of the 45 AFD subjects (62%) reported at least one of the following symptoms: shortness of breath on exertion, cough or wheeze. Eighteen of the 45 subjects (40%) noted shortness of breath on exertion, with 40% reporting cough and 31% (14 of 45) reporting wheeze.

3.5.3 Gender and ethnicity

The AFD group consisted of 20 (44%) males, the COPD group 9 (64%) males, and the healthy control group 5 (56%) males. Forty-four out of 45 (98%) of the AFD subjects were Caucasian, with the remaining subject being of African origin. All 14 of the COPD control subjects were Caucasian, as were 8 of the 9 (89%) healthy control subjects with the remaining one being of Asian extraction. Given the nature of AFD as an X-linked disease and not associated with ethnicity, it is likely that gender would be more relevant to the interpretation of results than race.

Table 3.5.1 Demographics of AFD, COPD and healthy control subjects recruited to study

Parameter (%)	AFD	Healthy control	COPD
n	45 (100)	9 (100)	14 (100)
Age [years] Mean (+/- SD)	46.5 (+/- 14.7)	41.7 (+/- 10.5)	65.8* (+/- 10.0)
Males, females	20 (44), 25 (55)	5 (56), 4 (44)	9 (64), 5 (36)
Smoking history >10 pack years	14 (31)	2 (22)	13 (93)
ERT	37 (82)	-	-
Caucasian	44 (98)	8 (89)	14 (100)
Beta blocker use	6 (13)	0	0
Beta agonist use	7 (16)	0	14 (100)

*$p < 0.001$ compared to AFD and healthy control group using 1 way ANOVA

3.5.4 Smoking history

Of the AFD group 31% (14 of the 45) had a smoking history of >10 pack years, whereas this was the case for 92% of the COPD subjects, and 22% of the healthy control group (Table 3.5.1). Of the AFD subjects, 5 were current smokers at the time of the study. None of the COPD subjects or healthy control subjects were ongoing smokers.

There was no history of occupational exposure to asbestos or other significant respirable agents in the AFD subjects or healthy controls.

3.5.5 Relevant Medication History

Six of the 45 AFD subjects (13%) were on beta blocker medication for cardiac co-morbidity, with 3 subjects on bisoprolol at a maximum dose of 2.5mg once daily, and 3 subjects on atenonol at a maximum of 50mg once daily. In

addition 7 of the AFD subjects (16%) had been prescribed an inhaled bronchodilator in the form of salbutamol to use on an as required basis (though on questioning none of them were using this regularly). All study subjects were asked to refrain from using any inhaled bronchodilators prior to their lung function assessments. Of the 45 participants in the AFD group, 82% were already receiving enzyme replacement therapy, with 8 of the 45 being enzyme naïve (Table 3.5.1).

3.5.6 Genotypes

Of the AFD subjects recruited, the genetic mutation in the α-galactosidase gene had been documented in all of the 45 subjects. This comprised 22 different mutations, with the most frequent mutation being the missense mutation N215S present in 11 of the 45 subjects, followed by R227X (6 subjects) and 1317T (4 subjects) (Table 3.5.6).

Table 3.5.6 Genotypes of Anderson Fabry Disease subjects recruited to the study

Genotype	Frequency n (%)
Total	45 (100)
N215S	11 (24)
R227X	6 (13)
1317T	4 (9)
exon 1 deletion	3 (7)
R310X	2 (4)
P205T	2 (4)
P293H	2 (4)
A309P	1 (2)
B/3316E	1 (2)
R342Q	1 (2)
I90.T	1 (2)
G261V	1 (2)
G208H	1 (2)
520-T	1 (2)
c.277G>T D93Y	1 (2)
c925G>C	1 (2)
c92C>T	1 (2)
c.334C>T	1 (2)
c.972-G in exon 6	1 (2)
c.748_801+8del62	1 (2)
c.802-3_802-2 del	1 (2)
c520delT	1 (2)

3.6 Results - Lung function data

3.6.1 Spirometry and diffusion capacity

Baseline lung function tests at recruitment were compared between the AFD and healthy control groups. Although 9 of the 45 AFD subjects were matched for age and gender to the 9 healthy controls, these subjects were kept in the group for the purposes of lung function data analysis as they were not matched on this parameter.

Significant differences were seen in spirometry between the AFD and healthy control group, including FEV_1 %predicted, FVC %predicted and FEV_1/FVC which were all found to be lower in the AFD group (Table 3.6.1). This was also the case for measurements of peak expiratory flow rate (PEFR).

In the AFD group 80% of subjects (36 out of 45) had evidence of smaller airway obstruction (based on the presence of an MEF_{50} <70%predicted), though no difference was seen in RV/TLC between the groups.

Diffusion capacity, measured by TLCO, was found to be lower in the AFD group compared to the healthy control group (p=0.04), though this was not evident when corrected for alveolar volume, KCO (Table 3.6.1)

On analysis of the age and gender matched sub-group of 9 AFD subjects and healthy controls, the same pattern of significant differences in airway calibre was found (Appendix 2, Table 1.0). However the difference in gas transfer as measured by TLCO was not evident in the age and gender matched groups.

As would be expected, all of the spirometric parameters and measures of diffusion capacity were significantly lower in the COPD group compared to the healthy control group (data shown here for descriptive purposes, comparison not shown). For the 14 AFD and matched COPD subjects, data are shown in Appendix 2, Table 2.0.

Table 3.6.1 Spirometry and diffusion capacity in AFD, healthy control and COPD subjects, with comparison between AFD and healthy control groups

% predicted Mean (+/- SD)	AFD	Healthy Control	COPD	p value (unpaired t test)
n	45	9	14	-
FEV_1	89.8 (+/- 11.9)	109.6 (+/- 16.3)	70.2 (+/- 13.1)	**<0.0001**
FVC	104.1 (+/- 12.2)	117.4 (+/- 18.6)	103.7 (+/- 18.0)	**0.009**
FEV_1 /FVC	0.7 (+/- 0.08)	0.8 (+/- 0.08)	0.6 (+/- 0.02)	**0.02**
PEFR	105.7 (+/- 15.6)	119.1 (+/- 19.7)	81.9 (+/- 18.2)	**0.03**
MEF50	57.8 (+/- 18.2)	93.9 (+/- 38.7)	25.5 (+/- 14.3)	**<0.001**
RV/TLC	88.6 (+/- 15.7)	86.3 (+/- 14.1)	117.7 (+/- 18.1)	0.5
TLCO	86.4 (+/- 12.2)	95.4 (+/- 12.1)	69.7 (+/- 16.3)	**0.04**
KCO	96.0 (+/- 13.3)	98.8 (+/- 12.6)	78.7 (+/- 19.4)	0.56

3.6.3 Reversibility to bronchodilators

Reversibility to bronchodilators was tested by repeating spirometry 15 minutes after the administration of 2.5mg of nebulised salbutamol at the baseline visit on 43 of the 45 AFD subjects recruited, with significance interpreted ≥ 15% improvement from baseline on the repeat test.

Only 5% of subjects tested (2 of the 43) had any significant improvement in FEV_1 %predicted and PEFR with a mean improvement of 18% and 19.5% respectively (Table 3.6.3).

More significant reversibility to bronchodilators was evident in 37% of AFD subjects showing significant improvement in MEF_{50} %predicted on repeat spirometry, though it is acknowledged that in general this measure may be subject to some variability (Table 3.6.3).

Table 3.6.3 Summary of positive bronchodilator reversibility tests in AFD subjects

Parameter (% predicted)	FEV_1	PEFR	MEF50
n (%)	2 (5)	2 (5)	16 (37)
Mean % improvement (+/- SD)	18.0 (+/- 4.2)	19.5 (+/- 4.6)	31.4 (+/- 17.6)

n=number of subjects out of total 43 with a significant bronchodilator response of ≥15%

3.6.4 Lung function in relation to symptoms

Of the 18 subjects who reported shortness of breath on exertion, 8 (44%) had evidence of airway obstruction with FEV_1/FVC <0.7, whilst 78% (14 of the 18) had MEF_{50} <70% predicted.

Of those reporting cough, 67% (12 of 18) had an FEV$_1$/FVC <0.7, as did 83% (10 of 12) of the AFD subjects reporting wheeze, with 85% and 88% of subjects who reported cough or wheeze having MEF$_{50}$ <70 %predicted.

3.6.5 Comparison of AFD subjects with and without airway obstruction

Within the AFD group, 21 out of the total 45 subjects (47%) had evidence of airway obstruction based on the criteria of FEV$_1$/FVC <0.7. The mean age of the AFD subjects with airways obstruction was 49.8 years, which was older than those AFD subjects without airways obstruction at 43.8 years, though this was not statistically significant (Table 3.6.5). In addition the AFD group with airway obstruction contained more male subjects (62% vs 29%) and more subjects with a smoking history of greater than 10 pack years (43% vs 21%) than the unobstructed AFD group.

Examining the overall disease severity of the groups using the Mainz Severity Score Index, it was apparent that the AFD subjects with airway obstruction had significantly more severe disease than those without airway obstruction (p=0.007) (Table 3.6.5). However, on using the Age Adjusted Severity Score, although the AFD group with airway obstruction scored higher than those without, this was not found to be statistically significant between the groups.

Table 3.6.5 Comparison of demographics of AFD subjects with and without evidence of airway obstruction as defined by $FEV_1/FVC <0.7$

Parameter	AFD with airway obstruction	AFD without airway obstruction	P value (unpaired t test)
n (%)	21 (47)	24 (53)	-
Age, years (mean +/-SD)	49.8 (+/- 14.0)	43.8 (+/- 15.0)	0.17
Males, females (% of n)	13 (62), 8 (38)	7 (29), 17 (71)	- -
Smokers >10 pack years (% of n)	9 (43%)	5 (21%)	**0.009**
On ERT (% of n)	18 (86%)	20 (83%)	-
MSSI	27.2 (+/- 12.2)	16.35 (+/- 10.6)	**0.007**
Age adjusted severity score	4.4 (+/- 8.4)	0.1 (+/- 6.9)	0.11

3.6.6 Gender differences in spirometry and diffusion capacity in AFD, COPD and healthy control subjects

Thirteen of the 20 AFD males (65%) had evidence of airway obstruction as defined by an $FEV_1/FVC <0.7$. There were significant differences in spirometric measures with FEV_1 %predicted, FVC %predicted, FEV_1/FVC and PEFR all being lower in AFD males compared to healthy control males (Table 3.6.6). In addition 16 of the 20 AFD males (80%) had MEF_{50} <70 %predicted, with measures of MEF_{50} %predicted being significantly lower than in healthy control males.

However, no differences in RV/TLC were seen between the two male groups (Table 3.6.6).

In AFD females, 7 of the 25 (28%) had evidence of airway obstruction with FEV_1/FVC <0.7, and FEV_1 %predicted was found to be significantly lower than in healthy control females (p=0.02). In 17 of the 25 AFD females (68%) MEF_{50} was found to be low at <70 %predicted (Table 3.6.6), and overall MEF_{50} %predicted was found to be significantly lower than that in healthy control females (p=0.02) (Table 3.6.6).

Between the genders in AFD there were some significant differences in spirometric parameters with FEV_1 %predicted and FVC %predicted being lower in AFD males than AFD females (p=0.03 and 0.008 respectively), though measures of diffusion capacity were similar (Table 3.6.6).

Measures of gas transfer were similar in AFD males compared to healthy control males, and in AFD females compared to healthy control females. No difference in diffusion capacity was seen between genders in AFD subjects.

There were no differences found in any of the parameters between males and females in the healthy control group (Table 3.6.6) or in the COPD group (Appendix 2, Table 3.0).

Table 3.6.6 Spirometry and diffusion capacity in AFD and healthy control males and females

% predicted Mean (+/SD)	AFD males	Healthy control males	p	AFD females	Healthy control females	p
n	**20**	5	**-**	**25**	4	-
FEV1	**85.7 §** **(+/- 9.7)**	107.7 (+/- 14.1)	**0.005**	**93.0** **(+/- 12.6)**	111.9 (+/- 20.7)	**0.04**
FVC	**98.8 §** **(+/- 9.1)**	112.3 (+/- 12.3)	**0.02**	**108.3** **(+/- 13.0)**	123.7 (+/- 25.0)	0.2
FEV$_1$ /FVC	**0.6** **(+/- 0.07)**	0.8 (+/- 0.1)	**0.04**	**0.7** **(+/- 0.08)**	0.8 (+/- 0.06)	0.27
PEFR	**102.8** **(+/- 16.1)**	125.6 (+/- 23.1)	**0.02**	**108.1** **(+/- 15.2)**	111.0 (+/- 12.9)	0.72
MEF50	**53.87** **(+/- 15.3)**	97.82 (+/- 50.3)	**0.01**	**60.96** **(+/- 20.1)**	89.13 (+/- 24.4)	**0.05**
RV/TLC	**92.53** **(+/- 16.6)**	88.76 (+/- 18.5)	0.66	**85.68** **(+/- 14.8)**	83.23 (+/- 7.3)	0.75
TLCO	**87.6** **(+/- 14.4)**	99.8 (+/- 13.8)	0.11	**85.4** **(+/- 10.4)**	89.9 (+/- 7.9)	0.41
KCO	**99.7** **(+/- 14.1)**	103.8 (+/- 14.6)	0.57	**93.1** **(+/- 12.2)**	92.5 (+/- 6.9)	0.93

Comparison of AFD groups and sex matched controls performed using Mann Whitney U test given small numbers

§ p<0.05 AFD males compared to AFD females using unpaired t test

3.6.7 Impact of smoking history on spirometry and diffusion capacity

Fourteen AFD subjects with >10 pack years of smoking and 31 AFD subjects with <10 pack years of smoking were compared to 7 healthy controls with <10 pack years smoking history.

In the 31 AFD subjects who had little in the way of a smoking history (<10 pack years) spirometric measures including FEV_1 %predicted, FVC %predicted, FEV_1/FVC and PEFR were all significantly lower than in the healthy control group (Table 3.6.7). MEF_{50} %predicted was also significantly lower in the AFD group compared to the healthy controls though no difference was seen in RV/TLC. No significant differences in gas transfer were evident between the groups with <10 pack years of smoking. However, in AFD subjects who had a >10 pack years smoking history there was a significant reduction in diffusion capacity as measured by TLCO and KCO (p=0.02 for both) (Table 3.6.7).

3.6.8 AFD lung function change over time

AFD subjects were followed up at 6 months intervals, and lung function data collected as frequently as possible during the study. For 12 of the 45 AFD subjects recruited, lung function was performed on 3 separate occasions at 6 month intervals (spirometry as shown in Figure 3.6.8). All of these subjects were receiving enzyme replacement therapy fortnightly. There was no significant variation in any of the measured parameter of large airways, small airways or gas transfer over a follow up period of 12 months (Table 3.0, Appendix 1).

For 15 of the 45 subjects, baseline and 6 month lung function follow up data were collected and for 4 subjects, baseline and 12 month lung function data were collected (data not shown).

Table 3.6.7 Spirometry and diffusion capacity in AFD subjects in relation to smoking history

% predicted Mean (+/- SD)	AFD >10 pack years	AFD <10 pack years	Healthy controls <10 pack years	p (unpaired t test)
n	14	31	7	-
FEV_1	88.7 (+/- 13.4)	90.6 (+/- 11.4)	112.4 (+/- 17.4)	**0.0002**
FVC	105.0 (+/- 13.1)	103.3 (+/- 12.3)	116.6 (+/- 20.9)	**0.03**
FEV_1/FVC	0.7 (+/- 0.07)	0.7 (+/- 0.07)	0.8 (+/- 0.07)	**0.01**
PEFR	104.9 (+/- 15.3)	106.1 (+/- 16.0)	121.1 (+/- 21.5)	**0.04**
MEF_{50}	52.2 (+/- 18.9)	60.3 (+/- 17.7)	103.8 (+/- 38.5)	**<0.0001**
RV/TLC	88.3 (+/- 18.6)	88.8 (+/- 14.5)	87.4 (+/- 16.1)	0.83
TLCO	79.9* (+/- 12.8)	89.3 (+/- 11.0)	98.1 (+/- 12.3)	0.07
KCO	89.2* (+/- 12.4)	99.1 (+/- 12.7)	101.7 (+/- 12.7)	0.62

p values shown for comparison between AFD <10 pack years of smoking and healthy control group using unpaired t test

* p=0.02 compared to AFD <10 pack years using unpaired t test, p >0.05 for all other parameters

Figure 3.6.8 Spirometry in AFD subjects receiving fortnightly enzyme replacement therapy at baseline, 6 months and 12 months follow up

i) FEV$_1$ % predicted
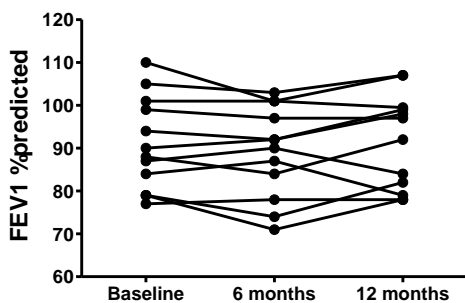
ii) FVC % predicted
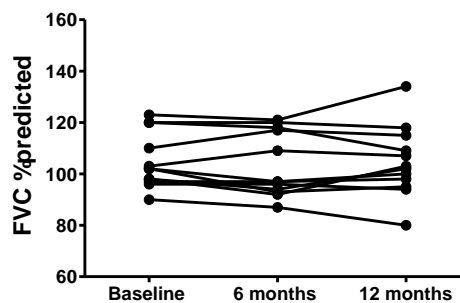
iii) FEV$_1$/FVC
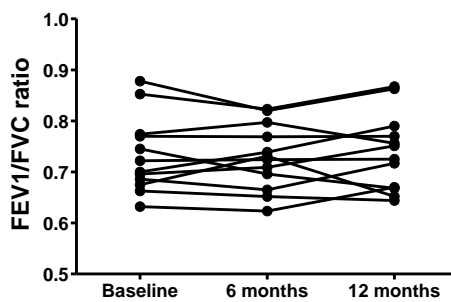
3.7 Results - Imaging

Plain chest radiographs (CXRs) were performed at baseline according to the protocol outlined in the Methods section 2.3.4.1 on the first study visit for 38 of the 45 AFD subjects, and were reported by standard radiology criteria by the Royal Free Hospital Radiology Department. Of these, 34 CXRs were reported as normal with clear lung fields. The remaining CXRs showed various abnormalities as outlined in Table 3.7. These changes were not thought to be related to pulmonary involvement of AFD though clearly some were likely related to cardiac involvement of AFD.

Seventeen of the 45 AFD subjects also had high resolution computed tomography (HRCT) scan of the chest (protocol described in Methods section 2.3.4.2), with inspiratory and expiratory images to highlight the presence of air trapping as an indicator of small airways disease. None of the 17 scans were reported to show evidence of air trapping, though abnormalities were noted on the scans from 3 male subjects in the form of scattered bullae in one, a small amount of emphysema on another with this being the only subject with a significant smoking history of 40 pack years, and an area of linear atelectasis in a third study. One of the HRCT scans was performed in inspiration only, and therefore a full assessment of the presence of air trapping could not be made, though no other abnormality was seen.

Table 3.7 Summary of Imaging studies in AFD subjects

	Normal n (%)	Abnormal n (%)	Abnormalities seen
CXR	34 (89%)	4 (11%)	1. cardiomegaly + ICD 2. prominent vasculature 3. blunted left costophrenic angle 4. bilateral apical fibrosis
HRCT chest	14 (82%)	3 (18%)	1. scattered bullae 2. emphysema 3. linear atelectasis

CXR – chest radiograph, HRCT – high resolution computed tomography scan, ICD – implantable cardiac device

3.8 AFD disease severity, enzyme replacement therapy and genotype

3.8.1 Enzyme replacement therapy (ERT) and disease severity

Of the AFD subjects 37 were on ERT with a mean duration of 53 months (SD +/-31.2) and 8 of the AFD subjects were enzyme naïve. There appeared to be no significant difference in spirometric parameters or diffusion capacity between those AFD subjects on ERT and those who were not on ERT (Table 3.8.1).

As might be expected, the disease severity of the AFD subjects on enzyme replacement therapy, as measured by both the Mainz Severity Score Index (MSSI) and the Age Adjusted Severity Score was significantly worse in the group on enzyme replacement therapy compared to the enzyme naïve group (p=0.001 and 0.009 respectively) (Table 3.8.1).

When analysing the data as continuous variables there appeared to be a weak but significant negative correlation between FEV_1/FVC ratio and MSSI (r = -0.41, p=0.01), though again this was not evident with the Age Adjusted Severity Score (Figure 3.8.1i) and ii)).

Figure 3.8.1 Correlation between disease severity scores and FEV$_1$ /FVC in AFD

i) MSSI
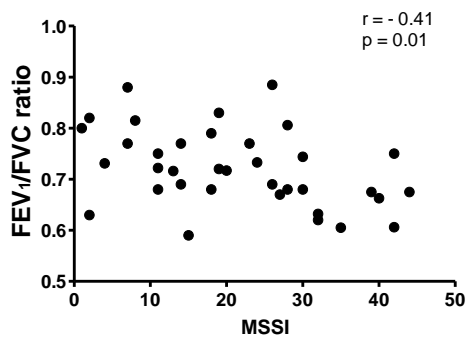
ii) Age Adjusted Severity Score
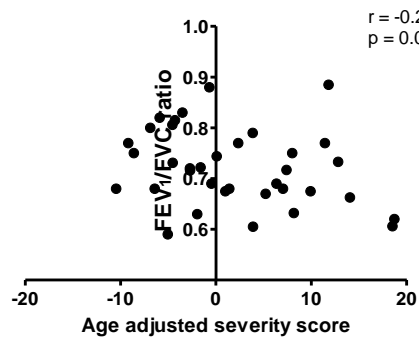
Table 3.8.1 Disease severity and enzyme replacement therapy in AFD subjects in relation to lung function

% predicted Mean (+/- SD)	ERT	No ERT
n	37	8
MSSI Median (range)	26.0 ** (14.0 - 32.0)	3.0 (1.75 - 13.5)
Age adjusted severity score Median (range)	3.89** (-2.74 - 9.93)	-4.790 (-6.148 - -2.52)
FEV$_1$	90.67 (+/- 11.9)	85.63 (+/- 11.4)
FVC	104.4 (+/- 12.4)	102.5 (+/- 12.4)
FEV$_1$/FVC	0.72 (+/- 0.07)	0.72 (+/- 0.08)
PEFR	107.5 (+/- 15.7)	97.38 (+/- 12.8)
MEF$_{50}$	58.4 (+/- 18.7)	55.0 (+/- 16.4)
RV/TLC	88.19 (+/- 15.0)	91.17 (+/- 21.3)
TLCO	85.41 (+/- 12.3)	90.75 (+/- 11.4)
KCO	95.81 (+/- 14.0)	96.86 (+/- 9.4)

**$p < 0.01$ compared to no ERT group using Mann Whitney U test

3.8.2 Lung function in relation to genotype

The spirometric parameters of the most common genotypes from the group were compared, and FEV_1/FVC ratio was seen to be lowest in subjects with the mutation R227X and highest in the subjects with exon 1 deletions, though the numbers within each group were very small (Figure 3.8.2) Between the genotype sub-groups, mean age was greatest in the N215S group, and overall disease severity as measured by MSSI was worst in the exon 1 deletion group, though with the Age Adjusted Severity Score, overall disease severity was worst in the 1317T sub-group (Table 3.8.2)

Table 3.8.2 Severity scores for AFD cohort by genotype

Genotype	N215S	R227X	Exon 1 deletion	1317T
n (%)	11 (24)	6 (13)	3 (7)	4 (9)
FEV_1/FVC Median (range)	0.75 (0.59 -0.81)	0.63 (0.59 – 0.72)	0.83 (0.74 – 0.89)	0.72 (0.68 – 0.85)
Age [years] Mean (+/- SD)	53.4 (+/- 15.8)	42.1 (+/- 12.4)	44.3 (+/- 17.5)	47.5 (+/- 21.8)
MSSI Median (range)	21.5 (1.0 – 44.0)	20.5 (2.0 – 32.0)	26.0 (19.0 – 30.0)	21.0 (18.24)
Age adjusted severity score Median (range)	-4.55 (-6.9 – 8.0)	2.7 (-2.0 – 8.2)	0.08 (-3.5 – 11.8)	7.1 (1.4 – 12.8)
Smoking history [pk years] Median (range)	15.0 (0 – 40)	2.5 (0 – 35)	0 (0)	17.5 (11.0 – 22.5)

3.8.3 Multivariate analysis

To investigate the impact of factors associated with FEV_1/FVC and MEF_{50} %predicted after controlling for other potential confounders, multivariate linear regression analyses were. The potential factors considered were gender, smoking, age, residual enzyme activity and disease severity as measured by MSSI. Both FEV_1/FVC and MEF_{50} %predicted were confirmed to be normally distributed, ensuring that performing a linear regression was reasonable. The models were performed using SPSS version 20 (IBM SPSS Statistics, USA). The findings of the analyses did not reveal any significant factors influencing FEV_1/FVC and MEF_{50} %predicted when taking into account all the potential confounders, though there was a suggestion that MSSI may be a more influential factor in FEV_1/FVC and gender in MEF_{50} %predicted.

Table 3.8.3.1 Multivariate analysis FEV_1/FVC in AFD

Factor	Comparator	Univariate			Multivariate		
		Regression coefficient	95% CI	P value	Regression coefficient	95% CI	p value
Gender	Male vs. Female	-0.04	-0.08, +0.01	0.13	-0.031	-0.12, 0.82	0.47
Smoking	>10 pyrs vs. <=10 pyrs	-0.05	-0.09, +0.002	0.06	-0.032	-0.09, 0.02	0.23
Age	Per 5 years older	-0.005	-0.013, 0.003	0.20	0.010	-0.004, 0.025	0.16
Residual enzyme activity	Per 1 higher	0.01	0.002, 0.02	0.02	-0.005	-0.028, 0.019	0.70
MSSI score	Per 1 higher	-0.002	-0.004, 0.000	0.02	-0.003	-0.005, 0.000	0.09

Table 3.8.3.2 Multivariate analysis of MEF 50% predicted in AFD

Factor	Comparator	Univariate			Multivariate		
		Regression coefficient	95% CI	p value	Regression coefficient	95% CI	p value
Gender	Male vs. Female	-6.82	-17.78, 4.14	0.22	-20.77	-45.03, 3.49	0.09
Smoking	>10 vs. <=10 pk yrs	-8.12	-19.84, +3.60	0.17	-5.47	-20.19, 9.25	0.45
Age	Per 5 years older	-0.98	-2.86, 0.90	0.30	2.84	-1.19, 6.88	0.16
Residual enzyme activity	Per 1 higher	1.67	-0.89, 4.22	0.20	-4.86	-11.48, 1.77	0.14
MSSI score	Per 1 higher	-0.42	-0.85, 0.02	0.06	-0.56	-1.37, 0.26	0.17

3.9 Discussion

Prior to this study pulmonary abnormalities have documented in Anderson Fabry disease within mainly case reports and the occasional clinical series. Here, we find that 62% of AFD subjects recruited had at least one respiratory symptom, and 40% of subjects two or more. Whilst it is possible that some of these symptoms are attributable to underlying cardiac involvement, given the findings from this study, it is difficult to discount pulmonary disease as being a significant factor. We confirm what has been suggested by the limited published data that pulmonary involvement in AFD in the form of airway obstruction is common. In this cohort 47% of the AFD subjects demonstrated an FEV_1/FVC of <0.7, the current widely used definition of obstructive airways disease in clinical respiratory medicine (195;196), with 80% of all AFD subjects showing some evidence of small airways obstruction on MEF_{50} measurement. This is similar to previous work by Magage et al (197)who found that only 16% of their AFD cohort showed no abnormality on spirometry when tested. Also, Brown et al (198) reported 36% of their cohort of 25 subjects demonstrated criteria in keeping with airway obstruction.

All 21 of the AFD subjects who had evidence of airway obstruction had either mild or moderate disease on spirometry, with 13 subjects classed as GOLD Stage I, and 8 classed as GOLD Stage II, similar to the degree of airway obstruction reported in the aforementioned studies, though there have been case reports of more severe airway obstruction (199). Our study goes on to show that differences in airway calibre in AFD compared to healthy controls are also evident using peak expiratory flow rate (PEFR) measurement. This is a simple bedside measure of mainly large airways, and can be easily performed and monitored by subjects at home. This may be useful for future studies as a method of evaluating respiratory symptoms and assessing pulmonary involvement in AFD.

In addition there was seen to be a reduction in one measure of gas transfer in the form of TLCO in AFD subjects compared to controls, though when corrected for alveolar volume (KCO) this was not consistently so. When carefully-

matched AFD and healthy control sub groups were compared, this difference was lost. Whether this reflects smaller sample size in the latter populations or the genuine effect of removing confounders is unclear. Previously Wang et al (200) reported a case of AFD with significantly reduced transfer factor and evidence of pulmonary infiltrates on CT imaging, which stabilised on commencement of ERT, whilst Bartimmo and colleagues (201) also reported reductions in diffusion capacity in a family with AFD, though it was suggested that the effects of smoking may have had a significant part to play in that particular case. The work reported within this study would tend to support the latter suggestion, given that smoking with a pack year history of >10 in AFD subjects appeared to be associated with statistically significant reductions in both TLCO and KCO compared to AFD subjects with a smoking history of <10 pack years.

Despite widespread evidence of small airway obstruction, no significant differences were demonstrated in static lung volumes in the AFD subjects compared to controls in this study. In previous case series where elevated residual volumes (RV) have been reported, these have been only in smokers with other evidence of emphysema, and therefore difficult to assign to changes related to AFD (202;203). In our series however there was no physiological evidence of significant air trapping with RV/TLC measures similar in AFD subjects and healthy controls; though as would be expected, this was elevated within the COPD cohort.

Very few of the AFD subjects demonstrated significant improvement in spirometry following nebulised bronchodilators, with only 2 subjects showing any increase in FEV_1 %predicted and PEFR out of the 43 tested (5%). More widespread improvement was seen in small airway parameters with 30-40% of AFD subjects showing a significant increase in MEF_{25}, MEF_{50} and MEF_{75} %predicted following administration of the drug. The clinical significance of this is less clear, given that there can be significant variability in the measurement of these latter parameters. In contrast, Brown et al (204) reported that 5 of 8 male subjects showed an improvement in FEV_1 following bronchodilator

administration, though this was using a cut off of 12% improvement as being significant, as opposed to 15% as in this and most other studies. They also performed methacholine challenge testing on their subjects, but did not report any positive responses in the 10 subjects tested suggesting a lack of bronchoconstrictive tendency. Magage et al (205) also reported only partial reversibility in some of their subjects who underwent bronchodilator testing.

We hypothesised that the changes seen in AFD in the lungs would be evident in both males and females, though more common and more severe in men. Analysis of lung function data by gender confirmed this, with two thirds of male subjects demonstrating evidence of significant airway obstruction (FEV_1/FVC of <0.7), whereas for females this was just under one third.

On further analysis it was apparent that the extent of the changes was greater in AFD males compared to sex matched controls, with changes in AFD females limited to FEV_1 %predicted and MEF_{50} %predicted, though the number of subjects in each of the control groups was small. As was anticipated, the extent of change in spirometry was more significant in AFD males compared to AFD females. No differences were seen in any of the lung function parameters when analysed by gender in either the COPD cohort or the healthy control cohort, suggesting that the difference seen between AFD males and females appears to result from AFD rather than other gender related factors. Older age did not appear to explain the degree of airway obstruction in AFD subjects.

This pattern of lung involvement by gender is similar to that found in a previous AFD study (206). This is of particular importance as in particular as in the past the extent of lung changes in AFD was mainly documented in male subjects (207). Data on AFD females prior to this have been largely confined to case reports (208;209). Data from the Fabry Outcome Survey (FOS) has highlighted that females are affected by the systemic disease in a similar manner to males, though clinical sequalea tend to occur up to a decade later (210). This may, in part, explain the difference in prevalence of airway involvement in the gender-divided groups of a similar age.

Stratifying AFD subjects by their smoking history enabled us to examine the potential impact of smoking on lung function. It was evident when comparing AFD subjects with little in the way of smoking history to healthy control subjects with a similar degree of smoking history that the changes seen in airway calibre in AFD cannot be explained by smoking alone. Significant reductions in both large and small airway parameters were found in AFD subjects with <10 pack year history of smoking when compared to healthy controls. No differences were seen in lung volumes or measures of diffusion capacity between these groups.

Further reductions in these parameters were present with >10 pack years of smoking, with significant worsening of spirometric measures compared to healthy controls, as well as AFD subjects with <10 pack years smoking. In addition, these AFD subjects with a heavier smoking history also showed significant reductions in diffusion capacity, both TLCO and KCO - making these changes (when present) more likely to be related to smoking than to AFD itself. From a clinical perspective this underlines the need to emphasise the importance of smoking cessation in this group of patients, given that it would appear to promote pulmonary change which may already be ongoing as a result of AFD.

Given the nature of AFD as a condition shown to progress over time (211), we were keen to determine whether this pattern also applied within the lungs. The maximum follow up for this study however was only 12 months, and over this time no significant changes to lung function parameters were noted. It is likely that this follow up period was not long enough to detect longitudinal change in individual lung function parameters. In previous studies with longer follow up periods (of maximally 60 months), there was a trend towards falling spirometric measures, though these did not reach significance (212). Given that the majority of subjects in this study were on enzyme replacement therapy, and the cohort in the previously mentioned study were enzyme naïve, it is difficult to argue that any lack of progression over time was as a result of a therapeutic intervention such as ERT.

Plain chest radiographs in the AFD subjects did not reveal obvious lung abnormalities. The great majority of these were reported as normal, apart from one where changes were thought likely secondary to previous undiagnosed bacterial infection. The other abnormalities seen on the remaining CXRs were largely cardiac in nature, and likely secondary to cardiac morbidity associated with AFD. This is similar to findings from Brown et al (213) who reported normal chest radiographs in 24 of their 25 male AFD subjects, with streaky shadowing bibasally in the one remaining individual.

High resolution computed tomography (HRCT) scanning in 17 AFD subjects revealed normal lung parenchyma in 82% of subjects, with evidence of bullae and emphysema in 2, and a small area of atelectasis in a third subject. Of note, the emphysematous changes were seen in one of the AFD subjects with a >20 pack year smoking history, though neither of the other 2 AFD subjects were current smokers. Inspiratory and expiratory HRCT images were performed specifically to assess for evidence of air trapping, a marker for small airways disease, demonstrated by mosaic changes of the lung parenchyma. In our series this was not evident in any of the AFD subjects. Evidence of air trapping with severe mosaic attenuation along with ground glass opacity have previously been documented in a case report of a female AFD subject with respiratory symptoms, whose air trapping persisted on CT despite symptomatic and physiological improvement following commencement of enzyme replacement therapy (214) as have bullous changes and pneumothoraces.

Although the majority of the AFD subjects recruited to the study were already established on ERT, there were a number who were enzyme naïve, mainly due to a lack of clinical indication for commencement of ERT. As a result it is not surprising that the disease severity scores of the AFD subjects on ERT using both the Mainz Severity Score Index (MSSI) (215) and the Age Adjusted Severity Score (216) were significantly worse in the group on ERT compared to enzyme naïve subjects. However, given there was no significant difference between the groups in any of the lung function parameters measured, one might propose that ERT has little demonstrable impact on lung function in AFD. However, further

longitudinal follow up and ideally larger numbers would be required to ascertain whether ERT might slow the rate of decline in lung function over time with increasing age that has been suggested is the natural history of lung involvement in AFD by previous studies (217;218).

Examining MSSI scores in AFD subjects with and without airway obstruction, it would appear that those with airway obstruction are likely to have more severe overall AFD pathology. However it is difficult to draw any firm conclusion given that there are a number of other confounding factors, such as age, smoking history and gender which may also influence this within our study.

By the same token, and given the limited numbers within the study, it is difficult to draw any firm conclusions regarding the influence of genotype on the presence and severity of pulmonary involvement in AFD. It would appear that within this small group, subjects with the mutation R227X had most significant pulmonary disease when assessed by FEV_1/FVC. Given that this group had the youngest subjects and was not found to be most severe by either the MSSI or the Age Adjusted Severity Scores, this finding is less likely to be attributable to age, overall disease severity or smoking as these patients had one of the lowest pack year histories. However, as some of the subjects within some of these groups were family members, one could argue that familial tendencies and common environmental factors need to be taken into consideration as they might influence the changes seen.

3.10 Conclusions

The data in this chapter demonstrates that respiratory symptoms and pulmonary involvement in AFD subjects is mild but common and can be summarized by the following:

1. The involvement of the lungs in AFD mainly takes the form of airway obstruction, and this can be further characterised as:

- affecting both large and small airways, and detectable on spirometry

- largely fixed, though with some bronchodilator reversibility evident in small airways

- more common and more severe in males compared to females

- having no impact on diffusion capacity

- more likely to be present with increasing disease severity as measured by the Mainz Severity Score Index

2. Both large and small airway obstruction are more severe in smokers with >10 pack yrs

3. No significant change in lung function parameters can be demonstrated over a twelve month follow up period

4. Pulmonary changes in AFD are not evident on chest radiograph or CT imaging

5. Genotype may influence the severity of airway involvement in AFD

Chapter 4 – Pulmonary α-galactosidase A

4.1 Introduction

In Anderson Fabry disease (AFD) α-galactosidase A activity in both plasma and peripheral blood leucocytes has been characterised (219). In males the diagnosis of AFD can be made solely on the finding of reduced α-galactosidase A activity, whereas in females plasma and peripheral blood leucocyte α-galactosidase activity can fall within the normal range, presumably as a result of lyonisation in the peripheral blood (220), and therefore genotyping is essential when the diagnosis is suspected.

The reference ranges for plasma and peripheral blood leucocyte α-galactosidase activity used at the Royal Free Molecular Diagnostics Laboratory are as below:

Plasma

Normal   4 – 21.9 nmol/hr/ml

Hemizygote AFD   0 – 0.9 nmol/hr/ml

Heterozygote AFD   0.22 – 7.4 nmol/hr/ml

Peripheral blood Leucocyte

Normal   33 – 134 nmol/hr/mg protein

Hemizygote AFD   0.4 – 5 nmol/hr/mg protein

Heterozygote AFD   4.3 – 73 nmol/hr/mg protein

Reduction in α-galactosidase A activity results in accumulation of globotriaosylceramide (Gb3) which can be demonstrated at a cellular level by electron microscopy, histological staining using toluidine blue, or immunohistochemistry. Total Gb3 in plasma, urine or tissue homogenate can be quantified using mass spectrometry or high performance liquid chromatography (HPLC). α-galactosidase A activity can also be measured in cellular homogenates

from various organs or body fluid including tears, though this is not performed routinely (221). Although storage material in sputum-derived cells has been demonstrated by light and electron microscopy (222), α-galactosidase A activity in sputum has not been quantified.

Evidence from clinical trials suggests that enzyme replacement therapy infused fortnightly by AFD subjects produces improvement in both the clinical manifestations of the disease and clearance of Gb3 on serial biopsy at end organ sites such as liver, kidney and skin (223;224). This has not been assessed in the lung. As α -galactosidase A activity within the lung has not been quantified, it is unknown to what degree enzyme replacement therapy penetrates lung sites, and if so what its impact might be, though there have been case reports of clinical benefit in the lung (225).

Therefore, for the purposes of examining the involvement of the pulmonary system in more detail, a method to measure α-galactosidase A activity in both the fluid phase and cellular phase of induced sputum was established (described in Methods Sections 2.3.6 and 2.3.7). Measurement of induced sputum α-galactosidase A activity allowed variations in lung function in AFD patients and controls to be related to local enzyme activity.

The presence of inclusion bodies representing Gb3 deposition within bronchial epithelial cells suggests the presence of intrinsic airway involvement and have been shown to be similar to those found in other organ systems involved in AFD (226). These "zebra" bodies have been documented in the kidney through renal biopsy (227) and examination of urine sediment (228). They have also been reported in the gastrointestinal tract (229), skin (230) as well as in cardiac myocytes (231) and nerves (232). Studies looking at the underlying pathology in the lung are limited to case reports of sputum and broncho-alveolar lavage findings of Gb3 inclusion bodies within respiratory derived cells (233;234).

4.2 Hypotheses

This chapter aimed to test the following hypotheses:

Hypothesis 1:

α-galactosidase A activity is measurable in induced sputum and is lower in AFD subjects than in controls

Rationale:

It is likely that in the lung the pattern of α-galactosidase A activity will follow that of blood in AFD

Hypothesis 2:

α-galactosidase A activity in induced sputum is lower in AFD males than AFD females

Rationale:

AFD females may have blood and plasma α-galactosidase A activity within the normal range though this is always low in males

Hypothesis 3:

There is a consistent relationship between blood and induced sputum α-galactosidase A activity

Rationale:

α-galactosidase A activity in sputum is likely to reflect that of peripheral blood given that sputum is consistent mainly of leucocytes originally from the blood

Hypothesis 4:

α-galactosidase A activity is lower in the induced sputum of AFD subjects with pulmonary involvement compared to those without

Rationale:

Pathology in AFD occurs as a result of deficient α-galactosidase A activity

Hypothesis 5:

α-galactosidase A activity will be similar in AFD subjects on enzyme replacement therapy (ERT) than those who are enzyme naïve

Rationale:

The half life of exogenous enzyme is short so that it is unlikely to be detectable for very long after infusion

4.3 Objectives

1. To establish a normal reference range for α-galactosidase A activity in induced sputum

2. To compare α-galactosidase A activity in the induced sputum of AFD subjects and controls

3. To compare α-galactosidase A activity in the induced sputum of AFD males and females

4. To delineate a relationship between peripheral blood and lung derived α-galactosidase A enzyme

5. To examine the effect of enzyme replacement therapy on α-galactosidase A activity in induced sputum

6. To examine the relationship between induced sputum α-galactosidase A enzyme activity and lung involvement in AFD

4.4 Methods

All collection of data, induced sputum and blood samples were performed by the same operator, Dr. Nadia Shafi. Paired samples of blood and induced sputum were obtained from control and AFD subjects and prepared as described in Methods Section 2.3.2 and 2.3.6.2. Samples were processed on the same day with sputum samples processed no more than 2 hours after being obtained, and stored for subsequent enzyme assays. Enzyme assays were performed according to the protocols outlined in Methods Section 2.3.3 and 2.3.7.

The further preparation and examination of induced sputum samples by electron microscopy were performed by Mr. Innes Clatworthy of the EM Department at the Royal Free Hospital London, as outlined in Methods Section 2.3.11.

Statistical analysis was performed using unpaired t test for normally distributed data, Mann Whitney U test for non-parametric data, with Wilcoxon Signed Rank test for paired non-parametric data.

4.5 Results

4.5.1 Overview

α-galactosidase A enzyme activity was measured in paired blood and induced sputum samples from 23 AFD subjects and 23 non-AFD controls. The non-AFD control group was made up of the 9 healthy control subjects and 14 COPD control subjects recruited to the study. Given that none of the control group had clinical features of AFD, and in addition all of the COPD controls and all females were genotyped to exclude the possibility of an underlying diagnosis of AFD, they were felt to be a valid control group for the purposes of comparison of enzyme activity. This was borne out on measurement of enzyme activity in blood.

Participants in the healthy control group were matched to AFD subjects by gender and age. Subjects in the COPD group were matched to AFD subjects by

gender and spirometry. As a result, because age was not a primary matching criteria in the COPD group, the mean age of the AFD subjects was significantly younger than of the non-AFD control subjects (p=0.02, Table 4.5.1.1). Both the AFD and control groups contained 13 males and 10 females.

The mean FEV_1/FVC ratio of the AFD and control groups was not significantly different at 0.68 for each group (Table 4.5.1.1). When analysed separately, as expected the healthy control group was found to have a significantly higher FEV_1/FVC ratio than the COPD control group (p<0.0001, Table 4.5.1.2). Of the AFD subjects 18 were receiving enzyme replacement therapy (ERT). Of the 5 subjects not receiving ERT, 2 were male.

A diagnosis of AFD was excluded in all members of the non-AFD control group by measurement of plasma α-galactosidase A activity, peripheral blood leucocyte α-galactosidase A activity and genetic analysis if necessary. For the majority of the control subjects the plasma enzyme activity fell within the normal reference range with only 1 subject from the COPD control group with a plasma enzyme activity outside the upper limit of the reference range at 31.0 nmol/hr/ml (Figure 4.5.1.1).

Peripheral blood leucocyte enzyme activity fell within the normal reference range of the RFH Molecular Diagnostics Laboratory for 16 of the 23 control subjects. In the remaining 7, peripheral blood leucocyte enzyme activity was between 22.31 and 32.44 nmol/hr/mg protein. Genetic analysis did not reveal the presence of any disease-causing mutations in the α-galactosidase A gene in these subjects.

When compared to the AFD subjects both plasma and peripheral blood leucocyte enzyme activity was significantly higher in the control groups, both when analysed as separate groups (1-way ANOVA, Kruskal-Wallis test p<0.05, Figure 4.5.1.1) and when combined (Mann Whitney U test p<0.0001, Table 4.5.1.1). As such, subsequent analysis of enzyme data was performed with the healthy controls and COPD control combined as one group of non-AFD controls.

Figure 4.5.1.1 Plasma and peripheral blood leucocyte α-galactosidase A activity in AFD subjects compared to control groups
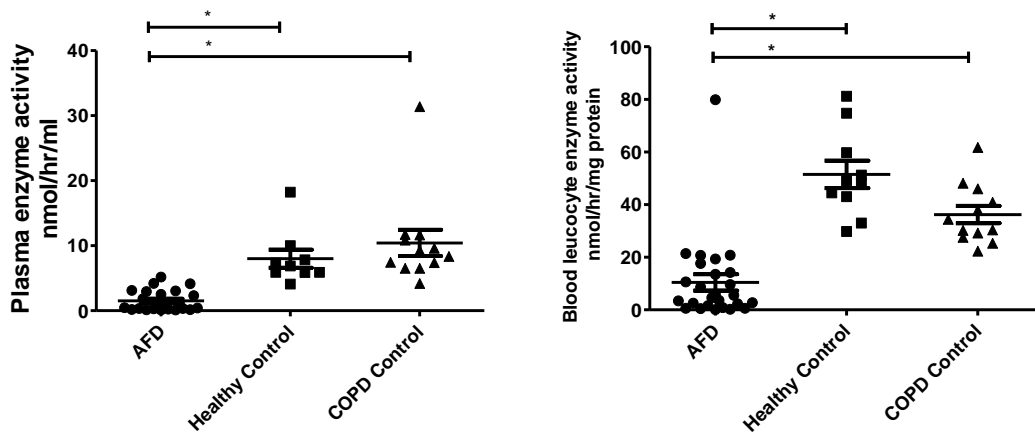
* $p < 0.05$ using 1 way ANOVA, Kruskal-Wallis test

Table 4.5.1.1 Demographics of AFD and non-AFD control groups used to examine differences in α-galactosidase A enzyme activity.

Parameter	AFD	Non-AFD Control	p value Unpaired t test
n (%)	23 (100)	23 (100)	-
Males, Females	13 (57), 10 (43)	13 (57), 10 (43)	-
Age [years] Mean (+/- SD)	45.7 (+/- 13.7)	55.7 (+/- 16.2)	**0.02**
FEV$_1$/FVC Mean (+/- SD)	0.68 (+/- 0.06)	0.68 (+/- 0.12)	0.68
Plasma nmol/hr/ml Median (range)	0.49 (0.1 – 5.2)	7.5 (4.1 – 31.4)	**<0.0001**
Blood leucocyte nmol/hr/mg protein Median (range)	3.60 (0.1 – 79.9)	41.9 (22.3 – 81.2)	**<0.0001**

Table 4.5.1.2 Demographics for healthy control and COPD control subgroups

Parameter	Healthy Control	COPD Control	p value Unpaired t test
n (%)	9 (39)	14 (61)	-
Age [years] Mean (+/- SD)	41.0 (+/- 10.0)	66.9 (+/- 9.4)	**<0.0001**
Males, females	5 (22), 4 (17)	9 (39), 5 (22)	-
FEV_1/FVC Mean (+/- SD)	0.82 (0.72 – 0.85)	0.61 (0.58 – 0.66)	**0.0003**

4.5.2 Blood and sputum α-galactosidase A activity in AFD and non-AFD control subjects

4.5.2.1 Plasma

As expected plasma α-galactosidase A activity was significantly lower in the AFD group compared to the control group (p<0.0001) (Table 4.5.2.1). Plasma enzyme activity was lower in AFD males than females (p<0.0001) and in each case lower than sex matched controls (p<0.0001 for males, p=0.002 for females, Table 4.5.2.2).

4.5.2.2 Peripheral blood leucocytes

Peripheral blood leucocyte α-galactosidase A activity in the AFD group was significantly lower than in the control group (p<0.0001, Table 4.5.2.1). Again enzyme activity in AFD males was significantly lower than in AFD females (p<0.01) and in each case lower than sex matched controls (p<0.0001 for males, p=0.002 for females, Table 4.5.2.2).

4.5.2.3 Induced sputum supernatant

α-galactosidase A activity in the fluid phase of the sputum was found to be similar in AFD subjects and controls in the group taken as a whole (Table 4.5.2.1). When analysed by gender, the sputum supernatant enzyme activity in AFD males was significantly lower compared to control males ($p<0.0001$) and AFD females ($p<0.01$, Table 4.5.2.2). However no difference was found between AFD females and control females.

4.5.2.4 Induced sputum derived leucocytes

The pattern of α-galactosidase A activity in sputum-derived leucocytes was similar to that seen in sputum supernatant: it was not significantly different between AFD and control subjects in the group taken as a whole (Table 4.5.2.1), though enzyme activity in AFD males was significantly lower than that in both control males ($p=0.003$) and AFD females ($p<0.01$, Table 4.5.2.2).

Table 4.5.2.1 α-galactosidase A activity in blood and sputum compartments in AFD and non-AFD control subjects

Sample type	AFD	Non-AFD control	p value (MWU test)
n	23	23	-
Plasma nmol/hr/ml Median (range)	0.5 (0.1 – 5.2)	7.5 (4.1 – 31.4)	**<0.0001**
Blood leucocyte nmol/hr/mg protein Median (range)	3.60 (0.1 – 79.9)	41.9 (22.3 – 81.2)	**<0.0001**
Sputum supernatant nmol/hr/ml Median (range)	2.3 (0.3 – 21.5)	2.8 (1.1 – 10.4)	0.8
Sputum leucocyte nmol/hr/mg protein Median (range)	54.1 (3.9 – 281.4)	82.8 (19.6 – 225.4)	0.2

Table 4.5.2.2 Blood and induced sputum α-galactosidase A activity in male and female AFD and non- AFD control subjects

Sample type Median (range)	AFD males	Non-AFD males	p	AFD females	Non-AFD females	p
n	13	13		10	10	
Plasma nmol/hr/ml	0.3# (0.1 – 0.8)	7.6 (4.2 – 31.4)	<0.0001	3.0 (1.2 – 5.2)	7.4 (4.1 – 10.9)	0.0002
Blood leucocyte nmol/hr/mg protein	1.6# (0.1 – 10.6)	43.0 (25.4 – 81.2)	<0.0001	13.9 (3.4 – 79.9)	34.4 (22.3 –59.8)	0.002
Sputum supernatant nmol/hr/ml	1.3# (0.3 – 3.9)	4.0 (1.5 – 10.4)	0.005	3.2 (0.7 – 21.5)	2.6 (1.1 – 3.2)	0.10
Sputum leucocyte nmol/hr/mg protein	33.9# (3.9 – 118.0)	78.5 (26.9 –225.4)	0.003	109.5 (24.4-281.4)	103.2 (19.6-177.0)	0.3

# p<0.01 compared to AFD females

4.5.3 Relationship between sputum and blood α-galactosidase A activity in AFD and non-AFD control subjects

The relationship between sputum and blood α-galactosidase A activity was examined using paired samples taken from the 23 AFD and non-AFD control subjects. Samples were taken at the same study visit for each subject and processed on the same day. The Wilcoxon signed rank test was used for the statistical analysis of paired samples of non-parametric data, with the Mann Whitney U test for unpaired data.

4.5.3.1 Sputum supernatant and plasma

In the non-AFD control group as a whole, the α-galactosidase A activity in induced sputum supernatant was significantly lower than that in plasma (p<0.0001). When examined by gender, both non-AFD control males and females had significantly higher enzyme activity in plasma compared to paired samples of sputum supernatant (p=0.001 and p=0.004, Table 4.5.3.1).

However in the AFD group the reverse appeared true with the sputum supernatant enzyme activity found to be significantly higher than that in plasma for the group as a whole (p=0.007). When examined by gender, AFD males had higher enzyme activity in sputum supernatant than plasma (p=0.0002, Table 4.5.3.1) whereas no significant difference was seen between enzyme activity in sputum supernatant and plasma in AFD females.

The ratio of sputum supernatant to plasma was shown to be consistent in the control group as a whole and when examined by gender. The sputum supernatant/plasma enzyme activity ratio was significantly higher in the AFD groups compared to their relevant control groups (p<0.001, Table 4.5.3.1).

Table 4.5.3.1 α-galactosidase A activity in paired samples of sputum supernatant and plasma in AFD and non-AFD controls

α-galactosidase A activity nmol/hr/ml Median (range)					
Sample type/ Group	n	Sputum supernatant	Plasma	p	Sputum supernatant/plasma enzyme ratio
Non-AFD controls	23	2.8 (1.1 – 10.4)	7.5 (4.1 – 31.4)	**<0.0001**	0.3 (0.1 – 0.9)
AFD	23	2.3 (0.3 – 21.5)	0.5 (0.1 – 5.2)	**0.007**	**1.8#** (0.4 – 15.4)
Non-AFD males	13	4.0 (1.5 – 10.4)	7.6 (4.2 – 31.4)	**0.001**	0.3 (0.2 – 0.9)
AFD males	13	1.3 (0.3 – 3.9)	0.3 (0.1 – 0.8)	**0.0002**	**2.1#** (0.6 – 15.4)
Non-AFD females	10	2.6 (1.1 – 3.2)	7.4 (4.1 – 10.9)	**0.004**	0.3 (0.1 – 0.4)
AFD females	10	3.2 (0.7 – 21.5)	3.0 (1.2 – 5.2)	0.25	**1.2#** (0.4 – 7.0)

# p<0.001 for each compared to matched non-AFD control group

4.5.3.2 Induced sputum derived leucocytes and peripheral blood leucocyte

In both the non-AFD control group and the AFD group the α-galactosidase A activity in sputum-derived leucocytes was significantly higher than that in peripheral blood derived leucocytes (p=0.002 and p<0.0001, Table 4.5.3.2). This finding persisted when analysed by gender for both non-AFD controls (males p=0.001, females p=0.02) and AFD subjects (males p=0.0005, females p=0.002) (Table 4.5.3.2).

The ratio of sputum to blood leucocyte α-galactosidase A activity was very similar in the non-AFD control group as a whole and in male and female control groups, and was significantly greater in AFD subjects compared to matched controls. The difference between the sputum/blood enzyme ratio between AFD males and AFD females was not statistically significant.

Table 4.5.3.2 α-galactosidase A activity in paired samples of induced sputum and peripheral blood leucocytes from AFD and non-AFD control subjects

Leucocyte α-galactosidase A activity nmol/hr/mg protein Median (range)					
Sample type/ Group	n	Sputum	Peripheral Blood	p	Sputum/blood leucocyte ratio
Non-AFD controls	23	82.8 (19.6 – 225.4)	41.9 (22.3 – 81.2)	**0.002**	1.9 (0.7 – 5.5)
AFD	23	54.1 (3.9 – 281.4)	3.6 (0.1 – 79.9)	**<0.0001**	**17.1#** (0.7 – 196.7)
Non-AFD control males	13	78.5 (26.9 – 225.4)	43.0 (25.4 – 81.8)	**0.001**	1.8 (1.0 – 5.5)
AFD males	13	33.9 (3.9 – 118.0)	1.6 (0.1 - 10.6)	**0.0005**	**19.9#** (0.7 – 361.0)
Non-AFD control females	10	103.2 (48.98 – 124.6)	34.44 (30.02 – 48.64)	**0.02**	2.7 (0.7 – 3.2)
AFD females	10	109.5 (24.4 – 281.4)	13.9 (3.4 – 79.9)	**0.002**	**7.2#** (2.7 – 47.0)

# p<0.001 for each compared to matched non-AFD control group

4.6 Impact of enzyme replacement therapy (ERT) and disease severity on α-galactosidase A activity

4.6.1 Plasma

In order to examine the impact of ERT, measurements of plasma α-galactosidase A activity taken at the time of diagnosis (prior to initiation of ERT) were compared to repeat plasma enzyme measurements at baseline study visit in 33 subjects who had been receiving ERT for different durations following diagnosis. The median duration of ERT for this group was 54.4 months, with a range 5 to 121 months. All subjects were currently receiving fortnightly enzyme infusions.

The median plasma α-galactosidase A activity of this group at diagnosis was 2.10 nmol/hr/ml (range 0.01 − 7.5) and at baseline study visit 1.82 nmol/hr/ml (range 0.1 − 7.3). There was no statistical difference demonstrated (p=0.1, Wilcoxon signed rank test, Figure 4.6.1 a))

To further examine the effect of ERT, plasma α-galactosidase A activity was compared in 36 AFD subjects receiving ERT (median 2.1 nmol/hr/ml, range 0.1 − 7.3) and 8 enzyme naïve subjects (median 3.5 nmol/hr/ml, range 0.1 − 4.3). Again, there was significant difference found (p=0.7, Figure 4.6.1 b)).

In a sub-group of 14 AFD subjects a composite plasma enzyme profile was made using multiple measurements of plasma α- galactosidase A at different time points over the two week period between one enzyme infusion and the next. These were compared to the same subject's plasma enzyme activity at diagnosis (Day 0) (Figure 4.6.1 c). Although measurements were taken at different infusion cycles within the same subject, it was ensured that there had been no changes in day of delivery of the infusion or dose between enzyme cycles. The largest variation of plasma α-galactosidase A activity within a study subject was 3.4 − 7.5 nmol/ml/hr, with the smallest being 0.11 − 0.16 nmol/hr/ml. Overall there was little variation in plasma α-galactosidase A activity within AFD subjects regardless of the timing of sampling following enzyme infusion (Figure 4.6.1c)).

Figure 4.6.1 Effect of ERT on enzyme activity in AFD plasma

a)

b)

c)
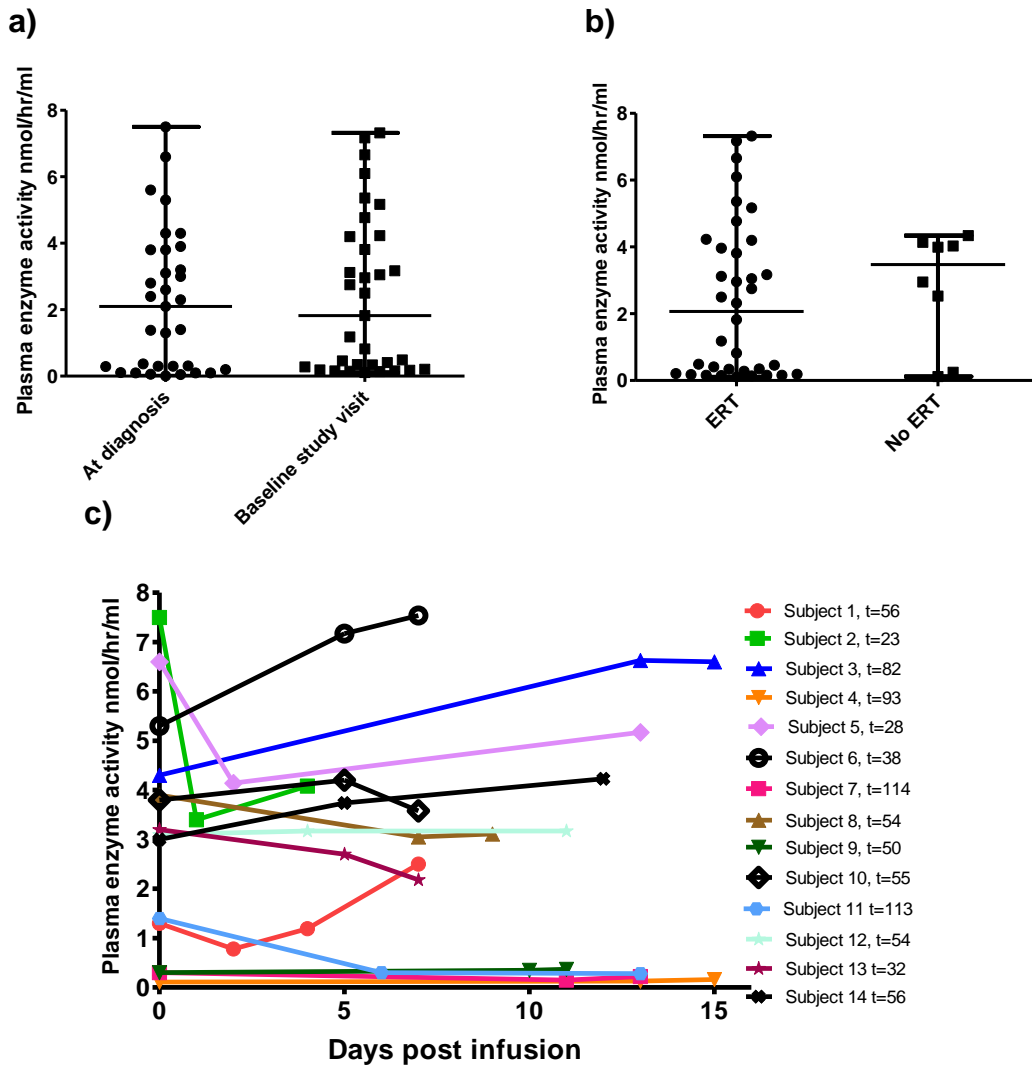
a) Plasma α-galactosidase A activity in AFD subjects receiving ERT, measured at the time of diagnosis and on the day of first study visit (median and range shown)

b) Plasma α-galactosidase A activity in AFD subjects receiving ERT and enzyme naïve subjects (median and range shown)

c) Profile of plasma α-galactosidase activity in 14 AFD subjects on ERT; measurements were performed at different time points during different infusion cycles in individual subjects with activity at diagnosis used for day 0 (t=duration of ERT in months)

4.6.2 Peripheral blood leucocytes

Peripheral blood leucocyte α-galactosidase A activity at time of diagnosis and at baseline study visit were compared in a similar manner in 15 AFD subjects receiving ERT. The median duration of ERT for this group was 54.0 months with a range of 7 to 121 months.

The median blood leucocyte α-galactosidase A activity at diagnosis was 12.0 nmol/hr/mg protein (range 0.2 – 88.0) and at baseline study visit was 5.6 nmol/hr/mg protein (range 0.2 – 79.9), again with no significant difference seen between the two (p=0.1, Wilcoxon signed rank test, Figure 4.6.2 a)).

Blood leucocyte α-galactosidase A activity was compared in 23 AFD subjects on ERT (median 3.6 nmol/hr/mg protein, range 0.1 – 79.9), and 5 enzyme naïve subjects (median 9.6 nmol/hr/mg protein, range 2.7 – 19.3) and was not found to be significantly different (p=0.3, Figure 4.6.2 b).

In 12 AFD subjects, repeat measurements of blood leucocyte α-galactosidase A activity were performed at different time points in different enzyme infusion cycles, and plotted as a composite profile alongside enzyme activity at diagnosis (Day 0) (Figure 4.6.2 c). The largest range of blood leucocyte α-galactosidase A activity within a study subject was 20.78 – 54.00 nmol/hr/mg protein (Subject 7); whilst the smallest range was 0.19 – 1.08 nmol/hr/mg protein (Subject 11).

Figure 4.6.2 Effect of ERT on enzyme activity in peripheral blood leucocytes in AFD
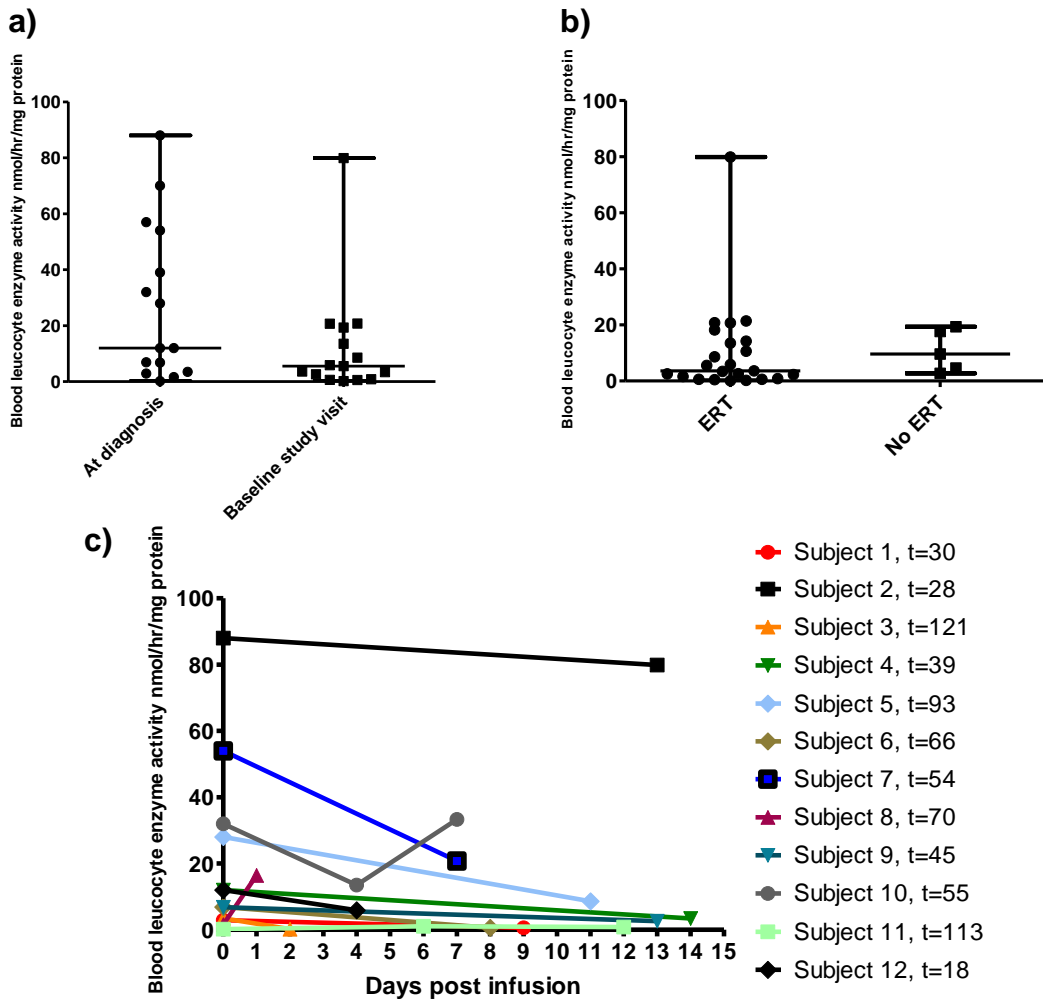
a) Blood leucocyte α-galactosidase A activity in AFD subjects on ERT measured at the time of diagnosis compared to blood leucocyte enzyme activity on the day of first study visit (median and range shown).

b) Blood leucocyte α-galactosidase A activity in AFD subjects on ERT and enzyme naïve subjects (median and range shown)

c) Profile of peripheral blood leucocyte α-galactosidase activity in 12 AFD subjects on ERT; measurements were performed at different time points during different infusion cycles in individual subjects with activity at diagnosis used for day 0 (t=duration of ERT in months)

4.6.3 Sputum

A-galactosidase A activity in sputum supernatant and from sputum-derived leucocytes was not significantly different in AFD subjects receiving ERT compared to those who had never received ERT, though numbers in the ERT naïve group were small (Table 4.6.3). Paired sputum and blood samples were used to calculate sputum supernatant/plasma and sputum leucocyte/blood leucocyte enzyme ratios. There appeared to be no significant difference in the ratios between subjects receiving ERT and those who were ERT naïve (p=0.9 and p=0.2 respectively).

4.6.4 Severity of disease in subjects on ERT and ERT naïve subjects

Disease severity scores in the form of the Mainz Severity Score Index (MSSI) and the Age-Adjusted Fabry Outcome Survey-MSSI were calculated for the AFD subjects on ERT and those who were enzyme naïve at the first study visit.

As disease severity would have been the trigger for the instigation of enzyme replacement therapy, severity scores of AFD subjects on ERT were significantly higher than enzyme naïve AFD subjects (p=0.001 for MSSI and p=0.02 for Age adjusted Severity Score using Mann Whitney U test, Table 4.6.3).

When examined further, no difference in sputum/blood enzyme ratio was seen in AFD subjects with milder disease (MSSI <20) compared to those with more severe disease (MSSI >20) (Table 4.6.4).

Table 4.6.3 Effect of overall disease severity and ERT on α-galactosidase A enzyme activity

Parameter	On ERT Median (range)	ERT naïve Median (range)	P value
n	23	6	-
MSSI	27.0 (8.0 - 44.0)	3.0 (1.0 – 15.0)	**0.001**
Age adjusted Severity Score	6.5 (-10.5 - 18.7)	-5.0 (-6.9 - -2.0)	**0.02**
Plasma α-galactosidase A activity nmol/hr/ml	0.5 (0.1 – 5.2)	2.5 (0.1 – 4.3)	0.7
Blood leucocyte α-galactosidase A activity nmol/hr/mg protein	3.6 (0.1 – 79.9)	9.6 (2.7 – 19.3)	0.3
Sputum supernatant α-galactosidase A activity nmol/hr/ml	2.4 (0.3 – 36.1)	3.6 (0.3 - 7.4)	0.8
Sputum leucocyte α-galactosidase A activity nmol/hr/mg protein	47.40 (3.9 – 371.0)	75.80 (21.0 – 120.6)	0.6
Sputum supernatant/plasma enzyme activity ratio	1.8 (0.4 – 15.4)	2.1 (1.0 – 2.9)	0.9
Sputum/blood leucocyte enzyme activity ratio	19.3 (0.7 – 361.0)	6.2 (4.5 – 19.9)	0.2

Table 4.6.4 Comparison of sputum/blood ratios in AFD subjects on ERT with milder disease compared to more severe disease as measured by Mainz Severity Score Index

	MSSI <20	MSSI >20	p value
n	13	5	-
Sputum supernatant/plasma ratio	1.5 (0.4 – 11.4)	6.0 (1.4 – 8.0)	0.2
Sputum/blood leucocyte ratio	19.9 (2.7 – 148.0)	18.8 (0.7 – 196.7)	0.7

4.7 A-galactosidase A activity in relation to airflow limitation

A-galactosidase A enzyme activity in plasma and peripheral blood derived leucocytes was found to be significantly lower in AFD subjects with airway obstruction (defined as FEV_1/FVC <0.7) than those without airway obstruction (p=0.03 for plasma, p=0.04 for peripheral blood leucocytes, Table 4.7.1).

Examination of α-galactosidase A activity in sputum from AFD subjects with and without airway obstruction did not reveal statistically significant differences either from the sputum supernatant or sputum derived leucocytes. However, there was a trend towards sputum leucocyte enzyme activity being lower in airway obstructed AFD subjects (p=0.08). Sputum supernatant/plasma ratio was significantly higher in the obstructed AFD subjects compared to the unobstructed, though there was no difference in sputum/blood leucocyte enzyme ratio between the groups (p=0.02 and p=0.5, Table 4.7.1).

No significant difference was seen in enzyme activity from plasma, peripheral blood derived leucocytes, sputum supernatant or sputum derived leucocytes when comparing obstructed and unobstructed AFD subjects by gender (see Appendix 1, Table 1.0). There was a trend towards lower blood leucocyte enzyme activity in AFD males with airway obstruction compared to AFD males without (p=0.07).

When analysed as continuous variables, there were no strong correlation found between FEV_1/FVC and α-galactosidase A activity in plasma, peripheral blood leucocytes, sputum supernatant and sputum derived leucocytes, or sputum/blood leucocyte enzyme ratio. Weak correlations were evident between FEV_1/FVC and plasma enzyme activity (r=0.3, p=0.03) and FEV_1/FVC and sputum supernatant/plasma ratio (r=0.4, p=0.02, Figure 4.7.2).

Table 4.7.1 α-galactosidase A activity in AFD subjects with and without airway obstruction

(airway obstruction defined as $FEV_1/FVC < 0.7$)

Sample type for α-galactosidase A activity	n	Obstructed Median (range)	n	Unobstructed Median (range)	p value
Plasma nmol/hr/ml	20	0.4 (0.1 – 6.1)	24	3.0 (0.91 - 4.66)	**0.03**
Blood leucocyte nmol/hr/mg protein	15	2.6 (0.1 – 20.8)	13	10.6 (1.6 - 79.9)	**0.04**
Sputum supernatant nmol/hr/ml	16	1.5 (0.3 – 21.5)	15	2.8 (0.3 – 36.1)	0.2
Sputum leucocyte nmol/hr/mg protein	18	43.5 (3.9 – 164.2)	11	80.0 (21.0 -371.2)	0.08
Sputum Supernatant/ plasma ratio	16	3.0 (0.4 – 15.4)	15	1.4 (0.6 – 7.6)	**0.02**
Sputum /blood leucocyte ratio	15	18.8 (0.7 – 361.0)	12	12.3 (2.0 – 115.6)	0.5

Figure 4.7.2 α-galactosidase A activity in relation to airflow limitation in a) plasma and b) sputum supernatant/plasma ratio
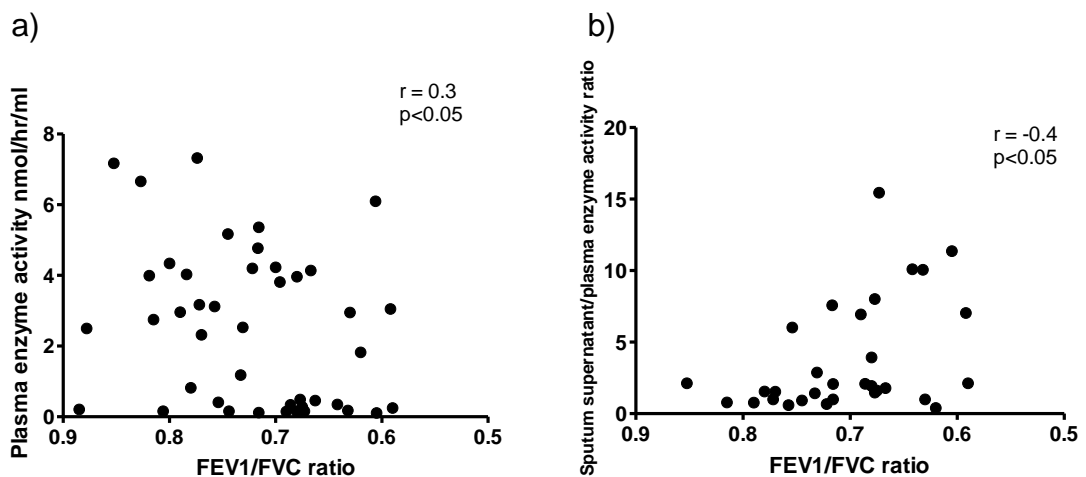
4.8 Induced sputum electron microscopy

Of the 3 induced sputum samples from AFD male subjects prepared and examined for evidence of storage by EM, inclusion bodies were evident in one of the samples. This sample was from a 32 year old male with the mutation P205T, who had been receiving ERT for a total duration of 30 months. This subject had an MSSI of 11 at recruitment, and had an FEV_1/FVC of 0.68, but no reported respiratory symptoms. As shown below in Figure 4.8.1 his sputum derived cell contained inclusion bodes which had the typical lamellar structure that has been previously documented in AFD (235).

Figure 4.8.1 Transmission electron microscopy of induced sputum cell from male AFD subject demonstrating inclusion bodies typical of Gb3 storage

a) magnification x31000   b) magnification x110000
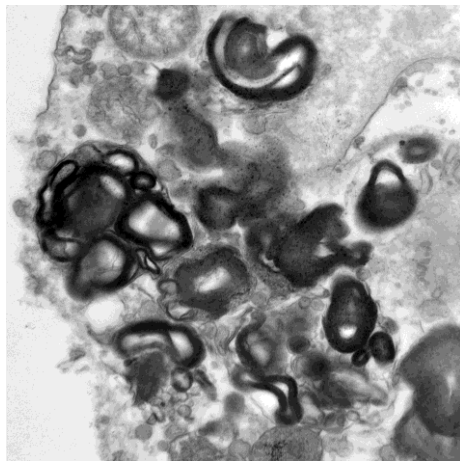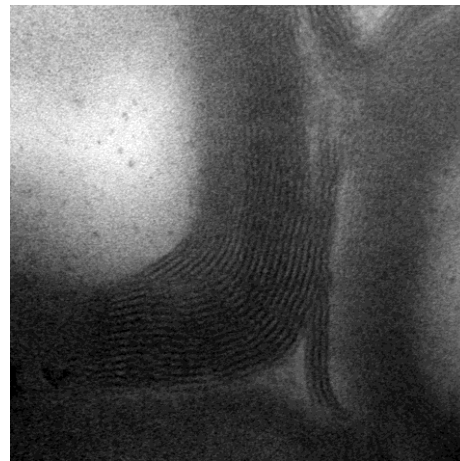
4.9 Discussion

α-galactosdase A activity has been quantified in a number of different sites in subjects with Anderson Fabry Disease, including liver, kidney, skin as well as blood and tears. Thus far, evidence of pulmonary involvement in AFD has been based on clinical parameters with obstructive changes in lung function in AFD subjects (236;237); and on the demonstration of storage material in lung-derived samples histologically, both on light and electron microscopy (238;239). Quantification of α-galactosidase A activity from lung derived samples has not been performed. Hence there is no normal reference range with which to compare the values found in AFD subjects.

To examine changes in the lung in AFD in more detail it was necessary to first establish that α-galactosidase A could be measured in sputum samples, and subsequently to build a reference range for sputum α-galactosidase A activity from non-AFD control subjects. For all non-AFD control subjects, plasma α-galactosidase A activity made it highly unlikely that any had undiagnosed AFD. However, in some control subjects blood leucocyte α-galactosidase A activity was below the reference range used for this assay. Despite this, however, there was still a clear and significant difference between blood leucocyte enzyme activity in the AFD and control groups. Further confirmation was obtained that these subjects did not have AFD by genotyping for known AFD-related mutations (standard practice in AFD females).

As outlined in Methods Section 2.3.6.1, experiments were performed to establish that generally accepted processing of sputum samples did not affect α-galactosidase A enzyme activity measurements. It was then possible to investigate differences between sputum enzyme activity in AFD and controls.

Taken as a whole, AFD results indicate that α-galactosidase A activity was similar in AFD subjects compared to non-AFD controls in both the fluid phase of sputum and induced sputum derived leucocytes. However, when divided by gender, it was clear that α-galactosidase A activity in AFD males was significantly

lower in both the fluid phase and cellular phase of sputum compared to non-AFD male controls, as well as being lower than in AFD females.

Enzyme activity in sputum from AFD females was not significantly different to that of control females. This is similar to reported findings from both plasma and blood leucocyte enzyme activity in AFD, where lyonisation is thought to play a part in the differences in enzyme activity between AFD males and females; and enzyme activity in AFD female subjects sometimes falls within the normal reference range despite clinical features and genotyping pointing towards AFD (240).

The relationship between plasma and sputum supernatant enzyme activity within AFD and non-AFD control subjects appeared to differ, with activity being higher in plasma than in the fluid phase of sputum in non-AFD control subjects, and the opposite in AFD subjects. This was statistically significant in the group as a whole and in AFD males, but not females. No gender differences were apparent in control subjects.

Examining the relationship between sputum and blood-derived leucocyte enzyme activity, more consistency was present between AFD and non-AFD control groups. Sputum leucocyte derived α- galactosidase A activity was higher than that of paired samples of blood derived leucocytes for both groups as a whole, and when divided by gender. The sputum/blood enzyme ratio was consistently higher in AFD groups compared to the matched non-AFD control groups. This was most marked in AFD males, implying that this group had the largest relative difference between sputum and blood enzyme activity. It is possible that enzyme replacement therapy may have an impact on sputum/blood leucocyte enzyme ratio in AFD subjects compared to non-AFD subjects, although this is unclear.

It is likely that α-galactosidase A enzyme detected in the fluid phase of sputum derives from both plasma α-galactosidase A within the pulmonary circulation, and enzyme leaching out of sputum cells which are suspended within it. A possible explanation for the differences seen in plasma and sputum

supernatant activity between non-AFD controls and AFD subjects may be that it is the latter mechanism which is responsible for the majority of enzyme activity being evaluated in the fluid phase of sputum. Given that plasma enzyme activity in the AFD male group was very low, the relatively large difference in sputum leucocyte enzyme activity compared to blood evident in this group may have caused the enzyme activity in the sputum supernatant to surpass that of the plasma in the AFD subjects.

As would be expected, the disease severity of AFD subjects on ERT was significantly greater than that of subjects who were enzyme naïve as assessed by both the Mainz severity score and the Age adjusted FOS-MSSI. However no significant difference was seen in α-galactosidase A activity in blood or sputum, although numbers of ERT naïve subject were small. Examination of the effect of ERT on plasma and blood leucocyte α-galactosidase A activity demonstrated that there is very little change from baseline activity at diagnosis, regardless of the duration of treatment or the time elapsed since the last enzyme infusion. This would be in keeping with pharmacokinetic data from clinical trials of enzyme replacement therapy which demonstrated the short half life of exogenous enzyme (241).

It is not clear, however, whether the half life of ERT may be different within the lung milieu, where the longer life span of leucocytes compared to that of blood white cells might allow an accumulation of lung derived cells containing exogenous enzyme compared to blood which provides an additional explanation for the gradient between sputum/blood enzyme activity in AFD. Clarification of this would only be possible by longitudinal assessment of sputum α-galactosidase A activity in subjects commenced on ERT, who had been sampled at baseline. The ratio of sputum/blood α-galactosidase A activity was no different between AFD subjects with mild or severe disease.

AFD subjects with evidence of airway obstruction had lower plasma and blood leucocyte α-galactosidase A activity compared to those AFD subjects with normal spirometry. This suggests that changes in spirometry may be related to

residual enzyme activity. Examination of sputum α-galactosidase A activity in these populations revealed that AFD subjects with airway obstruction also tended to have lower sputum leucocyte enzyme activity compared to those without airway obstruction, though this did not reach statistical significance (p=0.08), with the small numbers studied. The weak correlation between sputum supernatant/plasma ratio and FEV_1/FVC may imply that lung involvement in the form of airflow obstruction is more dependent on blood enzyme activity than that of sputum, but further sampling and work in this area would need to be done to clarify this.

4.10 Conclusions

The data collected and analysed in this chapter can be summarised as follows:

1. α-galactosidase A activity can be measured in induced sputum supernatant and from induced sputum derived leucocytes

2. α-galactosidase A activity is lower in induced sputum derived leucocytes from AFD males compared to non-AFD male controls

3. α-galactosidase A activity is lower in induced sputum derived leucocytes from AFD males compared to AFD females

4. Induced sputum α-galactosidase A activity is greater than blood α-galactosidase A activity in AFD subjects (statistically significant in all compartments except sputum supernatant in AFD females)

5. No demonstrable difference in α-galactosidase A activity in blood or induced sputum was seen in AFD subjects on ERT compared to those who were ERT naïve

6. There is no demonstrable relationship between overall disease severity as measured by MSSI and sputum α-galactosidase A activity

7. Blood and plasma α-galactosidase A activity are lower in AFD subjects with airway obstruction compared to those without

8. There is a suggestion that induced sputum leucocyte α-galactosidase A activity is lower in AFD subjects with airway obstruction compared to those without

Chapter 5 – Cell populations and cytokines in induced sputum

5.1 Introduction

The mechanisms underlying the development of airway involvement in AFD are as yet unclear. However, there is some evidence that changes occur at a cellular level within the lungs with the demonstration of lamellar inclusion bodies in bronchial epithelial cells both from bronchoscopy samples including bronchoalveolar lavage and brushings (242), as well as from induced sputum sampling (243). Additional studies have also examined induced sputum cell populations in normal individuals without underlying respiratory disease providing a point of reference for studies into other lung pathology (244)((245). To our knowledge, further examination of the sputum for the purposes of establishing the pulmonary impact of AFD has not been undertaken. Given that the changes noted in AFD have been related to the airways (246;247), sputum sampling is an appropriate method of further examining the AFD lungs. As the majority of subjects would be unable to produce spontaneous sputum, sputum induction would allow samples to be generated and to be standardised for comparison with those from control subjects.

Given that it is NKT cells which are responsible for lipid antigen recognition, it has also been suggested that abnormalities in NKT cell populations may exist in AFD, (248). A specific subset of invariant NKT cells (iNKT), are particularly responsible for glycolipid recognition. Indeed, studies in AFD mice, demonstrated increased tissue CD4+/CD8+ T cell ratios in the liver, and noted that lower numbers of invariant NKT cells were present, though did not see the same in the peripheral blood of human AFD subjects (249). It has also been demonstrated in mice that lipid accumulation reduces NKT cell numbers by impairing thymic selection and interferes with lipid antigen presentation in lysosomal storage disorders (132).

We therefore aimed to use flow cytometry to examine induced sputum samples from AFD subjects with and without lung involvement, and to compare these to both a COPD and healthy control group in order to determine the proportions of different cells present. We sought in particular to examine lymphocyte numbers and subtypes, including NKT cells. Given the cell numbers

obtained in induced sputum and the general paucity of iNKT cells we felt it would be difficult to examine differences in iNKT cell populations in a meaningful way using this method.

Pro-inflammatory cytokines such as interleukin-8 (IL-8) and interleukin-6 (IL-6) have been strongly implicated in airways disease. IL-8 is produced by monocytes/macrophages and airway epithelial and smooth muscle cells, and is known to function as a neutrophil chemoattractor and activator (250). It has been measured in induced sputum samples from COPD subjects and shown to be closely associated with the degree of airway obstruction present (251). IL-6, which is also produced by T cells and B cells, has been found to be elevated in induced sputum samples from subjects with severe asthma (252) as well as in COPD where it too has been associated with lung function decline (253;254).

Therefore, in order to gain further information on the inflammatory processes present in the airways of AFD patients, we also aimed to examine induced sputum concentrations of cytokines IL-8 and IL-6 in comparison to subjects with chronic airways disease and healthy individuals. Studies in airways disease have also noted that cytokine concentrations may be influenced by cigarette smoking (255) and given that existing studies in AFD subjects suggest that the degree of airway obstruction may be significantly worsened by smoking (256) and we therefore aimed to examine this also.

5.2 Hypotheses

Hypothesis 1:

Induced sputum samples from AFD subjects with airways obstruction would have a greater proportion of CD8+ T cells than AFD subjects without airway obstruction

Rationale:

Increased proportions of CD8+ cells are seen in COPD patients, which has some similar features to the airway obstruction in AFD

Hypothesis 2:

Induced sputum samples from AFD subjects with airways involvement would have a higher proportion of neutrophils than that of AFD subjects without airway obstruction

Rationale:

Neutrophilic inflammation is an important feature of airway inflammation in airways disease such as COPD

Hypothesis 3:

Higher concentrations of pro-inflammatory cytokines IL-8 and IL-6 would be present in the induced sputum of AFD subjects with airway obstruction compared to those without

Rationale:

Inflammatrory cytokines IL-8 and IL-6 are present in elevated concentrations in COPD subjects

5.3 Objectives

1. To delineate the cell populations present in the induced sputum of AFD subjects

2. To compare cell populations in the induced sputum of AFD subjects to COPD and healthy control subjects

2. To compare the cell populations present in the induced sputum of AFD subjects with airway obstruction and those without

3. To examine the inflammatory processes in the lung in AFD by examining cytokine profiles

4. To examine the impact of smoking on inflammatory cytokines in induced sputum in AFD

5.4 Methods

All induced sputum sampling, processing of samples and flow cytometry data acquisition and analysis were performed by the same operator, Dr. Nadia Shafi. Induced sputum samples were obtained and processed within 2 hours as per the protocol outlined in Methods Section 2.3.6.2. Antibody staining and flow cytometry data acquisition were performed on the same day as sputum sampling. Samples for cytokine assays were frozen at -80°C and enzyme linked immunosorbent assays (ELISAs) performed as a batch at a later date as per the protocol outlined in Methods Section 2.3.10.

Statistical analysis for comparison normally distributed data including of age and lung function data was performed using the unpaired t test. For non-parametric data including cell numbers and percentages, statistical analysis was performed using the Mann Whitney U test.

5.5 Study Population

5.5.1 Demographics

Flow cytometric examination was performed on induced sputum samples from 22 AFD, of whom 13 had evidence of airway obstruction with FEV_1/FVC <0.7, and 9 who did not. Alongside this were 12 COPD subjects matched as closely as possible to the AFD subjects with airway obstruction, and 7 healthy control subjects matched as closely as possible to AFD subjects without airway obstruction (Table 5.5.1). Priority for matching was given to spirometry and gender over age, and as a result of this the COPD group were significantly older than the AFD and healthy control groups with a mean age of 64.2 years (p<0.005, Table 5.5.1). There was no significant age difference between the AFD subjects and the healthy controls. Data were also included from 3 AFD subjects who were not matched to a control subject.

The AFD group with airway obstruction had a mean FEV_1/FVC and FEV_1 %predicted which was significantly lower than that of both the AFD group without airway obstruction and the healthy control group (p<0.05 and p<0.001,

Table 5.5.1), but not significantly different to the COPD group. However, when assessed by GOLD criteria, the AFD obstructed group as a whole had a milder degree of airway obstruction than the COPD group.

On assessment of the small airways, both the AFD obstructed group and COPD group had significantly lower MEF_{50} %predicted than the AFD unobstructed group and healthy control group though on comparison of RV/TLC as a measure of small airways disease, only a difference between the COPD group and these groups was seen (Table 5.5.1).

Table 5.5.1 Demographics of AFD, COPD and healthy control subjects used for flow cytometric analysis of induced sputum cell populations

Parameter (mean +/-SD)	AFD with airway obstruction	COPD	AFD without airway obstruction	Healthy Control
n	13	12	9	7
Age, years	49.5 (+/- 14.5)	64.3§ (+/- 9.5)	44.2 (+/- 13.7)	44.3 (+/- 10.3)
Male, Female	8, 5	8, 4	1, 8	3, 4
FEV_1/FVC	0.65**# (+/-0.04)	0.62**# (+/-0.05)	0.76 (+/-0.04)	0.77 (+/-0.09)
FEV_1 % predicted	82.2*# (+/- 8.0)	69.2**# (+/-11.7)	95.3 (+/-15.2)	111.6 (+/-18.0)
MEF_{50} % predicted	43.7* (+/- 9.8)	24.9**# (+/- 13.8)	63.8 (+/- 18.4)	68.6 (+/- 25.4)
RV/TLC % predicted	89.7 (+/- 17.5)	117.7**# (+/- 18.1)	77.2 (+/- 6.5)	81.3 (+/- 11.1)

*$p < 0.05$, **$p < 0.0001$ compared to AFD without airway obstruction

#$p < 0.0001$ compared to Healthy Controls, § $p < 0.005$ compared to all groups

5.5.2 Flow cytometry data plots

Populations of T lymphocytes, NKT cells, B lymphocytes, neutrophils and monocytes/macrophages were identified using flow cytometry as described in Methods Section 2.3.9. The number of events captured on the flow cytometry plots used for the comparison of cell populations was similar for each group (Table 5.5.2).

For the purposes of comparison between groups, data for each population examined was expressed as a percentage of the relevant total population, as described in each section to follow. This was to enable the proportion of cells representing each cell sub-type in the different conditions to be compared.

Table 5.5.2 Total number of events captured on flow cytometry plots used for analysis of cell populations in induced sputum of AFD, COPD and healthy control subjects

Flow plot event number x10³						
Median (range)						
Flow plot	**n**	**AFD**	**n**	**COPD**	**n**	**Healthy Control**
T lymphocyte	18	137.7 (45.7- 819.9)	12	251.2 (105.6- 690.4)	7	252.9 (66.8 – 454.5)
NKT cell	21	162.6 (17.4 – 711.0)	12	242.8 (89.2 – 718.5)	8	234.1 (101.5–559.4)
B lymphocyte	8	229.9 (14.8- 574.6)	6	194.5 (146.2 -362.2)	5	235.1 (163.2 -363.9)
Neutrophil, Monocyte/ Macrophage	19	100.0 (14.6- 650.0)	13	171.1 (38.8 – 404.3)	6	210.7 (12.9 – 265.9)

5.6 Lymphocytes

5.6.1 T lymphocyte populations

Induced sputum samples were stained with antibodies for CD3, CD4, CD8 after processing, using the method previously outlined in Methods Section 2.3.9. A standardised lymphocyte gate was applied to the samples analysed, whose position was established using peripheral blood samples on forward scatter vs side scatter plots (Figure 5.6.1i). T lymphocytes were defined as CD3 positive (CD3+) events falling within the lymphocyte gate, and CD3+CD4+ and CD3+CD8+ events were identified from events within this gate (Figure 5.6.1ii).

From what is known about induced sputum cell populations, it was likely that lymphocytes would only make up a very small proportion of total cell numbers (257;258), only induced sputum samples with a minimum of 2000 events in the lymphocyte gate were included in the data analysis for lymphocyte populations. This enabled data from 15 AFD subjects, 12 COPD subjects and 7 healthy controls to be compared. On analysis of the flow plots, the percentage of the total events that fell inside the defined lymphocyte gate for each sample was not significantly different for each of the group (Table 5.6.1).

Comparing the groups, the COPD subjects had the highest percentage of CD3+ T cells. This was significantly higher than that of the AFD and healthy control subjects (p<0.05, Table 5.6.1). No difference was seen in T cell percentage between AFD and healthy control subjects.

Table 5.6.1 T cell populations in induced sputum of AFD, COPD and Healthy control subjects

Lymphocyte cell type	AFD	COPD	Healthy control
	Median (range)		
n	15	12	7
% of total events falling within lymphocyte gate	2.30 (0.52 – 7.26)	1.54 (0.42 – 3.19)	1.79 (0.59 – 3.50)
CD3+ T cells % of lymphocyte gate	64.7 (15.4 – 89.9)	74.5* (53.5 – 92.5)	40.1 (21.3 – 85.3)
CD3+CD4 + % of T cells	68.6 (30.8 – 86.1)	77.90 (27.0 – 93.1)	73.22 (39.8 – 86.8)
CD3+CD8+ % of T cells	31.4 (13.9 – 69.9)	22.10 (6.9 – 73.0)	26.78 (13.3 – 60.2)
CD4+/CD8+ T cell ratio	2.2 (0.43 – 6.2)	3.54 (0.37 – 13.52)	2.73 (0.66 – 6.56)
NKT cell % of T cells	1.60 (0.72– 5.3)	0.94 (0.1 – 3.0)	1.2 (0.1 – 2.2)

*$p < 0.05$ compared to AFD and Healthy control

Sub-populations of T lymphocytes were analysed, with CD4+ and CD8+ lymphocyte populations expressed as a percentage of the total CD3+ T cells within the lymphocyte gate. The percentages of CD4+ T lymphocytes and CD8+ T lymphocytes were not significantly different in the AFD, COPD and healthy control groups, with the CD4/CD8 ratio also consistent between the groups (Table 5.6.1).

Figure 5.6.1 Example flow cytometry cell plot for lymphocytes in induced sputum

Induced sputum flow cytometry cell plot of forward scatter vs side scatter, with i) gate used for lymphocyte population analysis of all samples and ii) example of CD3+CD4+ and CD3+CD8 T cell populations generated from the lymphocyte gate events and shown as a percentage of events from that gate (here CD4+ 34.19% and CD8+ 7.88%)
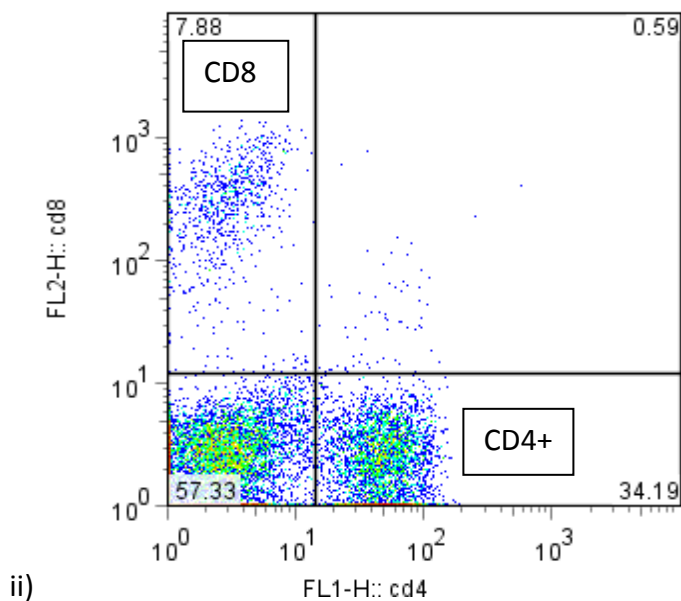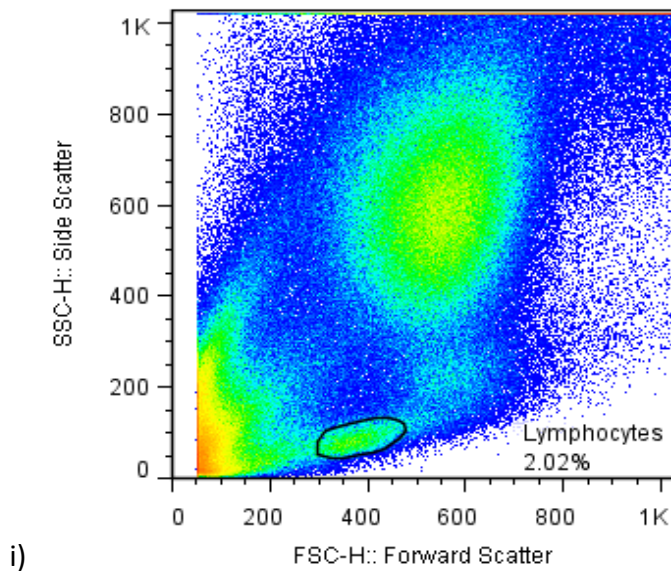
5.6.2 NKT cell populations

NKT cells were defined as CD3+CD16+CD56+ events within the lymphocyte gate previously described. Again, the percentage of the total events falling within the lymphocyte gate was similar between all three groups, and no significant difference was seen in the percentage of NKT cells found in the induced sputum of AFD, COPD and healthy control subjects (Table 5.6.1).

5.6.3 B lymphocytes

Induced sputum samples for B lymphocyte populations were analysed from 8 AFD, 6 COPD and 5 healthy control subjects having been stained with antibodies to CD45 and CD19. The same lymphocyte gate as seen in Figure 5.6.1i) was applied to all the flow cytometry plots of only CD45+ events. B lymphocytes were defined as all CD45+CD19+ events falling within the lymphocyte gate.

The percentage of the total events from each sample contained within the lymphocyte gate was similar between the groups. No significant differences in the B cell populations were seen between the groups (Table 5.6.3).

Table 5.6.3 B lymphocyte populations in induced sputum of AFD, COPD and healthy control subjects

Cell type	AFD	COPD	Healthy Control
	Median (range)		
n	8	6	5
% of CD45+ events within lymphocyte gate	1.94 (0.39 – 2.62)	1.95 (0.64 – 2.74)	1.47 (0.68 – 2.71)
B cell % of lymphocyte gate	0.37 (0.12 – 1.37)	0.71 (0.13 – 1.42)	0.33 (0.24 – 2.06)

5.7 Neutrophils and Monocytes/macrophages

Induced sputum samples from 19 AFD, 12 COPD and 6 healthy control subjects were stained with CD45, CD14 and CD15 antibodies to examine the neutrophil and monocyte/macrophage populations. From CD45 vs side scatter plots, CD45+ events were identified and used to delineate neutrophil and monocyte/macrophage populations.

Analysis of the flow plots revealed that the total percentage of the CD45+ cells within the groups was similar (Table 5.6.1). Neutrophil and monocyte/macrophage cell populations were expressed as a percentage of all the CD45+ events. Statistical analysis was performed using Mann Whitney U test for non-parametric data.

5.7.1 Neutrophil populations

Neutrophil populations were identified from the CD45+ cells by their strongly CD15+ properties (Figure 5.7.1ii). On statistical analysis neutrophil populations were not significantly different between the groups, with only trends towards significant differences between AFD group and healthy control groups with the percentage of neutrophils being lower in AFD (p = 0.09, power 66%, (Table 5.7.1).

5.7.2 Monocyte/macrophage populations

Monocyte/macrophages percentages were quantified by identification of CD14+ events after the subtraction of neutrophil and lymphocyte populations from the total CD45+ population, with the lymphocytes being identified by their position on the CD45 vs side scatter plots (Figure 5.7.1i) (259). In AFD subjects and COPD subjects the monocyte/macrophage population appeared to be the predominant cell population in induced sputum, though this was not the case for healthy controls where neutrophil and macrophage numbers appeared more even. However although monocyte/macrophage populations were not statistically significantly different between the groups, there was a trend towards significance between the AFD and healthy control groups (p = 0.07, power 79%, Table 5.7.1). Further differentiation of monocytes and macrophages was not

possible due to the degree of auto-fluorescence generated by alveolar macrophages.

Figure 5.7.1 Example flow cytometry cell plot for neutrophils and monocytes/macrophages in induced sputum

Induced sputum flow cytometry plot with CD45, CD15 staining used to identify i) CD45+ cells above line 44.2% of events on plot, and lymphocyte population (outlined) and ii) neutrophil population identified as CD45+CD15+ cells, here 65.6.1% of CD45+ population (outlined)
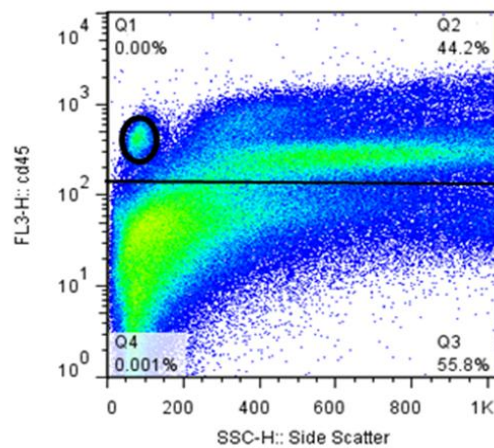
i)
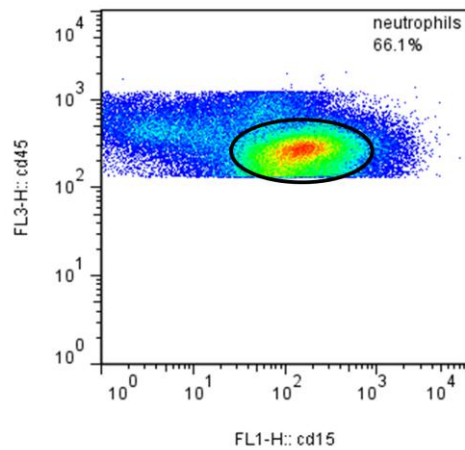
ii)

Table 5.7.1 Neutrophil and monocyte/macrophage populations in induced sputum from AFD, COPD and healthy control subjects

Cell type	AFD	COPD	Healthy control
	Median (range)		
n	19	12	6
CD45+ % of total events	47.1 (36.92– 76.44)	51.5 (26.7 – 74.5)	51.3 (37.3 – 62.5)
Neutrophil % of CD45+ events	21.7 (7.6 – 40.7)	18.3 (3.5 – 80.3)	43.3 (15.9 – 88.0)
Monocyte /macrophage % of CD45+ events	76.4 (53.1 – 88.2)	76.4 (17.3 – 95.6)	49.4 (11.0 – 80.9)

5.8 Sputum cell populations compared to blood

The percentage populations of lymphocytes, neutrophils and monocytes/macrophages in AFD subjects were compared between the induced sputum and peripheral blood. Peripheral blood differential white cell percentages were taken from the results of full blood count samples taken on the day of the AFD subjects study visit. Samples were processed by the Royal Free Hospital Heamatology Laboratory as per standard protocol.

Comparison of blood and sputum lecuocyte populations revealed that the predominant leucocyte population in peripheral blood was that of neutrophils, which were present at significantly higher percentage than that of induced sputum ($p < 0.0001$), whilst the percentage of lymphocytes in the peripheral blood was also significantly higher than that of sputum ($p < 0.0001$). However, the percentage monocyte population was found to be significantly higher in induced sputum samples compared to peripheral blood ($p < 0.0001$) (Table 5.8)

Table 5.8 Comparison of peripheral blood and induced sputum percentage lymphocyte, neutrophil and monocyte populations in AFD subjects

% population Median (range)	Peripheral blood	Induced sputum	p value
Lymphocytes	29.8 (19.2 – 40.8)	2.3 (0.5 – 7.3)	**<0.0001**
Neutrophils	60.8 (47.7 – 73.6)	21.7 (7.6 – 40.7)	**<0.0001**
Monocytes	7.0 (4.8 – 11.9)	76.4 (53.1 – 88.2)	**<0.0001**

5. 9 Airway Obstruction in AFD and cell populations

Airway obstruction was defined as FEV_1 /FVC <0.7. T cell populations in 10 AFD subjects with airway obstruction were compared to 5 AFD subjects without airways obstruction alongside the 12 COPD and 7 healthy control subjects.

5.9.1 Lymphocytes in relation to airflow obstruction

Comparison of T cell percentages revealed a trend towards higher percentages of T cells within the lymphocyte gate in AFD subjects with airway obstruction compared to AFD subjects without airway obstruction, though this did not reach significance (p=0.07, Table 5.9.1). No significant differences were seen in T cell populations between AFD subjects in either group and healthy controls. The COPD group had significantly higher T cell percentages than both the unobstructed AFD subjects and the healthy control group (p<0.05, Table 5.9.1).

On further analysis, the percentage of CD3+CD4+ T cells and CD3+CD8+ T cell were similar in each group, as were the CD4+/CD8+ ratios (Table 5.9.1) No significant differences were seen in the NKT cell population between the groups (Table 5.9.1).

5.9.2 Neutrophil and monocyte/macrophages in relation to airflow obstruction

Neutrophil and monocyte/macrophage populations from 10 AFD subjects with airway obstruction were compared to the 9 AFD subjects without airway obstruction alongside the COPD subjects and healthy control subjects. Comparison of the neutrophil and monocyte/macrophage populations between the groups did not reveal any significant differences (Table 5.9.2).

Table 5.9.1 T lymphocyte populations in relation to airflow obstruction

Group	AFD with airway obstruction	AFD without airway obstruction	COPD	Healthy control
	Median (range)			
n	10	5	12	7
FEV1/FVC Mean (+/- SD)	0.65* (+/- 0.04)	0.74 (+/- 0.04)	0.62* (+/- 0.05)	0.77 (+/- 0.09)
CD3+ T cells % lymphocyte gate	69.6 # (18.8 – 89.9)	30.9 (15.2 – 64.7)	74.5* (53.5 – 92.5)	40.1 (21.3 – 85.3)
CD3+CD4 + % of T cells	68.4 (30.8 – 80.5)	80.1 (59.1 – 86.1)	77.9 (27.0 – 93.1)	73.2 (39.8 – 86.8)
CD3+CD8+ % of T cells	31.6 (19.5 – 69.9)	19.9 (13.9 – 40.9)	22.10 (6.9 – 73.0)	26.8 (13.3 – 60.2)
CD4+/CD8+ T cell ratio	2.2 (0.4 – 4.1)	4.0 (1.1 – 9.2)	3.5 (0.4– 13.5)	2.7 (0.7 – 6.6)
NKT cell % of T cells	1.0 (0.7 – 3.0)	1.7 (0.8 – 5.3)	0.9 (0.1 – 3.0)	1.2 (0.1 – 2.2)

* $p < 0.05$ compared to AFD without obstruction and healthy controls, # $p = 0.07$ compared to AFD without airway obstruction

Table 5.9.2 Neutrophil and monocyte/macrophage populations in relation to airflow obstruction

Group	AFD with airway obstruction	AFD without airway obstruction	COPD	Healthy control
	Median (range)			
n	10	9	12	6
FEV1/FVC Mean(+/-SD)	0.65 (+/- 0.04)	0.76 (+/- 0.04)	0.62 (+/- 0.05)	0.77 (+/- 0.09)
Neutrophil % of CD45+ events	19.5 (7.64 – 34.9)	21.0 (0.58 – 40.7)	18.3 (3.47 – 80.3)	43.3 (15.9 – 88.0)
Monocyte / macrophage % of CD45+ events	74.3 (60.4 – 88.2)	76.71 (53.1 – 88.2)	76.35 (17.3 – 95.6)	49.38 (11.0 – 80.9)

5.10 Sputum Cytokine assays

Pro-inflammatory cytokine interleukin-8 (IL-8) and interleukin-6 (IL-6) concentrations were measured in sputum derived leucocyte lysate samples from 10 AFD subjects, 5 COPD subjects and 5 healthy control subjects using the procedure described in Methods Section 2.3.6. These groups were not matched at recruitment. Cytokine assays were performed using standard ELISA kits as described in Methods Section 2.3.10.

In the groups tested for cytokines as a whole, COPD subjects were significantly older than both the AFD subjects and healthy control subjects (Table 5.10.1). Lung function parameters for both large and small airways including FEV_1 /FVC, FEV_1, RV/TLC and MEF_{50} % predicted were all significantly lower in the COPD group compared to both the AFD group as a whole and the healthy control group (Table 5.10.1). There were no differences in lung function parameters between the AFD group and the healthy control group.

5.10.1 Induced sputum leucocyte lysate IL-8 and IL-6 concentrations in AFD, COPD and healthy Control subjects

Induced sputum leucocyte lysate IL-8 concentration was found to be significantly higher in both AFD subjects and COPD subjects compared to healthy controls (p<0.01, Table 5.10.1). No significant differences were seen in IL-6 concentrations between the AFD, COPD and healthy control groups (Table 5.10.1).

Table 5.10.1 Sputum IL-8 and IL-6 concentrations in AFD, COPD and Healthy Control subjects

Parameter Mean (+/- SD)	AFD	COPD	Healthy Control
n	10	5	5
Age [years]	47.2* (+/- 15.5)	68.1 (+/- 10.9)	42.2*** (+/- 8.6)
Pack year smoking history **Median (range)**	1.0* (0 – 20.0)	17.1 (0 – 75.0)	0 (0 – 16.0)
FEV_1/FVC	0.73** (+/- 0.07)	0.59 (+/- 0.06)	0.75** (+/- 0.08)
FEV_1 %predicted	92.0* (+/- 12.6)	78.6 (+/- 10.4)	103.2* (+/- 13.5)
RV/TLC	81.2** (+/- 12.0)	107.4 (+/- 17.9)	85.8** (+/- 6.9)
MEF 50 % predicted	60.8* (+/- 19.8)	30.1 (+/- 17.7)	72.1* (+/- 23.1)
Sputum leucocyte lysate IL-8 pg/ml/mg protein **Median (range)**	1764 # (519.6 – 11,409)	1166 # (334.4 – 9764)	200.6 (94.3 – 333.1)
Sputum leucocyte lysate IL-6 pg/ml/mg protein **Median (range)**	21.4 (6.2 – 132.4)	15.1 (4.8 –19.5)	13.8 (9.6 –28.2)

* $p<0.05$, ** $p<0.01$, *** $p<0.005$ compared to COPD group

# $p<0.01$ compared to healthy control group

5.10.2 Induced sputum leucocyte lysate IL-8 and IL-6 concentration in relation to airway obstruction

Of the 10 AFD subjects in this group, 4 had evidence of airway obstruction as defined by $FEV_1/FVC < 0.7$. However, the differences in lung function parameters between the AFD group with and without obstruction did not reach statistical significance (Table 5.10.2). The COPD group had significantly lower FEV_1/FVC, RV/TLC and MEF_{50} %predicted compared to the unobstructed AFD group and healthy controls, and a significantly lower FEV_1 %predicted compared to healthy controls (Table 5.10.2).

The sputum leucocyte lysate IL-8 concentration was found to be significantly higher in both the obstructed and unobstructed AFD groups and the COPD group compared to healthy controls ($p < 0.05$ for AFD obstructed and COPD, $p < 0.01$ for AFD unobstructed, Table 5.10.2). IL-6 concentrations were not found to be significantly different between any of the groups (Table 5.10.2).

Analysis of IL-8 and IL-6 concentration in sputum leucocyte lysate of AFD subjects in relation to FEV_1/FVC as continuous variables did not reveal any significant correlations (not shown).

Table 5.10.2 Sputum IL-8 and IL-6 concentration in leucocyte cell lysate in relation to airflow obstruction

Parameter median (range)	AFD obstructed	AFD unobstructed	COPD	Healthy control
n	4	6	5	5
FEV$_1$/FVC	0.67 (+/- 0.03)	**0.78*** **(+/- 0.05)**	0.58 (+/- 0.06)	**0.75*** **(+/- 0.08)**
FEV$_1$ % predicted	86.5 (+/- 9.5)	95.7 (+/- 13.9)	78.6 (+/- 10.4)	**103.2*** **(+/- 13.5)**
RV/TLC	92.0 (+/- 10.9)	**74.1*** **(+/- 5.8)**	107.4 (+/- 17.9)	**85.8*** **(+/- 6.9)**
MEF$_{50}$% predicted	45.8 (+/- 6.9)	**70.8*** **(+/- 19.4)**	30.6 (+/- 17.7)	**72.1*** **(+/- 23.1)**
Sputum leucocyte lysate IL-8 pg/ml/mg protein Median (range)	**761.0*** **(585.3 – 2341)**	**2497**** **(519.6 - 11,409)**	**1166*** **(334.4 – 9764)**	200.6 (94.3 – 333.1)
Sputum leucocyte lysate IL-6 pg/ml/mg protein Median (range)	6.7 (6.2 – 37.7)	23.5 (7.7 – 132.4)	15.1 (4.8 – 19.5)	13.8 (9.6 – 28.2)

*p<0.05 compared to COPD

**p<0.01 compared to healthy control

5.10.3 Influence of smoking history on induced sputum IL-8 and IL-6 concentration in AFD

Of the AFD subjects, only 2 had a smoking history of greater than 10 pack years, and none were current smokers. There was a significant difference between sputum leucocyte lysate IL-8 concentration in the 8 AFD subjects with <10 pack years of smoking compared to the 5 healthy controls; and also between the 5 COPD subjects and healthy controls, with the lowest values found in the healthy control group.

There were no significant differences in sputum IL-6 concentration in AFD subjects with greater than or less than 10 pack years of smoking compared to each other or to COPD or healthy control subjects.

Table 5.10.3 Induced sputum cell lysate IL-8 and IL-6 concentration in relation to smoking history

Sputum leucocyte lysate Median (range)	AFD >10 pack years	AFD <10 pack years	COPD	Healthy controls
n	2	8	5	5
IL-8 pg/ml/mg protein	1431 (519.6 – 2341)	**1764*** (677.2 – 5176)**	**1166** (593.8 – 5736)**	200.6 (95.26 – 331.8)
IL-6 pg/ml/mg protein	7.04 (6.35 – 7.72)	23.45 (10.49 – 83.54)	15.09 (8.35 – 18.79)	13.81 (9.72 – 21.44)

** $p < 0.05$, *** $p < 0.005$ compared to healthy controls

5.11 Discussion

Induced sputum sampling provides an additional method for examining the lungs that is non-invasive, tolerable and repeatable. In the context of Anderson Fabry Disease, with a group of patients who undergo multiple regular routine investigations of varying complexity already, it provided a suitable, straightforward alternative to more invasive methods of sampling the lung, such as bronchoscopy. In light of what we know about the changes in the lungs in AFD thus far being mainly changes that are airway related, the origin of induced sputum samples was also well suited to this purpose.

Samples obtained for the investigation of specific cell populations in each group were generally adequate in terms of cell yield. The number of events captured from each sample in the different groups was similar overall. However, for the purposes of examining the lymphocyte population, it was important that enough events within the set lymphocyte gate were captured in order to enable further dissection of the lymphocyte populations present. This resulted in 3 of the AFD samples obtained being unsuitable for use for this purpose.

Examination of the lymphocyte populations in the induced sputum of AFD subjects compared to healthy control subjects did not reveal any significant differences in T cell populations in the group as a whole. The COPD subjects demonstrated a higher percentage of T cells overall in induced sputum compared to AFD subjects and healthy controls. However, AFD subjects with airways obstruction appeared to have a higher percentage of T cells in induced sputum when compared to AFD subjects without airway obstruction though this did not reach statistical significance,

Further examination of T cell subsets did not reveal any differences between the CD4+ and CD8+ T cell percentages between the three groups. It has been previously shown by others that there may be a predominance of CD8+ T cells in the sputum of COPD subjects (260), but this did not appear to be the case in our cohort. The relatively small sample size may be a factor here; and with further sampling, differences in T cell subsets in the AFD subjects may become

apparent. An additional explanation for this is that the COPD subjects recruited for this study were from a COPD study cohort who are carefully and optimally monitored and managed and therefore may not be entirely representative of a typical COPD population. It was also ensured that all COPD subjects were sampled at baseline, at least 6 weeks away from their last exacerbation, which may also have influenced the sputum cell profiles obtained.

Examination of NKT cell populations did not reveal any differences between the groups, with the percentage of NKT cells in each of the groups being similar and small in number (0.94 -1.6% of the lymphocyte gate). Given this, it is likely that identification of specific invariant NKT cells would have been difficult. It would however be of interest to try and explore changes in iNKT cell populations at a cellular level within the lung, given the documentation of accumulated storage material within lung derived cells in previous studies (261).

Assessment of B lymphocyte populations did not reveal any differences between AFD subjects and healthy controls or COPD subjects. This would be consistent with the evidence that AFD has not been shown to particularly affect B cell populations. In contrast, there has been some suggestion that B cells may be relevant in the pathophysiology of COPD, with findings of increased B cell numbers in bronchial biopsies (262).

Overall, from the examination of lymphocyte populations in these groups there is some evidence to suggest that changes in lymphocyte cell numbers warrant further investigation in the context of lung involvement in AFD, and that changes seen in COPD airways may be relevant. Overlap with pathophysiology of COPD may guide potential treatments of value to AFD subjects who are symptomatic as a result of their pulmonary involvement.

The examination of neutrophil populations did not reveal any significant differences between AFD, COPD and healthy control subjects. However, on calculating the power of this part of the study, it is apparent that it was under-powered with the sample size recruited. From this analysis the healthy control group had the highest percentage of neutrophils in their induced sputum at

43.3%. This is similar to other studies in healthy populations (263;264). In comparison, the AFD subjects had lower percentages of neutrophils, coming close to statistical significance (p=0.09), though with no difference between AFD subjects with and without airway obstruction, implying that perhaps in AFD neutrophilic inflammation does not play a big part in the underlying pathophysiology.

For monocytes/macrophage populations there was a strong suggestion that the percentage populations in AFD and COPD subjects were greater than that of healthy control subjects (p=0.07). Here, too, larger numbers of study subjects may help to clarify this point. The median percentage macrophage population of 49.3% in healthy control subjects is not dissimilar to previous studies examining induced sputum cell populations (265;266), though was slightly lower than seen in others (267).

Comparison of sputum populations with those seen in the peripheral blood revealed significant differences, with as expected the percentages of lymphocytes and neutrophils much higher in blood compared to sputum, and that of monocytes significantly higher in sputum. This finding supports the idea that the differences demonstrated in induced sputum leucocyte and peripheral blood leucocyte α-galactosidase A activity may be due to differences in the leucocyte populations present.

The most significant limitation to this aspect of the study is the number of samples obtained from study subjects for the purpose of examining cell populations. This was limited by the yield from one sputum induction procedure being insufficient to consistently provide enough cells for the purposes of both enzyme activity assessment and examination of cell populations. Therefore subjects had to be sampled on consecutive study visits to achieve both. In addition to this, the yield from induced sputum sampling from subjects who are largely non-producers of sputum, such as healthy controls and AFD subjects, was on occasion insufficiently low in cell number for the population under

investigation, and therefore not suitable for use. In practice this was only the case in the lymphocyte plots in this study.

Analysis of cytokines in the induced sputum of AFD subjects revealed elevated concentrations of IL-8 when compared to healthy control subjects. This increase was present in both AFD subjects with and those without airway obstruction, suggesting that the trigger for this is related to AFD. Given that IL-8 is produced by alveolar macrophages, this is also in keeping with our finding that AFD subjects had a greater percentage population of macrophages in induced sputum compared to healthy controls. The same was true for the COPD group in this study, where IL-8 concentrations were also greater than in healthy controls, (as were increases in monocyte/macrophage populations). No difference was seen in IL-6 concentration between the groups.

The role of cytokines in the pathophysiology of AFD is as yet unclear, though there are suggestions that cytokines may play a role in the presentation and severity of changes seen in AFD (268). One study has raised the question as to whether genetic polymorphisms of IL-6 may influence the severity of ischaemic events in AFD subjects (269).

5.12 Conclusions

The new findings from this chapter can be summarised in the following points:

1. No significant differences were seen in T lymphocyte populations or T cell subsets between AFD subjects and healthy controls

2. There was a suggestion that AFD subjects with airway obstruction have a higher percentage of T lymphocytes in induced sputum compared to AFD subjects without airway obstruction, as noted in the COPD group

3. No significant differences were seen in neutrophil populations between AFD and healthy control subjects

4. There was the suggestion of increased monocyte/macrophage percentages in the induced sputum of AFD subjects and COPD subjects compared to healthy controls

5. There was an elevated concentration of interleukin-8 in the induced sputum of AFD subjects compared to healthy controls

Chapter 6 – Discussion

6.1 Summary of study findings

This study has demonstrated that both respiratory symptoms and airways disease are common in patients with Anderson Fabry Disease. We have shown that it is generally mild in nature, and has features of small airways airflow limitation on lung function testing. However, we have also found evidence of more marked airway involvement in almost half the AFD subjects recruited. This was most frequent in males though was also present in a significant proportion of females (32%). We have demonstrated that the majority of this airflow limitation appears to be fixed, with little reversibility to bronchodilators, and is not seen to progress significantly over a 12 month period. We did not find any specific changes in measures of diffusion capacity in AFD subjects, other than in the context of a significant smoking history. In addition we did not demonstrate any consistent radiological changes in AFD subjects on CXR or CT scanning of the chest.

Our study showed that AFD subjects with worse overall disease as measured by MSSI were more likely to suffer from pulmonary involvement, and demonstrated weak negative correlations between both MSSI and Age adjusted Severity Score with airway obstruction. We have also demonstrated that the changes in airflow limitation seen in our cohort of AFD subjects could not be explained by smoking alone, with subjects who had minimal smoking history showing significant evidence of airway obstruction compared to healthy controls. We did not reveal any differences in lung function in AFD subjects on ERT compared to those who were naïve of ERT. Comparison of lung function data of some of the more frequent genotypes in this cohort indicated that the most obstructed subjects had the genotype R227X, whilst the least obstructed had an exon 1 deletion, though the numbers of subjects were too small to analyse this statistically for the purpose of exploring genotype-phenotype correlation.

In our study we established a method for measuring α-galactosidase A activity in induced sputum samples, and showed that activity of the enzyme was low in AFD males compared to controls and AFD females. We demonstrated that

for the most part, α-galactosidase A activity was greater in the sputum than in the blood in both AFD and non-AFD subjects, apart from in the plasma and fluid phase of the sputum where the opposite appeared to be true in non-AFD subjects only.

Measurements of α-galactosidase A activity in blood or sputum were found to be no different in AFD subjects on ERT when compared to those who were enzyme naïve. In addition, we found no differences in sputum enzyme activity in subjects with mild compared to more severe disease as measured by an MSSI of less or more than 20. Whilst we were able to demonstrate that plasma and blood leucocyte α-galactosidase A activity was significantly lower in AFD subjects with airway obstruction compared to those without, sputum α-galactosidase A activity was also lower in this group but this did not reach statistical significance (p=0.08).

Examination of induced sputum samples from one AFD male subject out of 3 examined revealed the presence of inclusion bodies in keeping with those reported previously in AFD subjects (270), indicating detectable storage material in sputum derived cells.

This study also sought to investigate cell populations in the induced sputum of AFD subjects, and demonstrated that the predominant cell population in the induced sputum of AFD subjects was monocytes and macrophages followed by neutrophils, in similar percentages to that seen in the COPD subjects in this study. We did not find any differences in the populations of these cells in AFD subjects with airways disease compared to those without.

Examination of populations of T lymphocytes in AFD subjects compared to healthy controls revealed no difference. T lymphocytes were proportionally higher in the sputum of COPD subjects compared to both AFD subjects and controls. On examining sputum T lymphocyte populations in AFD subjects with airway obstruction there was a suggestion that this population was greater than that seen in sputum from AFD subjects without airway obstruction, though this did not reach statistical significance (p=0.07). No differences in the populations

of T cell CD4+ and CD8+ subsets were found between AFD, COPD and healthy control subjects. No differences in NKT cell populations were revealed between the groups.

On examining cytokines in the induced sputum from AFD subjects we found elevated concentrations of IL-8 in sputum in AFD subjects, both in those with and without airway obstruction, compared to healthy controls. COPD subjects also had demonstrably high IL-8 concentrations in comparison to healthy controls. No differences were noted in IL-6 concentrations between the groups studied.

6.2 Possible Model of AFD lung disease

From what is known about AFD as a whole, and from the limited evidence that has been gathered regarding how AFD affects the lungs, one could propose the following model of AFD pathophysiology in the lungs:

As in other organ systems, there is a gradually increasing accumulation of substrate throughout the lung in AFD over time. This is deposited in airway wall and in the airway and vascular epithelium as well as in pneumocytes, as has been demonstrated on bronchoscopy and lung biopsy samples (271;272). The impact of this is first noted in testing the small airways as they are of the smallest calibre and contribute most to airway resistance (273). The deposition and accumulation of Gb3 results in hypertrophy of the smooth muscle in the airway wall, as seen in the smooth muscle of the vascular wall in AFD vasculopathy (274) which triggers an ongoing low grade inflammatory process, in turn driving further immunopathology, including pro-inflammatory cytokines (275). At this stage in the presence of airway smooth muscle hypertrophy, changes in airway calibre are reversible and there is a measurable response to bronchodilators. However, with ongoing tissue activity, local cell death and necrosis from sphingolipid-driven apoptosis and abnormalities in iNKT cells and lipid antigen processing (276;277) plus cytokine production, more established and irreversible change in the airway

wall develop such as peribronchial and peribronchiolar fibrosis. These have been reported in open lung biopsy samples from an AFD patient with significant respiratory symptoms (278). Over time, with the ongoing accumulation of substrate and the responses that this triggers, this process reaches a threshold where its effects becomes evident in the larger calibre airways also, thereby being detectable on lung function testing. The slow, chronicity of the process means that it presents in older AFD subjects, and correlates with other factors that would indicate worsening disease.

Whilst some of the work from this study has provided support for this proposed model, there remain gaps which require further investigation to determine just how closely this reflects the pathophysiology of AFD lung involvement.

6.3 Changes seen with pulmonary involvement

6.3.1 Small airways

The findings from this study and from the majority of the previous literature on pulmonary involvement in Anderson Fabry Disease, point towards lung pathology that is mainly airway-related. We documented in our cohort mild airway obstruction, with widespread changes in particular in the calibre of the smaller airways affecting 80% of the subjects. More global measures of both large and small airways involvement were present in almost half of those assessed.

It is recognized that the assessment of small airway pathology is difficult using spirometry given the degree of variability that can occur with measures such as MEF_{25-75} (279;280). However, on the basis of these findings it is important to investigate this further in AFD. It has been documented in respiratory diseases such as cystic fibrosis that early changes occur in the smaller airways, with considerable damage often present at this site prior to a detectable fall in FEV_1 (281). In view of this there has been some focus recently on the use of lung

clearance index (LCI), a technique based on multi-breath washout of tracer, to examine small airway function in more detail and to detect early changes in the airways (282). This may be useful in AFD to test out the hypothesis, to ascertain the extent of changes in airway calibre more accurately, and to document its possible progression.

6.3.2 Progression of airway involvement

Our study findings support the concept that gradual progression of lung involvement occurs over time, involving the small airways first before affecting the larger caliber airways. Those subjects with lower FEV_1/FVC <0.7 were older, though this did not reach significance in the number of subjects investigated here. This is in line with previous work (283). Whilst we did not demonstrate changes in lung function parameters over time, it is acknowledged that the period of 12 months in the context of AFD is perhaps not long enough to assess this accurately. With more sensitive measures of airway function such as LCI, it is possible that even small but genuine changes may be detected. If this were the case it may assist with determining the rate of change of lung function over time, and enable one to predict individual projection of lung function decline. Any alterations to this in subjects subsequently commenced on ERT would also be of interest.

6.3.3 Reversibility

Of note in AFD is the lack of reversibility, for the most part, to bronchodilators. This suggests that the airway obstruction in AFD is largely fixed. Considering the model outlined above, this irreversibility may result from peribronchial fibrosis. If this were the case it would raise the question whether ERT if started early, may prevent the development of fixed airway obstruction in AFD through reducing substrate accumulation and thus limiting the process that results in fibrotic change.

Similar concepts have been postulated in heart disease, where autopsy findings of AFD subjects diagnosed relatively late, but who had received ERT, showed ongoing evidence of substrate within the myocardium and significant

fibrosis (284). It was suggested that once established fibrotic change is present, the comparative effectiveness of ERT on clearing substrate from the myocardium may be diminished (285). This theory is supported by studies involving serial myocardial biopsies to examine the effectiveness of ERT in clearing substrate from the heart, which found that although there is clearance of Gb3 from the coronary vasculature, persistence of substrate in cardiomyocytes is seen (286). This implies that the persistence of substrate in muscle cells can result in fibrosis. In the kidney, differences in the degree of substrate clearance from different cell types has also been reported on serial biopsy findings after receiving ERT for less than a year. Substrate persists predominantly in podocytes and the distal tubular epithelium (287).

From a clinical perspective one might question whether AFD subjects with airway obstruction would benefit from regular inhaled bronchodilator therapy for symptoms of cough and wheeze. This warrants further investigation using trials of treatment and validated respiratory questionnaires to assess the efficacy of such drugs.

6.3.4 Radiology

The physiological changes present in the airway were not demonstrable on radiological examination, despite the use of CT imaging protocols that should highlight changes in small airways, where the most widespread changes were seen. The knowledge that plain CXRs are likely to yield as much information in most cases as a CT scan is helpful in that it may avoid unnecessary radiation in a patient group who already acquire a significant cumulative exposure to radiation, as they undergo a large number of radiological investigations. However, for the purposes of further exploration into the airway changes seen in AFD, recent studies in airways disease have used new advances in CT imaging techniques and software to examine the airway wall, providing detailed assessments of airway dimensions and wall thickness, and their relationship to airflow limitation (288-290). In AFD the use of CT imaging may help to assess the degree of smooth muscle hypertrophy in the airway wall and to compare the pattern of airflow limitation in more detail to that seen airways disease.

6.4 Pattern of disease in the lungs

6.4.1 Gender

As with other aspects of AFD, we showed that lung involvement in AFD was worse in males, though spirometric abnormalities were also evident in females. Interestingly there was no difference in the average age of male and female AFD subjects with airway obstruction. One might have expected the females with evidence of lung involvement to have been older, as is the case in other AFD pathology (291). This raises the question whether other factors such as smoking are relevant. In our study the majority of spirometric changes appeared to be independent of smoking, though it did impact on diffusion capacity in AFD subjects with >10 pack years of smoking. Indeed, the results of the multivariable analysis suggest that smoking was not associated with FEV_1/FVC and MEF_{50} %predicted. AFD itself appeared to have little effect on diffusion capacity.

6.4.2 Disease severity

Some of the data from our study suggested that AFD subjects with worse generalised systemic disease as measured by MSSI, were more likely to have evidence of airway obstruction. This would also fit with our proposed model, in that changes in the lung are related to the same factors influencing severity of disease in other organs in AFD. When other possible confounders were controlled for within a multivariable analysis, however, the effect of MSSI on FEV_1/FVC did not reach statistical significance - even though the data suggest that with larger numbers of subjects this may have been the case. From a clinical perspective, this has relevance in the context of symptoms and well being, as it is the AFD subjects already suffering from the most marked disease who are likely to also be affected by the respiratory component of AFD. Obviously in the case of significant cardiac involvement symptoms such as shortness of breath and reductions in exercise tolerance may be compounded; though this highlights the need for further investigation into the response of such symptoms to respiratory interventions.

6.4.3 Genotype

As with other aspects of AFD, it was not possible to establish any firm association between genotype and lung involvement in AFD. It has been previously noted in AFD that relationships do exist between phenotype and the residual enzyme activity associated with different types of mutations, particularly in renal disease where the presence of detectable residual α-galactosidase A activity is linked to later onset renal disease, lower Gb3 content in renal cells and lower scores for histological damage (292). However we did not find any relationship between residual enzyme activity in plasma and airway obstruction, measured by FEV_1/FVC or MEF_{50} %predicted.

6.5 α-galactosidase A activity

6.5.1 Induced sputum enzyme activity

In our investigation into α-galactosidase A activity in the lung we were able to determine that one can measure enzyme activity in lung derived samples using similar methods to that in blood. The pattern in sputum, as hypothesised, was lower in AFD males than control males and AFD females. This was seen to be true for both the fluid phase and the cellular phase of the sputum.

In the context of our hypothesed model of lung involvement in AFD, this pattern of α-galactosidase A activity would be consistent, as it is the same group of subjects with low enzyme activity compared to controls who demonstrate lung involvement, which would have occurred as a result of greater Gb3 accumulation in the airways and the processes that this triggers.

6.5.2 Induced sputum and blood enzyme activity

In paired samples from the same subject α-galactosidase A activity in induced sputum in both controls and AFD subjects was higher than in blood, with the exception of the sputum supernatant compared to the plasma in non-AFD controls. It is possible that this may in part explain why the lungs in AFD are not a major cause of morbidity; and why overall pulmonary involvement is not a prominent feature of AFD presentation.

The finding that α-galactosidase A activity in induced sputum leucocytes was greater than that of blood leucocytes in the control population as well as in the AFD population was interesting as it suggests that this may be related to differences in the composition of lung derived samples compared to blood. In peripheral blood, the predominant white cell populations are generally neutrophils, followed by lymphocytes and then monocytes, which was shown to be true for our cohort by their differential white cell counts. In induced sputum, however, there is evidence to suggest that the main cell populations from healthy subjects are macrophages and neutrophils (293;294). This was verified in our study, with the monocyte/macrophage population predominant in sputum samples from all the groups, followed by neutrophils, both of which were significantly higher than the percentage populations in blood. In AFD there has been work showing that α-galactosidase A activity varies between different leucocyte cell types, being greatest in monocytes, Thus the presence of a predominant macrophage population in the sputum of AFD and healthy control subjects may explain the difference in enzyme activity seen (295).

Cell populations from bronchoalveolar lavage samples have been shown to differ from induced sputum, with the finding of much lower numbers of neutrophils in BAL (296). This raises the question whether the induced sputum process itself contributes to airway neutrophil recruitment. To examine the enzyme changes in the lung in AFD further, it may be useful to analyse BAL samples and determine the relationship between cell populations and α-galactosidase A activity. Although the majority of changes so far evident in the lung in AFD have been related to the airways rather than at alveolar level, storage material has also been demonstrated in pneumocytes (226;297), therefore this may be of great relevance.

α-galactosidase A activity has been quantified in a number of different sites in subjects with Anderson Fabry Disease, including liver, kidney, skin as well as blood and tears. We have been able to demonstrate activity from induced sputum samples, which in the main comprise lung derived leucocytes. However it is not known what is the enzyme activity in other cell types derived from the

lung, and whether this differs. This would clearly be relevant to further lung based work.

It was notable that the range for sputum leucocyte-derived enzyme activity was wide in both controls and AFD subjects, with the highest measures in sputum being much greater than those in blood. Whilst this again may result from the different cell populations present in sputum, it could also reflect the quality of the sample acquired. As, whilst all the samples processed for the purpose of measuring α-galactosidase A activity had a minimum of 1x10^6 leucocytes with a cell viability of >80%, some samples may have been more cellular than others. Furthermore the extent of salivary contamination differed from sample to sample, though all samples contained <20% squamous cells. Sampling the lung by bronchoscopy may be one way of avoiding salivary contamination, though this is more invasive.

6.5.3 Enzyme replacement therapy

It was difficult to assess the impact of ERT on the lung from this study, as the majority of subjects were already receiving it, resulting in a small enzyme naïve population. There were no significant differences noted in the sputum or blood enzyme activity of AFD subjects who were receiving ERT and those who were not. This is most likely due to the pharmacokinetcs of ERT, which have demonstrated that at doses similar to that administered for treatment over a 2 hour infusion period, 80% of peak concentration was 60 minutes into the infusion, and plasma concentration fell back to half peak 15 minutes after the infusion had been completed (298).

It is difficult to estimate penetration of ERT into the lung. Dosing studies in a mouse model prior to the clinical trials of ERT did demonstrate some recovery of human α galactosidase A glycoforms from the lungs after intravenous administration of enzyme, though at lower levels than was evident from the liver, heart and kidneys (299). However there has been no demonstration of Gb3 clearance from the lung with ERT, as shown in the liver, kidney, heart and skin (300), though such examination may be helpful.

The lack of difference in sputum enzyme activity between those on ERT and those who are enzyme naïve may also reflect higher residual α-galactosidase A activity in enzyme naïve subjects. However, when tested in blood no significant difference between the two groups was found. In our study almost all AFD subjects on ERT were receiving fortnightly Replagal®, with only 3 using Fabrazyme®. It is therefore not possible to comment on the effect of different enzyme preparations when interpreting these results, though clearly this and the differences in dosing may be relevant.

6.6 Sputum cell populations and cytokines

6.6.1 Lymphocytes

The examination of cell populations by flow cytometry suggest that further investigation into lymphocyte populations may of value in AFD subjects. AFD subjects with airway obstruction seemed to have a higher T cell percentage in their sputum than those without documented airway obstruction, though this did not reach statistical significance (p=0.07, power 72%). This is similar to that seen in the COPD control group. It may be proposed therefore that with a larger sample size a significant difference in T lymphocyte populations may be evident between AFD subjects with and without airways disease.

The finding of changes in CD3+ T cell population has been demonstrated in AFD mouse models by Balreira et al who reported lower numbers of hepatic T cells in the liver of Fabry mice, and were able to demonstrate that this was largely due to reduced numbers of CD3^{int} T relative to CD3^{high} T cells, and a subsequent reduction in the percentage CD3+CD4+ population (301). There is therefore an implication that T cells may play a part in the cascade that occurs as a result of the presence of lipid antigen.

In airways disease it has been shown that the percentage lymphocyte population present in bronchoalveolar lavage is significantly greater than that of induced sputum from both COPD and control subjects (302). This may therefore

be one way of examining the influence of lymphocytes in the AFD lung changes in greater detail. Sampling the lung in this way would potentially allow further examination of the lymphocyte subsets present, as with larger numbers of lymphocytes it would be easier to delineate CD4+ and CD8+ T cell populations.

6.6.2 NKT cells

In our study we did not demonstrate any difference in NKT cell populations in the induced sputum of AFD subjects compared to COPD subjects or healthy controls. However, when examining this population it must be acknowledged that the number of cells being analysed becomes very small, and that therefore results must be interpreted in light of this. By the same token it would have been unfeasible to further examine the flow cytometry plots for an iNKT cell population using CD markers for the invariant chain as these are likely to be an even smaller subset on the overall T cell population.

Given its higher yield of lymphocytes, sampling by BAL may allow better assessment of the invariant NKT cell population. This would be of interest given the anomalies in iNKT cells that have been described in regards to AFD, with reports of reduced numbers due to impaired thymic selection (133). Studies have been performed in airways disease to examine the prevalence of iNKT cells in BAL samples and shown that iNKT cells are present in low numbers in COPD, asthma and control subjects (303). In addition, peripheral blood iNKT cell percentages have been found to be low in COPD subjects, with further reductions seen at exacerbation (304).

6.6.3 Monocytes/macrophages

We found that the predominant cell population in the induced sputum of AFD subjects was the monocytes/macrophage population. This was also the case in our COPD and healthy control cohorts, though there was perhaps a lower percentage in the healthy control group. In COPD, increased numbers of alveolar macrophages are of significance in the pathophysiology of the inflammatory process due to their production of cytokines and chemokines, including IL-8, particularly on activation by cigarette smoke (305;306). Given that our data point

towards increased numbers of monocyte/macrophages in AFD sputum, this may be of relevance as it has been suggested that the impact of smoking on the lungs in AFD is disproportionate to smoking history alone (307).

Macrophage numbers have also been shown to correlate with worsening lung function in COPD (308). Here, no difference was seen in monocyte/macrophage populations between the AFD subjects with and without airways disease, suggesting that the cause for a higher proportion of macrophages in sputum may be inherent to AFD. Again the examination of induced sputum in more subjects in both groups is necessary to determine any differences in the AFD populations. Longitudinal follow up would allow us to assess whether subjects with higher macrophage numbers were more likely to develop airway obstruction over time.

6.6.4 Neutrophils

The neutrophil populations in induced sputum were also similar between the groups. However, when calculating the power of this part of the study to detect a difference, it is apparent that it was under-powered with the sample size recruited. Sputum neutrophilia has been documented in COPD; with increasing numbers found to correlate with decline in lung function (309;310). The recruitment of neutrophils to the airways in COPD has also been related to current smoking and exacerbations (311;312). In our COPD cohort the low percentages of neutrophils may be explained by the mild degree of airway obstruction recruited to the cohort, with the subjects being GOLD Stage I or II only, with no current smokers and sampling being performed in a stable state outside of an exacerbation.

An additional limitation when delineating sputum populations of monocytes and macrophages is the autofluorescence associated in particular with alveolar macrophages. This is a common problem, and is present with several CD antibody markers (313;314). Proposed methods to overcome this difficulty include using alternative antibodies. We did not pursue this here as we

felt it was beyond our particular study where the focus was the relative size of the populations (315;316).

6.6.5 Cytokines

The sputum cytokine measurements performed in our study revealed elevated concentrations of IL-8 in AFD and COPD subjects when compared to healthy controls, but no difference in IL-6 concentrations. Elevated concentrations of IL-8 have been demonstrated in the sputum in the sputum of both subjects with COPD and asthma in the past, and shown to have a negative correlation with FEV_1/FVC in COPD subjects (317). IL-8 is associated with neutrophil activation and chemotaxis, and has been shown to be elevated in smokers compared to non-smokers (318), though we did not see any difference in neutrophil populations between our groups. In our study it was difficult to draw any conclusions regarding the influence of smoking on the IL-8 concentrations in AFD as numbers were small. However there appeared to be a trend towards a significant difference in IL-8 in AFD subjects with >10 pack years of smoking compared to healthy controls (p=0.09, power 52%), suggesting that more data may be required to examine this.

IL-8 may be relevant, regardless of smoking, in that sputum concentrations were elevated in non-obstructed AFD subjects as well as those with airway obstruction. It is important to note that there are significant concentrations of IL-8 present in saliva, and this could have influenced our findings. The effect of this should have been minimized by the sputum processing method, which sought to minimise salivary contamination of the samples. Given that all the samples from each of the groups were obtained and processed in the same way, this should not be a significant factor. However sampling by BAL may further bypass salivary contamination and so help to clarify this.

Previous studies in AFD have demonstrated elevated plasma concentrations of IL-10, IL-1β and IL-1α (319). We did not, in our study measure concentrations of cytokines in paired lung and plasma samples, though this may

be of some value in determining whether IL-8 has a possible role in systemic AFD, or whether this finding is only evident in the lungs.

6.7 Clinical relevance

From a clinical perspective our study demonstrates that greater emphasis should be placed on respiratory aspects of AFD, as symptoms are common, and more frequent in those with more severe overall disease, in males and smokers. It is possible that symptoms including shortness of breath and reductions in exercise tolerance are being attributed solely to cardiac involvement, whereas there may be a significant contribution from the lungs in some cases. Certainly smoking cessation should be emphasized.

Given that there appears to be some overlap with airways diseases such as COPD, further investigation into the benefit of airway treatments such as inhalers is warranted to assess whether these might have a role in improving symptoms. Although little reversibility to bronchodilators was evident, one could propose that inhaled corticosteroids may be of potential benefit if the basis of the changes seen are inflammatory and due to processes affecting the bronchial epithelium and airway wall. When considering this it would be important to remember the distribution of the airways abnormalities noted in AFD, with the small airways perhaps being the largest contributing component. Therefore the choice of agents and delivery device used should allow particles sized small enough to reach this part of the lungs (320).

Certainly it may be also important to consider the choice of drugs used in the treatment of cardiac aspects of AFD, which may impact on the airways in a detrimental manner in subjects with possible reversible airway obstruction, such as β-blockers.

The role of ERT in respiratory aspects of AFD requires further evaluation. Whilst it is unlikely that respiratory involvement per se in AFD would commonly drive the need to commence ERT, it is possible that in the long term it may have a role in preventing the progression of airflow limitation becoming clinically

relevant. This may be helpful given that it appears to be those with overall worse disease severity who are more likely to have airway obstruction as a result of AFD.

6.8 Limitations

6.8.1 Numbers and demographics of subjects

As with all studies investigating rare diseases, one of the drawbacks of this work is the limited number of study subjects assessed. A total of 45 AFD patients were prospectively recruited, as described in Methods Section 2.2.1. They were therefore not subject to selection bias based on symptoms or signs suggestive of respiratory involvement but were a pragmatically-recruited group attending our service.

Given that all study subjects were clinically assessed and all sampling and processing was performed by the same operator, the number of subjects seen per day was limited by the practicalities of this in the time available. Study subjects often lived some distance from the hospital and were therefore only able to perform study investigations at their scheduled 6 monthly appointment. Hence all investigations had to be co-ordinated to have minimal impact on their clinical care.

The COPD cohort recruited consisted of 14 subjects matched to AFD subjects with evidence of airflow obstruction first by gender and then GOLD stage. The COPD control population was significantly older than the AFD subjects; however, as lung function data were reported and compared adjusted for age, this is likely to have had little impact on the results. However the evaluation of cell populations in sputum may have been affected by the changes to cellular immunity that occurs with age. This may have therefore been relevant.

Differences were present in the individual lung function parameters in the COPD cohort as despite being matched on GOLD stage these were significantly lower in the COPD group when compared to the AFD group. This is because each

stage of the GOLD criteria encompasses a range for the percentage predicted value of FEV_1. Therefore despite being classified in the same GOLD stage, the absolute value of an FEV_1 towards the higher end of the category can be up to 29% more than one at the lower end.

The healthy control cohort was small though these subjects were closely matched to a subset of the AFD cohort. The study would benefit from a larger healthy control cohort, particularly as the nature of AFD's genetic inheritance demands that affected males and females are analysed separately.

6.8.2 The nature of sputum and cell yield

Whilst induced sputum sampling is quick and non-invasive, it must be recognised that the yield from induced sputum sampling may be variable depending on the population being sampled. In sputum producers such as COPD subjects the cellularity of samples produced was much greater than in the non-sputum producing groups of AFD subjects and healthy controls. Whilst it was ensured that adequate numbers of cells were used for each aspect of sputum analysis, this did on occasion limit the number of investigations that could be performed on an obtained sample.

In addition, it is not known what the contribution of other lung derived cells is to the measurements of α-galactosidase A activity performed on sputum samples. Although the majority of cells within samples were leucocytes, the presence of squamous cells, bronchial epithelial cells and their contribution to measured enzyme activity is unknown. It is possible that this contribution is minimal, however the cellular protein from these cell types would be having an effect on the calculation of enzyme activity with the fluorometric assay used in this study at the very least. Alternative methods of measuring enzyme such as mass spectrometry may provide a way around this for future research.

6.8.3 Freeze/thawing of samples

Samples of blood and sputum were processed whilst fresh on the day of obtaining them and then stored at - 80°C prior to being thawed for enzyme and cytokine measurement. It is possible that there may have been some impact on

samples from this process and from the duration stored, which may be one explanation for findings outside of the normal range on α-galactosidase A samples from non-AFD control subjects.

6.8.4 Flow cytometry

The majority of the flow cytometry performed in this study was on a 3 colour Beckton Dickinson (BD) FACScan flow cytometer. When combined with limitations of cell yield from samples, this meant that the combinations of antibodies used were dictated by these factors, and therefore although able to identify cells adequately were limited in precision to delineate cell populations in more detail. The difficulty of autofluorescence in samples as a result of cellular debris and from macrophages was also a limiting factor in this. It is possible that the use of 4 colour machine for the addition of other antibodies may assist in further description of cell populations in AFD sputum samples.

6.9 Potential areas for ongoing work from this study

6.9.1 Additional recruitment and follow up

Clearly the examination of larger numbers of AFD subjects would help to further dissect out the impact of the potential cofounders such as smoking, gender and age. It would provide further data on the relationship between lung involvement and disease severity. The impact of ERT would also be better assessed with a larger cohort of enzyme naïve subjects, and ideally with the longitudinal assessment of subjects before and after the commencement of ERT, with sampling of the lungs in those subjects for changes in enzyme activity. Further follow up over a longer period of time would also be useful to examine ongoing changes in lung function which may progress at a slower rate compared to changes in other organ systems.

6.9.2 Cardiopulmonary exercise testing

Having delineated their lung function in detail, to further determine the relative contribution of the lungs and heart to symptoms in our cohort of AFD subjects the additional examination of data from cardiopulmonary exercise

testing would be of use. It would provide more information on the functional contribution of pulmonary aspects of AFD alongside cardiac involvement.

6.9.3 Detailed examination of lung derived cells

Whilst so far we have examined cell populations by flow cytometry, during this study, cytospins of each sputum sample obtained were made and fixed, with a view to further examination of lung derived cells. Further work examining lipid storage in lung derived cells may be performed by immunohistochemical staining with anti-CD77, as used to examine urinary sediment (321). This may help to clarify which cell types from the lung are most affected by storage material, and perhaps if examined longitudinally in subjects commenced on ERT may give some indication of ERT penetration and effect on the lung. It would also be of value to examine these in the context of the proportions of other less prominent cell populations which have not been examined, such as eosinophils and basophils.

6.10 Further work in the field of pulmonary involvement in AFD

6.10.1 Pulmonary sampling by bronchoalveolar lavage

It is possible that more precise information could be gained from pulmonary cells by obtaining samples using bronchoalveolar lavage - though with the recognition that such samples would be from a different lung compartment. Sampling the lung in this manner would provide a bigger and more consistent cell yield than induced sputum sampling, with less contamination by cellular debris and squamous cells. Given that in the alveolar compartment a higher yield of lymphocytes would be expected this may also further assist in future research into the lungs in AFD given the similarities to COPD that have been highlighted in this study.

Flow cytometric examination of BAL samples is generally easier and cleaner, though autofluorescence is still problematic, particularly when examining macrophage populations (259). A more cellular sample would also

allow the use of additional CD markers that might help to further differentiate cell populations, such as CD68 to delineate macrophages as distinct from the monocyte population (322).

6.10.2 Airway smooth muscle

Further evaluation of the changes in airway smooth muscle should be pursued. It is likely as we have suggested in our model of airway involvement, and based on the demonstration of storage material in airway smooth muscle cells in previous studies (323) that changes in airway smooth muscle are key to the pathogenesis of the lung changes observed in AFD. It is possible that exploring the inflammatory cytokines involved in airway smooth muscle hypertrophy in asthma such as IL-1β and TNF-α may provide insight as to changes that ensue from Gb3 deposition in AFD (324).

Evaluating airway wall thickness and the smooth muscle component of this by radiological means may also be useful as previously discussed. However it may also be of interest to explore whether ERT has an effect on clearance of Gb3 in airway smooth muscle cells histologically by serial bronchial biopsy.

6.10.3 Differential enzyme activity in sputum and blood

This study has highlighted that the α-galactosidase A activity in lung-derived samples appears to be higher than that of blood in paired samples from the same subjects, and raises the question as to what the cause of this differential in enzyme activity is. It may be of interest to look into this further as to whether there is a factor in the lung milieu which causes enzyme to accumulate, and if so if this could be exploited in some way to improve tissue activity of exogenous enzyme.

6.10.4 Future therapies

Research is ongoing into oral small molecules such as 1-Deoxygalactonojirymicin (DGJ), which has been shown to assist with the folding and trafficking of enzyme in AFD resulting from missense mutations (325) as well as other site specific chaperones that may enhance the efficacy of ERT by similar mechanisms (326). For such therapies that are currently under

development, their impact on lung aspects of the condition should be evaluated alongside their other effects. Small molecule therapies which have been introduced in other genetic disorders, such as cystic fibrosis, are proving to be revolutionary in subjects where specific gene mutations result in protein abnormalities. Some of these have been shown to be amenable to correction, such as the G551D mutation of the CF gene with the drug Ivacaftor in cystic fibrosis patients who carry least one copy of this mutation (327).

The use of agents which may cause reduced accumulation of Gb3 are being investigated in mouse models (328) either alone or potentially co-administered with chaperone therapy to increase effectiveness (329).

The possibility of gene transfer via viral vectors continues to be explored in AFD murine models, and show promising results in the context of LSDs, where it has been demonstrated that even modest increases in enzyme actitivity may be sufficient to significantly improve disease severity (330;331). Research is ongoing into the possibility of haemopeitic stem cell transplants in LSDs, as well as gene therapy as potential future therapies that may be of significant impact (332).

6.11 Conclusions

The findings from this study suggest that lung involvement in AFD is common, though relatively mild, slowly progressive and follows a similar pattern of involvement to that seen in other organ systems with regards to gender. The changes appear to mainly involve the development of airflow limitation, which is for the most part fixed, and appears to be independent of smoking. Involvement of the lung is more likely to be present in those with worse overall disease severity.

We have shown it is possible to measure α-galactosidase A activity in lung derived samples, and this is lower in AFD males than control males and AFD females. There is a suggestion that it is lower in AFD subjects with airway obstruction compared to those without, though this did not reach statistical significance. We have also shown that α-galactosidase A activity is for the most part greater in sputum-derived samples than blood; and that this may result from the difference in the predominant cell population in these samples. We have not shown any difference in sputum α-galactosidase A activity associated with ERT use.

The cell populations present in the induced sputum of AFD subjects with airway obstruction demonstrated similarities to those in COPD subjects. There were increases in total T cell percentage and a predominance of monocytes/macrophages though these did not reach significance when compared to healthy controls. No differences were seen in B cell, neutrophil or NKT cell populations between the AFD, COPD and healthy control groups. We also showed that sputum IL-8 concentrations were elevated in AFD, similar to that seen in the COPD group.

Appendix 1

1.0 Participant Invitation Letter

Dear

I would like to invite you to participate in the study:

"Investigation of pulmonary involvement in patients with Anderson-Fabry Disease"

This is a 12 month prospective study, at the Lysosomal Storage Disease Unit, Royal Free Hospital. The aims of this study are:

1. To investigate the causative factors in the development of lung involvement in Anderson Fabry's disease

2. To assess the impact of Enzyme Replacement Therapy on lung involvement in Anderson Fabry's Disease

3. To investigate if lung involvement correlates with cardiac and renal involvement in AFD patients

4. To elucidate the processes responsible for the pulmonary changes in Anderson-Fabry Disease, and how these compare to other lung diseases

I have enclosed a copy of the patient information sheet regarding this study.

If you would like more information please do not hesitate to contact us.

Yours sincerely

Dr. Nadia Shafi, MBBS, MRCP.

Clinical Research Fellow

Lysosomal Storage Disorders Unit

2.0 Patient Information Sheet: Anderson Fabry Disease (AFD) group

Re: Investigation of pulmonary involvement in patients with Anderson-Fabry Disease

You are being asked to take part in a study which is to investigate the effect of Anderson-Fabry disease on the lungs and airways. Before you decide to take part, it is important for you to understand why this study is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask your doctor or people listed at the end of this sheet if there is anything that is not clear or if you would like more information.

Why have I been chosen?

You have been asked to participate in this study because you have been diagnosed with Anderson-Fabry Disease.

What is the purpose of this study?

Lung involvement and airways disease in Anderson-Fabry patients has not been studied much. The purpose of this study is to try and investigate who out of the patients with Anderson-Fabry Disease is likely to develop lung involvement, and how and what causes this. This study should provide information that may be used to assess and monitor Anderson-Fabry patients, and to see if there is benefit from enzyme replacement therapy in this aspect of the disease. The lung findings will be compared to those in patients with Chronic Obstructive Pulmonary Disease (COPD), a condition with affects the lungs and causes narrowing of the airways, and with healthy volunteers with no lung disease.

Do I have to take part?

It is up to you whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are free to withdraw at any time and without giving a reason.

What will happen to me if I take part?

At the moment it is routine for you to have 6 monthly blood and urine tests, 6 monthly nuclear medicine kidney assessments and annual heart assessments. For this study the extra tests would be:

1) Two extra blood samples taken at each 6 monthly assessment
2) Lung function testing at each 6 monthly assessment
      - this takes approximately 30mins, and is done on site at RFH

3) Induced sputum sample at each 6 monthly assessment - this takes 30minutes
4) Additional blood samples on days 1, 4, 7 and 10 after your first ever enzyme infusion, for a few patients participating in an additional study (tick if participating in enzyme profiling)
5) A chest CT scan – we are offering this to all AFD patients to try and further clarify the changes the condition may have on the lung, by looking at the appearances of the lung tissue and airways.

What are the possible disadvantages and risks of taking part?

We do not envisage any significant risk to you if you decide to participate in this study, though if you have a CT scan of your chest this will obviously involve exposure to a small amount of radiation.

What are the possible benefits of taking part?

As an individual you may not benefit directly from the research carried out. However research from this type of study will help us understand how the

respiratory system is affected in Anderson-Fabry Disease and how to better assess and monitor the signs and symptoms of the disease. In addition, these tests may provide extra information on your own response to therapy.

What will happen to the results of the research study?

Results of your tests will be communicated to your doctor at the Lysosomal Storage Disease Unit, Royal Free Hospital.

Will any additional information be collected?

To enable us to accurately interpret the results we would like to collect information about your treatment details and progress, and any additional information particularly in relation to your lungs.

Who will have access to information about me?

All data collected will be held at a database at the Royal Free Hospital. Only the doctors, nurses and technicians involved in running this study will have access to it. Your GP will be informed (with your consent) about your participation in the study.

Who is organising and funding the research?

The study is being organised by the team at the Lysosomal Storage Disease Unit, here at the Royal Free Hospital. It is being funded by an educational grant from Shire Human Genetic Therapies Inc

Who has reviewed the study?

Dr Derralynn Hughes, Dr. Marc Lipman, Dr. Atul Mehta, Dr. J. Hurst

Contacts for further information?

If you require any further information regarding the study at any stage, or in the event of an emergency please contact the following doctor or nurse.

Dr Nadia Shafi Tel: 020 7472 6409

OR

Nurses: Linda Richfield/ Alan Milligan Tel: 020 7472 6409

What if I have any other concerns or wish to seek independent advice about the study?

If you have any complaints about the way the investigator has carried the study please contact the Patient Advocacy Liason Service at the Royal Free Hospital; telephone number 0207 4726447

Thank you for taking the time to read this information sheet.

You will be given a copy of this information sheet and a signed consent form to keep.

3.0 Patient Information Sheet: COPD group

Re: **Investigation of pulmonary involvement in patients with Anderson-Fabry Disease**

Anderson-Fabry Disease is a genetic condition, which belongs to a group of conditions known as Lysosomal Storage Disorders. It affects both men and women, and causes disease in multiple organ systems, including the lungs and airways. You are being asked to take part in a study which is going to investigate this further, by comparing the changes in the lung in AFD with those seen in Chronic Obstructive Pulmonary Disease (COPD), and people without any evidence of lung disease. Before you decide to take part, it is important for you to understand why this study is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask your doctor or people listed at the end of this sheet if there is anything that is not clear or if you would like more information.

Why have I been chosen?

You have been asked to participate in this study because you have been diagnosed with COPD, which is one of the groups we have chosen for comparison in the study.

What is the purpose of this study?

Lung involvement and airways disease in Anderson-Fabry patients has not been studied much. The purpose of this study is to try and investigate who out of the patients with Anderson-Fabry Disease is likely to develop lung involvement, and how and what causes this. We aim to do this by looking at the processes of inflammation and different cells found in the lungs, and comparing it to more common conditions where more is known, such as COPD.

Do I have to take part?

Appendix 1

It is up to you whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are free to withdraw at any time and without giving a reason.

What will happen to me if I take part?

If you choose to take part, we will need to confirm that you definitely do not have AFD by doing blood tests to check your plasma enzyme levels and genetics. Obviously, if we were to find that you do have AFD there may be implications for your future medical health, and that of your family, and potentially an impact on any future financial/insurance undertakings, which would be addressed with the help of the LSDU at the Royal Free. However, we will not exclude you from taking part if you do not wish to have this genetic test, if we think it is very unlikely that you have undiagnosed AFD.

At the moment it is routine for you to have regular follow up and lung function tests. We would aim to do the tests for the study when you come for your routine appointments, so avoiding any extra trips to the hospital. For this study the extra tests would be:

1) Blood tests for: Haematology, biochemistry, cytokine profiles, plasma enzyme levels, genotyping

2) Induced sputum sample
3) A chest Xray if you have not had one in the last 3 months

What are the possible disadvantages and risks of taking part?

We do not envisage any additional risk to you if you decide to participate in this study.

What are the possible benefits of taking part?

As an individual you are unlikely to benefit directly from the research carried out.

Appendix 1

What will happen to the results of the research study?

Results of your tests will be communicated to your doctor at the Royal Free Hospital, and the results of the research will be published once it is completed.

Will any additional information be collected?

To enable us to accurately interpret the results we would like to collect information about your treatment details and progress, and any additional information particularly in relation to your lungs.

Who will have access to information about me?

All data collected will be held at a database at the Royal Free Hospital. Only the doctors, nurses and technicians involved in running this study will have access to it. Your GP will be informed (with your consent) about your participation in the study.

Who is organising and funding the research?

The study is being organised by the team at the Lysosomal Storage Disease Unit, here at the Royal Free Hospital. It is being funded by an educational grant from Shire Human Genetic Therapies Inc

Who has reviewed the study?

Dr Derralynn Hughes, Dr. Marc Lipman, Dr. Atul Mehta, Dr. J. Hurst

Contacts for further information?

If you require any further information regarding the study at any stage, or in the event of an emergency please contact the following doctor or nurse.

Dr Nadia Shafi Tel: 020 7472 6409

OR

Clinical Specialist Nurses: Tel: 020 7472 6409

Appendix 1

What if I have any other concerns or wish to seek independent advice about the study?

If you have any complaints about the way the investigator has carried the study please contact the Patient Advocacy Liason Service at the Royal Free Hospital; telephone number 0207 4726447

Thank you for taking the time to read this information sheet.

You will be given a copy of this information sheet and a signed consent form to keep

Appendix 1

4.0 Patient Information Sheet: Healthy Volunteer group

Re: **Investigation of pulmonary involvement in patients with Anderson-Fabry Disease**

Anderson-Fabry Disease is a genetic condition, which belongs to a group of conditions known as Lysosomal Storage Disorders. It affects both men and women, and causes disease in multiple organ systems, including the lungs and airways. You are being asked to take part in a study which is going to investigate this further, by comparing the changes in the lung in AFD with those seen in Chronic Obstructive Pulmonary Disease (COPD), a condition with affects the lungs and causes narrowing of the airways, and people without any evidence of lung disease. Before you decide to take part, it is important for you to understand why this study is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask your doctor or people listed at the end of this sheet if there is anything that is not clear or if you would like more information.

Why have I been chosen?

You have been asked to participate in this study because you do not have any evidence that you are affected by Anderson Fabry Disease or lung disease, and so would be ideal to act as a member of one comparison group in the study.

What is the purpose of this study?

Lung involvement and airways disease in Anderson-Fabry patients has not been studied much. The purpose of this study is to try and investigate who out of the patients with Anderson-Fabry Disease is likely to develop lung involvement, and how and what causes this. We aim to do this by looking at the processes of inflammation and different cells found in the lungs, and comparing it to more

common conditions where more is known, such as COPD.

Do I have to take part?It is up to you whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are free to withdraw at any time and without giving a reason.

What will happen to me if I take part?

If you choose to take part, we will ideally need to confirm that you definitely do not have AFD by doing blood tests to check your plasma enzyme levels and genetics. Obviously, if we were to find that you do have AFD there may be implications for your future medical health, and that of your family, and potentially an impact on any future financial/insurance undertakings, which would be addressed with the help of the LSDU at the Royal Free. However, we will not exclude you from taking part if you do not wish to have this genetic test, if we think it is very unlikely that you have undiagnosed AFD.

In the first instance you would have a set of lung function tests, and once we have confirmed that these are normal, the further tests would be as follows:

1) Blood tests for: Haematology, biochemistry, cytokine profiles, plasma enzyme levels, genotyping

2) Induced sputum sample
3) A chest Xray if you have not had one in the last 3 months
   What are the possible disadvantages and risks of taking part?

We do not envisage any additional risk to you if you decide to participate in this study.

What are the possible benefits of taking part?

As an individual you are unlikely to benefit directly from the research carried out.

What will happen to the results of the research study?

Results of your tests will be communicated to your doctor at the Royal Free Hospital, and the results of the research will be published once it is completed.

Will any additional information be collected?

To enable us to accurately interpret the results we would like to collect information about your treatment details and progress, and any additional information particularly in relation to your lungs.

Who will have access to information about me?

All data collected will be held at a database at the Royal Free Hospital. Only the doctors, nurses and technicians involved in running this study will have access to it. Your GP will be informed (with your consent) about your participation in the study.

Who is organising and funding the research?

The study is being organised by the team at the Lysosomal Storage Disease Unit, here at the Royal Free Hospital. It is being funded by an educational grant from Shire Human Genetic Therapies Inc

Who has reviewed the study?

Dr Derralynn Hughes, Dr. Marc Lipman, Dr. Atul Mehta, Dr. J. Hurst

Contacts for further information?

If you require any further information regarding the study at any stage, or in the event of an emergency please contact the following doctor or nurse.

Dr Nadia Shafi Tel: 020 7472 6409

OR

Clinical Specialist Nurses: Tel: 020 7472 6409

What if I have any other concerns or wish to seek independent advice about the study?

If you have any complaints about the way the investigator has carried the study please contact the Patient Advocacy Liason Service at the Royal Free Hospital; telephone number 0207 4726447

Thank you for taking the time to read this information sheet.

You will be given a copy of this information sheet and a signed consent form to keep

6.0 Consent Form

Re: Investigation of pulmonary involvement in patients with Anderson-Fabry Disease

Name:

Date of Birth:

Hospital Number:

Centre:

Study Number:

Researcher:

Appendix 1

The patient should complete this sheet him/herself. (Please circle one)

1. I confirm that I have read and understood the information contained in the information sheet   Yes/No

2. I have had the opportunity to ask questions and had satisfactory answers to them   Yes/No

3. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason.   Yes/No

4. Do you give permission for your information to be collected, stored, and used for research in a database held at the Lysosomal Storage Disorder Unit at the Royal Free Hospital?   Yes/No

5. Do you give permission for your left-over samples to be stored and used for future ethically approved studies?   Yes/No

6. Do you give permission for your general practitioner to be informed of your participation in this study?   Yes/No

7. Do you agree to take part in the Investigation of pulmonary involvement in patients with Anderson-Fabry Disease?   Yes/No

Name (block letters) …………………………………………….

Signed …………………………………………………………

Date ………………………………………….

DOCTOR TAKING CONSENT (please delete)

Name (block letters) …………………………………………

Signed …………………………………………………….

Date ………………………………………….

7.0 GP Letter

Dear Dr.

Re:

I would like to inform you that the above patient has consented to be enrolled in the study:

"Investigation of pulmonary involvement in patients with Anderson-Fabry Disease"

This is a 12 month prospective study, at the Lysosomal Storage Disease Unit, Royal Free Hospital. The aims of this study are:

1. To investigate the causative factors in the development of lung involvement in Anderson Fabry's disease

2. To assess the impact of Enzyme Replacement Therapy on lung involvement in Anderson Fabry's Disease

3. To investigate if lung involvement correlates with cardiac and renal involvement in AFD patients

4. To elucidate the processes responsible for the pulmonary changes in Anderson-Fabry Disease, and how these compare to other lung diseases

If you would like more information please do not hesitate to contact us.

Yours sincerely

Dr. Nadia Shafi, MBBS, MRCP.

Clinical Research Fellow

Lysosomal Storage Disorders Unit

Appendix 1

8.0 The Royal Free Fabry and Gaucher Clinic: FABRY ASSESSMENT

Name: DOB: Hospital No:		Date: Visit Number: Referral Route:	
Major Events or Concerns since last visit:			
<u>**General**</u>		<u>**GI**</u> (indicate if mild, moderate or severe)	
Fatigue	Y/N	**Abdo pain**	Y/N
Reduced activity level	Y/N	**Diarrhoea** (# of mvmts/day, stool consistency)	Y/N
Thromboembolic events	Y/N		
Lymphoedema	Y/N	**Constipation**	Y/N
Varicose Veins	Y/N	**Bloating** (indicate severity**)**	Y/N
Raynauds	Y/N	**Nausea**	Y/N
Claudication	Y/N	**Vomitting**	Y/N
Heat / Cold intolerance	Y/N	**Haemorhhoids**	
Currently pregnant?	Y/N		
<u>**Neuro**</u>		<u>**Reproductive/ Endocrine**</u>	Y/N
Acroparasthesia	Y/N	**Erectile Dys**	Y/N
Fever pain crisis	Y/N	**Priapism**	Y/N
Chronic Pain	Y/N	**Delayed Puberty**	Y/N
Hypohydrosis	Y/N	**Growth impairment**	Y/N
Hyperhidrosis	Y/N	**Sexual Dysfunction**	
Anhidrosis	Y/N		
Temperature insens	Y/N		
Headache	Y/N	<u>**Dermatological**</u>	Y/N
Seizures	Y/N	**Rash**	Y/N
Syncope	Y/N	**Angiokeratomas**	Y/N
Neuropathy	Y/N	**Telangiectasia**	
<u>**Cardiac/ Resp**</u>		<u>**Eye**</u>	Y/N
Chest pain	Y/N	**Cataract**	Y/N
SOB	Y/N	**Torturous vessels**	Y/N
SOBoE	Y/N	**Visual impairment**	
Palpitations	Y/N		
Oedema	Y/N	<u>**Muscoloskeletal**</u>	
Cough	Y/N	(Indicate pain frequency, ie daily, wkly, monthly, <monthly)	Y/N
Wheeze	Y/N		Y/N
		Acute pain in past yr	Y/N
<u>**Renal/ Urinary**</u>		**Muscle Pain**	Y/N
Dysuria	Y/N	**Body Pain**	
Nocturia	Y/N	**Joint Pain Joint Stiffness**	
Macrohaematuria	Y/N		Y/N
		<u>**Ear**</u>	Y/N
		Tinnitus	Y/N
		Vertigo	Y/N
		Sudden Deafness	
		Hearing impairment	

Appendix 1

PAST MEDICAL HISTORY:

TIA / PRIND / Stroke
Depression / Dementia / Anxiety Dis
LVH / MI / Angina / Heart Failure / Valve Dis / Conduction ab / Arrhythmia /
Congenital HD
Hypertension
Asthma / COPD / Chronic Bronchitis
Dialysis / Transplant / Chronic Renal Failure
Peptic Ulcer / Pancreatitis / Other GI
Diabetes Insipidus / Diabetes Mellitus
Infections – Pneumonia / Septicaemia / Meningitis
Malignancy
Others

MEDICATIONS: **Allergies:**

ENZYME:

Name: Dose: (Number of vials:) Interval: days
Missed infusions (4 weeks)   Y / N   Number:
Delayed infusions (3 weeks)   Y / N   Number:
Premedication: Y / N

Reactions: **Administration:**
                                            Home / Hospital
                                            Self / Nurse / Relative

SOCIAL HISTORY: **FAMILY HISTORY:**

Alcohol intake:
Smoking:
Occupation:

EXAMINATION:	CNS:	RIGHT	LEFT
HEIGHT (cm):	CN:		
WEIGHT (kg):	II		
BMI (kg/m^2):	III,IV,VI		
URINALYSIS:	V		
TEMP:	VII		
General Cond: Good/ Mod/ Poor	VIII		
Heart Failure: Y/N NYHA Grade:	IX,X		
RASH:	XI,XII		
CVS:	UL		
Pulse:	TONE		
BP: Lying Standing	POWER		
JVP:	SENSATION		
HS:	COORDINATION		
SoA:	Reflex - Biceps		
Oedema:	Triceps		
	Supinator		
RS:	LL		
O$_2$ Sat	TONE		
	POWER		
	SENSATION		
ABDOMEN:	COORDINATION		
	Reflex - Knee		
	Ankle		
	Plantar		
Musculoskeletal			
Joint Swelling			
Joint Tenderness			

Checklist:	
Microalbuminuria:	Consider:
Urine Protein:	Cholesterol?
GFR:	Anti-platelet? (neuro event)
Homocysteine	Folic acid? (elevated homocysteine)
Audiology:	Anti-hypertensive? (>130/80)
Opthalmology:	Other Investigations:
Cardiac Assess:	
MAJOR CRITERIA: (tick)	MINOR CRITERIA:
1.Uncontrolled Pain	1. Abnormal brain MRI
2. >300mg/24hr proteinuria	2. Episodic vertigo
3. Ischaemic Hear disease/ cardiac dysfunction	3. Intraventricular conduction defect
4. LVH	4. Gastric symptoms
5. Cardiac arrhythmia	5. Severe asthenia
6. Significant neurovascular disease	6. Hearing Loss

CHANGE OR INITIATION OF THERAPY:	
FOLLOW UP:	GP LETTER:
PATIENT LETTER:	REFER TO:

Appendix 2

Table 1.0 Comparison of spirometry and diffusion capacity in AFD and healthy control matched sub-group

% predicted Mean (+/- SD)	AFD	Matched healthy control	p value (unpaired t test)
n	9	9	-
Age [years]	46.07 (+/- 10.4)	41.56 (+/- 10.5)	0.4
Males, females	5, 4	5, 4	-
FEV_1	82.77 (+/- 7.9)	109.6 (+/- 16.3)	0.0004
FVC	108.2 (+/- 10.0)	117.4 (+/- 18.6)	0.2
FEV_1 /FVC	63.70 (+/- 3.7)	78.91 (+/- 8.3)	0.0001
PEFR	98.67 (+/- 13.8)	119.1 (+/- 19.7)	0.02
MEF50	40.82 (+/- 9.0)	93.96 (+/- 38.8)	0.001
RV/TLC	90.97 (+/- 14.7)	86.30 (+/- 14.1)	0.5
TLCO	89.00 (+/- 18.9)	95.40 (+/- 12.1)	0.4
KCO	93.00 (+/- 19.0)	98.8 (+/- 12.6)	0.5

Table 2.0 Demographics and lung function of AFD subjects and matched COPD subjects

% predicted Mean (+/- SD)	COPD males	COPD females
n	9	5
FEV_1	67.5 (+/- 14.0)	74.3 (+/- 10.1)
FVC	99.4 (+/- 16.3)	115.1 (+/- 20.4)
FEV_1/FVC	0.6 (+/-0.08)	0.6 (+/- 0.07)
PEFR	76.7 (+/- 17.5)	95.7 (+/-13.6)
MEF_{50}	24.24 (+/- 15.6)	29.00 (+/- 11.9)
RV/TLC	112.3 (+/- 16.0)	128.4 (+/- 19.3)
TLCO	68.2 (+/- 19.3)	73.8 (+/- 0.45)
kCO	79.6 (+/- 22.5)	76.3 (+/- 9.6)

Table 3.0 Spiromtery and diffusion capacity in COPD males and females

% predicted Mean (+/- SD)	AFD	COPD	p value (unpaired t test)
n (%)	14 (100)	14 (100)	-
Age [years]	50.7 (+/- 11.4)	65.8 (+/- 10.0)	**0.001**
Males, females (%)	9 (64), 5 (36)	9 (64), 5 (36)	-
GOLD stage I (%) GOLD stage II (%)	4 (29) 10 (31)	4 (29) 10 (31)	-
FEV_1	82.5 (+/- 9.2)	70.2 (+/- 13.1)	**0.01**
FVC	105.7 (+/- 11.9)	103.7 (+/- 18.0)	0.75
FEV_1/FVC	0.64 (+/- 0.04)	0.60 (+/- 0.02)	**0.04**
PEFR	99.1 (+/- 11.3)	81.9 (+/- 18.2)	**0.01**
MEF_{50}	41.5 (+/- 9.3)	24.9 (+/- 13.8)	**0.002**
RV/TLC	88.3 (+/- 17.2)	117.1 (+/- 18.1)	**0.0005**
TLCO	87.2 (+/- 17.0)	69.7 (+/- 16.3)	**0.02**
KCO	94.2 (+/- 16.1)	78.7 (+/- 19.4)	**0.04**

Appendix 2

Table 4.0 Lung function parameters in AFD subjects at baseline, 6 months and 12 months follow up

% predicted Mean (+/- SD)	Baseline	6 months	12 months
n	12	12	12
FEV_1	91.08 (+/- 10.8)	89.17 (+/- 10.7)	91.71 (+/- 11.0)
FVC	104.9 (+/- 10.8)	103.4 (+/- 12.6)	104.6 (+/- 13.7)
FEV_1/FVC	0.73 (+/- 0.07)	0.73 (+/- 0.06)	0.74 (+/- 0.08)
PEFR	107.2 (+/- 9.6)	98.25 (+/- 13.6)	107.9 (+/- 14.3)
MEF_{50}	60.28 (+/- 15.7)	56.92 (+/- 13.4)	61.38 (+/- 17.6)
RV/TLC	87.69 (+/- 10.9)	83.83 (+/- 7.1)	92.86 (+/- 15.6)
TLCO	85.83 (+/- 13.4)	83.17 (+/- 11.8)	83.70 (+/- 12.2)
KCO	96.92 (+/- 13.7)	94.33 (+/- 11.6)	94.88 (+/- 13.8)

Table 5.0 Comparison of α-galactosidase A activity in AFD males and females with and without airway obstruction

Sample type for α-galactosidase A activity	AFD males Median (range)				AFD females Median (range)			
	n	Obstructed	n	Unobstructed	n	Obstructed	n	Unobstructed
Plasma nmol/hr/ml	12	0.2 (0.1 – 0.5)	7	0.2 (0.1 – 4.0)	8	3.9 (1.8 – 6.1)	17	3.9 (1.2 – 7.3)
Blood leucocyte nmol/hr/mg protein	10	0.7 (0.1 – 5.6)	4	3.6 (1.6 – 10.6) p=0.07	5	19.3 (5.9 – 20.8)	9	14.2 (3.4 – 79.9)
Sputum supernatant nmol/hr/ml	11	1.0 (0.3 – 3.9)	3	1.3 (0.3 – 2.5)	5	7.4 (0.7 – 21.5)	12	3.4 (1.7 – 36.1)
Sputum leucocyte nmol/hr/mg protein	12	33.5 (3.9 -118.0)	4	34.2 (21.0 -79.0)	6	99.4 (37.8 - 164.2)	7	169.2 (24.4 – 371.2)
Sputum supernatant/ plasma enzyme activity ratio	11	6.9 (1.5 – 15.4)	3	2.1 (1.6 – 6.0)	5	1.8 (0.4 – 7.0)	12	1.0 (0.6 – 7.6)
Sputum /blood leucocyte enzyme activity ratio	10	36.4 (0.7–361.0)	3	4.6 (2.0 – 29.6)	5	6.2 (1.8 – 27.9)	9	17.4 (2.7 –115.6)

Appendix 2

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