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Vascular Events in Fabry and Gaucher Disease

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Declaration

I, Alison Sian Buchanan Thomas, 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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Abstract

Fabry (FD) and Gaucher (GD) disease are lysosomal storage disorders, caused by single enzyme deficiencies on the glycosphingolipid degradation pathway as a result of genetic mutations in the *GLA* and *GBA* genes respectively. These result in a functional enzyme deficiency within the lysosome and accumulation of un-degraded substrate. GD is characterised by a bleeding tendency and bone infarction. Patients with FD suffer from a vasculopathy with strokes, proteinuric renal failure and cardiac conduction defects, but both disorders are highly heterogeneous. Abnormal cytokine profiles and a pro-inflammatory state have been found in both FD and GD, leading to the hypothesis that abnormalities at the blood-endothelial interface affecting coagulation and leucocyte adhesion contribute to the pathology of these disorders.

This thesis demonstrates the importance of vascular manifestations in the presentation of both GD and FD with failure to identify the underlying cause of these manifestations resulting in delays between the onset of clinical manifestations and arrival at the correct diagnosis. Abnormalities at the blood-endothelial interface identified in GD include up-regulation of adhesion molecules on lymphocytes that may be of importance in the pathogenesis of bone disease, and increased thrombin generation in an endothelial cell model of GD. In FD, whilst cardiac and renal manifestations occur at earlier onset and with greater severity in men, cerebrovascular disease seems to affect both sexes to a similar degree. Monocytes from females with FD exhibit an age-dependent increase in adhesion mimicking the age-dependent increase in cardiac and renal disease seen in these patients but the mechanisms underlying cerebrovascular disease remain uncertain. Initial investigations of platelet prothrombinase activity suggest this may be enhanced in FD. Further investigation of these abnormalities at the cellular level may shed new insights on, and open up new therapeutic options, for the management of the vascular complications of these disorders.

Table of Contents

Table of Figures

Table of Tables

List of Abbreviations

ACE   Angiotensin converting enzyme

acLDL   Acetylated low density lipoprotein

AHS   Autologous human serum

APC   Activated protein C

APTT   Activated partial thromboplastin time

AT   Anti-thrombin

ATCC   American Tissue Culture Collection

ATP   Adenosine triphosphate

BD   Becton Dickinson

BMB   Bone marrow burden score

BSA   Bovine serum albumin

CBE   Conduritol β epoxide

CO2   Carbon dioxide

COX2   Cyclo-oxygenase 2

CME   Clathrin mediated endocytosis

CR3   Complement receptor 3

DGJ   1-deoxygalactonorijimycin

DMEM   Dulbecco's modified Eagle medium

DMSO   Dimethyl sulfoxide

eNOS	Endothelial nitric oxide synthase
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
EPCR	Endothelial protein C receptor
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
ERT	Enzyme replacement therapy
ESRF	End stage renal failure
FBS	Fetal bovine serum
FD	Fabry disease
FITC	Fluorescein isothiocyanate
FV(a)	Factor V (activated)
FVIII	Factor VIII
FX(a)	Factor X (activated)
FXI	Factor XI
Gal	Galactose
GalNAc	N-acetylgalactosamine
GalT2	β-1-4-galactosyltransferase
Gb3	Globotriaosylceramide
GBA	Glucocerebrosidase

GD	Gaucher disease
GFP	Green fluorescent protein
GFR	Glomerular filtration rate
GI	Gastrointestinal
GLA	Alpha-galactosidase
Glc	Glucose
GlcNAc	N-acetylglucosamine
GluC	Glucosylceramide
GSP	Glucosylsphingosine
HDL	High density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSP	Heat shock protein
HUVEC	Human umbilical vein cells
ICAM	Intercellular adhesion molecule
ICH	Institute of Child Health (UCL)
IFNγ	Interferon gamma
IBS	Irritable bowel syndrome
ICD	Implantable cardiac defibrillator
IL	Interleukin
LacC	Lactosylceramide

LDL	Low density lipoprotein
LFA-1	Lymphocyte function-associated antigen 1
Lp(a)	Lipoprotein a
LSD	Lysosomal storage disorder
LysoGb3	Globotriaosylsphingosine
LVH	Left ventricular hypertrophy
LVMI	Left ventricular mass index
M	Molar
M6P	Mannose-6-phosphate
Man	Mannose
mM	Millimolar
MFI	Median fluorescent intensity
MPR	Mannose-6-phosphate receptor
MPV	Mean platelet volume
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MSSI	Mainz severity score index
MTT	Thiazolyl blue tetrazolium bromide
NaCl	Sodium chloride
NaOH	Sodium hydroxide

PAI	Plasminogen activator inhibitor
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC	Protein C
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein complex
PHS	Pooled human serum
PPM	Permanent pacemaker
PRP	Platelet rich plasma
PT	Prothrombin time
RFH	Royal Free Hospital
RPMI	Roswell Park Memorial Institute medium
PS	Protein S
S1P	Sphingosine-1-phosphate
SOB	Shortness of breath
SRT	Substrate reduction therapy
SSI	Severity score index (Zimran severity score – Gaucher disease)
TAFI	Thrombin activatable fibrinolysis inhibitor
TAT	Thrombin-anti-thrombin complex
TCP	Tissue culture plastic

TF   Tissue factor

TFPI   Tissue factor pathway inhibitor

TGFβ   Transforming growth factor beta-1

TIA   Transient ischaemic attack

TM   Thrombomodulin

TNFα   Tumour necrosis factor alpha

tPA   Tissue plasminogen activator

UDP   Uridine 5-disphosphate

UPR   Unfolded protein response

WPB   Weibel Palade bodies

VCAM   Vascular cell adhesion molecule

VEGF   Vascular endothelial growth factor

VLDL   Very low density lipoprotein

VT   Ventricular tachycardia

VWD   Von Willebrand disease

VWF   Von Willebrand factor

WML   White matter lesion

Chapter 1   Introduction

Fabry disease (FD) and Gaucher disease (GD) are lysosomal storage disorders, caused by inherited deficiency of specific hydrolases required for the degradation of glycosphingolipids in the lysosome. GD was first described by Phillipe Gaucher in 1882[1], denoting the presence of distinctive abnormal cells in a female with splenomegaly. Over subsequent decades a clinical syndrome characterised by abnormal macrophages in the reticuloendothelial system causing hepatosplenomegaly, peripheral blood cytopenias, bone disease and, in some cases, neurological involvement, was given the eponymous name, Gaucher disease. In 1898, William Anderson[2] and Johannes Fabry[3] independently described a widespread rash, angiokeratoma corporis diffusum and FD is now recognised as a multi-system disorder complicated by renal failure, cardiomyopathy and stroke. It was not until the 1960s that the underlying enzyme deficiencies were identified: glucocerebrosidase (GBA) in GD[4] and alpha galactosidase A (GLA) in FD[5]. This discovery led to the development of specific therapies in the form of enzyme replacement, licensed first for GD in 1991 and then for FD in 2001.

Despite these advances much still remains unknown about these disorders and in particular the mechanisms by which the presence of mutant enzyme results in the clinical manifestations. Although at first glance GD and FD may seem clinically disparate disorders, abnormalities involving interactions between blood constituents and the vessel wall have been implicated in the disease processes of both. This thesis investigates the role of vascular pathology in GD and FD, examining the prevalence of vascular manifestations in the patient cohort at the Royal Free Hospital, their relation to other disease manifestations and through laboratory investigation of blood-endothelial interactions. This chapter provides an overview of GD and FD and reviews the evidence base for vascular abnormalities in these disorders before outlining an investigative approach to further explore this area.

1.1   Glycosphingolipids

Glycosphingolipids comprise one or more sugar residues linked to the 1-hydroxyl group of ceramide by a β linkage. Along with cholesterol, they are key components of

eukaryotic cell membranes, where they are thought to segregate into discrete microdomains termed lipid rafts[6]. The ABO blood group determinants are complex glycosphingolipids and cell membrane glycosphingolipids have functional roles within the innate immune system; for example, globotriaosylceramide (Gb3) specifically binds the verotoxins of *E.coli* and *Shiga* toxin, whilst lactosylceramide (LacC) forms microdomains on the plasma membrane of neutrophils and macrophages which recognise, engulf and eliminate pathogens. Some glycosphingolipids, including LacC and sphingosine-1-phosphate (S1P) also function as bioactive signalling molecules. Regulated turnover of glycosphingolipids is therefore important for normal cellular functioning.

1.1.1 Glycosphingolipid synthesis

Glycosphingolipids needed to meet a cell's requirements can either be synthesised *de novo* or recycled from the catabolism of other glycosphingolipids. These catabolic reactions are more rapid and energy efficient than *de novo* synthesis and are stimulated by cytokines including tumour necrosis factor alpha (TNFα) and interferon gamma (IFNγ)[7]. *De novo* synthesis of glycosphingolipids starts with synthesis of ceramide, a long chain amino alcohol (sphingosine) acylated with a fatty acid, within the endoplasmic reticulum (ER)[8]. Ceramides are then usually transported to the Golgi where they are converted rapidly to glycosphingolipids by step-wise addition of monosaccharide residues through the action of glycosyltransferases[9].

Galactosylceramide (Galβ1-1'Cer) and glucosylceramide (Glcβ1-1'Cer, GluC) are the initial glycosphingolipids formed and are synthesised by direct transfer of the carbohydrate moiety from a sugar nucleotide (e.g. uridine 5-diphosphate (UDP)-glucose) to ceramide. Galactosylceramide is the principle glycosphingolipid in brain tissue. GluC, the main lipid which accumulates in GD, is found predominately in peripheral tissues but is also present at low levels in the brain. GluC is the precursor of lactosylceramide (LacC) and hence most of the complex neutral glycolipids and gangliosides. LacC (Galβ1-4-Glcβ1Cer) is synthesised by the addition of a galactose to GluC, catalysed by β-1-4-galactosyltransferases (GalT2). LacC is also regenerated by the catabolism of many of the lipids for which it is a biosynthetic precursor. In human endothelial cells, TNFα

activates GalT2, resulting in increased LacC[10]. More complex glycosphingolipids are formed by the step-wise addition of further monosaccharides to the terminal end of the oligosaccharide chain: glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc) or mannose (Man). Further addition of a galactose residue to lactosylceramide yields globotriaosylceramide (Gb3), the predominant lipid which accumulates in FD.

Bioactive small molecules from sphingosine and ceramide

As well as forming the backbone of sphingolipids, ceramide and sphingosine, and their phosphorylated derivatives also function as bioactive small molecules. Ceramide and sphingosine-1-phosphate (S1P) are interconvertible via sphingosine as an intermediate and the balance of ceramide and S1P is highly biologically important[11]. Ceramide has anti-proliferative and pro-apoptotic effects whilst S1P is pro-proliferative.

S1P is synthesised from sphingosine by two sphingosine kinases 1 and 2 (SphK1 and SphK2). Binding of S1P to specific G-protein coupled receptors, activates a number of cell signalling pathways with downstream effects including activation of endothelial nitric oxide synthase (eNOS) stimulating nitric oxide production[12], angiogenesis[13] and up-regulation of cyclooxygenase-2[14].

Non-acylated derivatives

Deacylated derivatives of glycopshingolipids are thought to arise either subsequent to deacylation reactions or by synthesis on a ceramide backbone. These lysosphingolipids are water soluble as opposed to their water-insoluble acylated counterparts. Glucosylsphingosine (GluS) accumulates in GD[15;16] and globotriaosylsphingosine (lysoGb3) accumulates in FD[17].

1.1.2 Glycosphingolipid degradation

Glycosphingolipids for degradation are transported to the lysosome, predominately via the endocytic pathway and broken down by specific hydrolases. Similarly to their synthesis, their degradation is by step-wise removal of the terminal monosaccharide from the end of the oligosaccharide chain (**Figure 1-1**). Glycosphingolipids with less than four sugar residues are only degraded in the presence of sphingolipid activator proteins

(saposins)[18]. Saposins A, B, C and D are derived by proteolytic processing from a single precursor prosaposin; saposin B is required by GLA and saposin C by GBA.

1.2   Lysosomes

Lysosomes are intracytoplasmic membrane bound organelles whose primary function is the degradation and recycling of macromolecules, both of intracellular and extracellular origin. Discovered by Christian De Duve[19], lysosomes have a lipid bilayer, cholesterol-poor membrane, lined internally with a thick glycocalyx to prevent its degradation by lysosomal enzymes. Lysosomes have an internal acidic pH, maintained by an ATP-dependent proton pump, at which lysosomal hydrolases are optimally functional.

Macromolecules are delivered to lysosomes by complex pathways including autophagocytic pathways (for intracellular material) and endocytic pathways (for extracellular material). Inside the lysosome, macromolecules are degraded in a step-wise manner with specific enzymes for each macromolecule catalysing each step. The breakdown products are then released from the lysosome either to the cytosol by diffusion or transporter proteins or to the extracellular space by exocytosis. Salvage of simple macromolecules, not yet fully degraded to their core components, and their transport back to the Golgi apparatus additionally allows efficient production of more complex macromolecules to meet the cells requirements without the need for energy expensive *de novo* synthesis. Egress of molecules from the lysosome involves multiple lysosomal proteins that act as transporters including cystinosin, sialin, cobalamin transporter and NPC1 protein. Lysosomal exocytosis, involving the fusion of lysosomal vesicles with the plasma membrane, enables repair of the plasma membrane and release of lysososomal enzymes and breakdown products into the extracellular space, e.g. enabling degradation of the extracellular matrix.
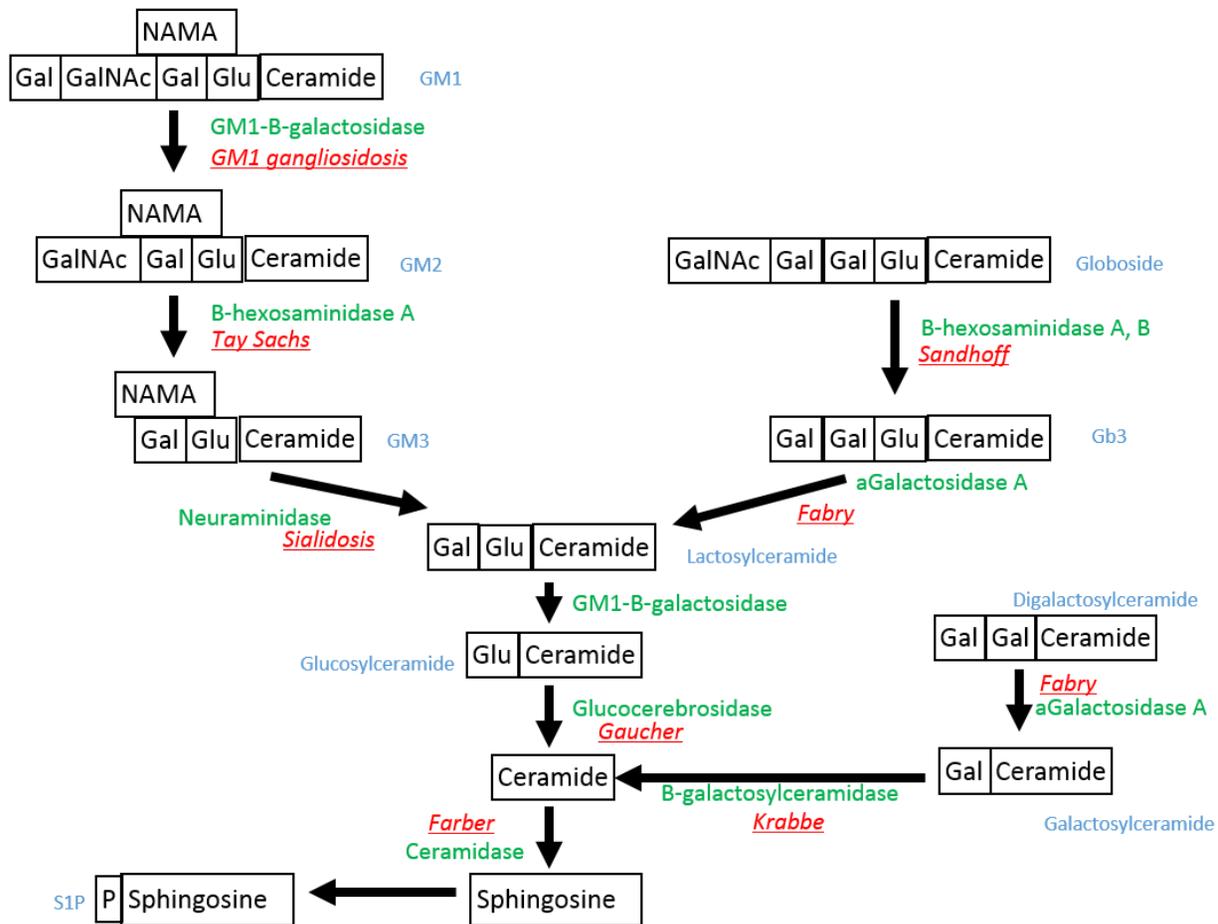
Figure 1-1: Degradation pathway for glycosphingolipids. Blue = lipids,; green = enzymes, red = disease caused by enzyme deficiency.

Gal = galactose; glu = glucose; NAMA = N-acetyl neurominic acid; p = phosphate; GalNAc = N-acetylgalactosamine[20].
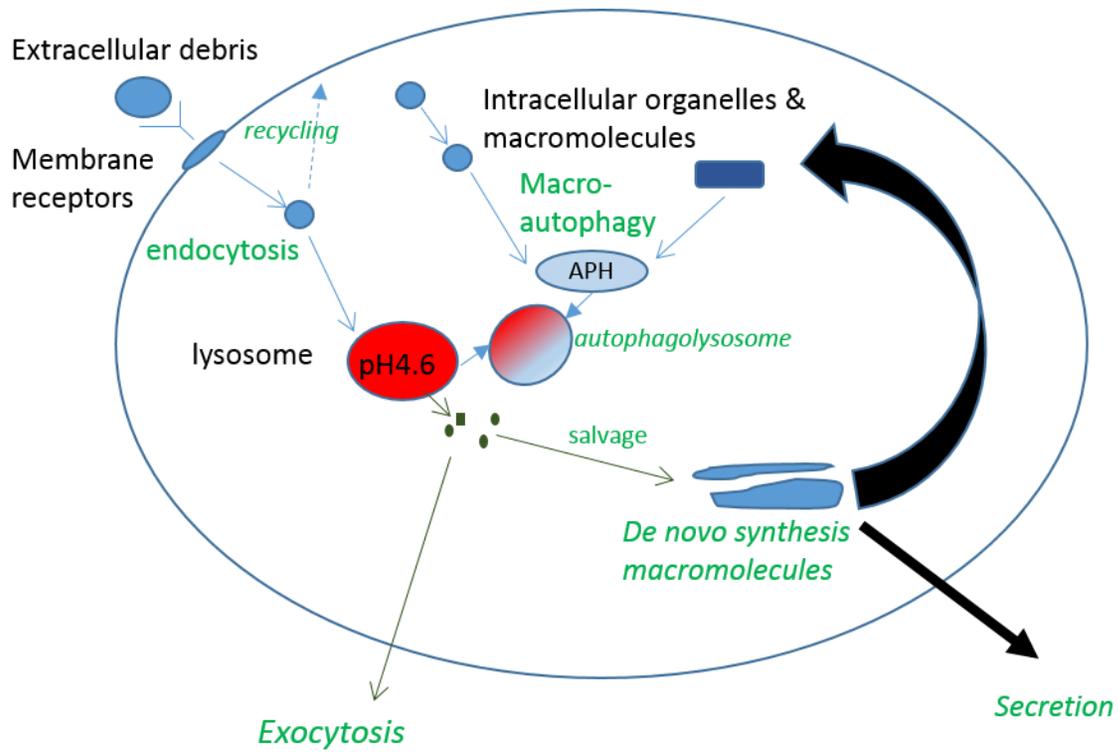
Figure 1-2: Trafficking pathways to and from the lysosome

1.2.1 Trafficking of material for degradation to lysosomes

A number of pathways are involved in the delivery of material to lysosomes including endocytosis, phagocytosis and autophagy (see Figure 1-2).

Endocytosis

Capture of extracellular material and integral membrane proteins occurs via specific endocytic mechanisms, with the precise mechanism depending on the nature of the material. Clathrin-mediated endocytosis (CME) occurs when clathrin-coated pits on the plasmalemma internalise membrane receptor-ligand complexes into vesicles. Clathrin-independent endocytosis most often occurs at flask-like invaginations along the plasmalemma called caveolae – long-lived plasma membrane microdomains composed of caveolins, cholesterol, sphingolipids, GPI-anchored proteins and various receptor proteins.

Early endosomes can be recruited back to the plasmalemma or to other organelles as sorting/recycling endosomes, allowing for the recycling and re-insertion of cell surface receptors, delivery of signalling ligands throughout the cell and internalisation of cell membrane components to be re-organised. Endosome to lysosome maturation involves a progressive decrease of the internal pH to about 5 in a mature lysosome generated primarily by the vacuolar-H+ ATPase.

Autophagy

Intracellular materials reach the lysosome through autophagocytic pathways which target material including organelles for degradation. Autophagy occurs at a basal level and is also activated by a broad range of cellular stressors and mediates degradation of protein aggregates, oxidised lipids, damaged organelles and intracellular pathogens. There are three distinct sub-types: microautophagy, chaperone-mediated autophagy and macroautophagy.

Microautophagy involves direct engulfment of cytosolic material by the lysosome, either by direct invagination of the lysosomal membrane or projected arm-like extensions that sequester material into intralysosomal vesicles.

Chaperone-mediated autophagy is selective for soluble monomeric proteins containing a binding motif for heat shock protein 70 (HSP70) which, with co-chaperones, then promotes protein unfolding and translocation across the lysosomal membrane via the lysosomal membrane protein LAMP2A[21].

When *macroautophagy* is stimulated, double-membrane bound vesicles known as autophagosomes form to engulf cytosolic material. This material, including dysfunctional organelles and oligomerised proteins is selectively recognised by chaperone complexes and adapter molecules including heat shock proteins, ubiquitin and p62/SQSTMI which allow for specific uptake of theses substrates within autophagosomes[22]. Autophagosomes then fuse with lysosomes. The enveloped material and its enveloping membrane are degraded by lysosomal hydrolases.

1.2.2   Lysosomal enzyme synthesis and trafficking to lysosomes

Soluble lysosomal enzymes are synthesised as precursor polypeptides and contain a 20-25 amino acid N-terminal sequence which directs their co-translational translocation into the lumen of the ER. Within the ER, the signal peptide is cleaved and the nascent protein undergoes glycosylation of selected asparagine residues (N-linked glycosylation). These residues play a key role in protein folding and quality control mechanisms which are important for onward transport of the protein to the Golgi apparatus.

Protein folding occurs within the ER: hydrophobic residues mainly face the interior of the protein whilst polar residues mainly occur on the external surface. Protein mis-folding with exposure of hydrophobic residues on the external surface of the protein results in formation of protein aggregates. Molecular chaperones within the ER inhibit formation of aggregates by binding exposed hydrophobic residues. Persistently mis-folded proteins are targeted for degradation by the ER-associated pathways (ERAD), of which the ubiquitin-proteasome system is a key component. The lysosomal enzymes are then transported from the ER to the cis-Golgi via a vesicular transport mechanism.

Targeting of lysosomal proteins from Golgi to the lysosomes

Lysosomal enzymes are targeted to lysosomes via a number of different pathways. Historically, the main mechanism for most lysosomal enzymes, including GLA, was

thought to be mediated by a mannose-6-phosphate (M6P) modification that occurs in the late Golgi compartment, catalysed by GlcNAc-1-phosphotransferase. There are two types of transmembrane mannose-6-phosphate receptors: cation-dependent (CD-MPR) and cation-independent (CI-MPR) which shuttle between trans-Golgi and late endosomes and target enzyme to lysosomes. Both MPR are also present on the plasma membrane but only CI-MPR endocytoses M6P containing ligands, enabling uptake of enzymes with the M6P modification in the extracellular space to be taken up by surrounding cells.

MPR bind M6P-containing proteins in the Golgi apparatus and these complexes exit the Golgi in clathrin-coated vesicles which fuse with endosomes. The lower pH in the endosomes results in dissociation of the protein-receptor complex and release of the protein into the endosomal lumen from where they are transported onwards to the lysosome. The MPR recycles back to the Golgi.

More recently, it has been discovered that some lysosomal enzymes are targeted to the lysosome by MPR-independent mechanisms. Sortilin, a transmembrane glycoprotein, has been shown to traffic a number of lysosomal enzymes which also use the MPR mechanism including GLA[23]. Glucocerebrosidase is not targeted via the MPR mechanism but is instead targeted via the integral lysosomal membrane protein LIMP2[24].

Under normal conditions, 5-20% of many lysosomal enzymes escape the M6P trafficking pathway and are secreted from the cell. These enzymes can then be taken up by MPR on distant cells, termed "secretion-recapture". GLA undergoes secretion-recapture[25], GBA does not.

1.2.3   Lysosomal storage disorders

Lysosomal storage disorders (LSDs) comprise a group of over fifty monogenic disorders affecting lysosomal function. The majority of LSDs are the result of deficiency of a soluble lysosomal hydrolase but others results from a defect in an activator protein, membrane protein or part of a transport pathway (see **Table 1-1**). Rarely, GD may arise due to a mutation in the prosaposin gene encoding saposin C.

Table 1-1: Examples of lysosomal storage disorders

Role	Gene	Protein	Function	Disease
Lysosomal hydrolases	*GBA1*	Glucocerebrosidase	Hydrolysis of terminal β-linked glucose residue from glucosylceramide	Gaucher disease
	GLA	Alpha galactosidase A	Hydrolysis of terminal α-linked galactose from glycosphingolipids	Fabry disease
Activator proteins	*PSAP*	Prosaposin, cleaved to saposins A, B, C & D	Saposin C: activator of glucoceridosidase	Gaucher disease (Saposin C)
	GM2A	GM2 activator protein	Activator protein for degradation of GM2 by hexosaminidase A	GM2 gangliosidosis (AB variant)
Membrane ion channels	*MCOLN1*	Mucolipin I	Ion channel involved in calcium signalling during lysosomal membrane fusion	Mucolipidosis type IV
	CLCN7	CIC-7, chloride ion channel	Contributes to lysosomal acidification	Osteopetrosis
Membrane transporters	*LAMP2A*	Lysosome-associated membrane glycoprotein 2	Mediates chaperone mediated autophagy by binding cytosolic protein substrate on the lysosomal membrane so they can be internalised and degraded	Danon disease
	NPC1	Neimann-Pick C1 protein	Exports cholesterol from endolysosomal compartment	Neiman-Pick C
Enzymes involved in post-translational modifications	*SUMF1*	Sulfatase-modifying factor 1	Oxidises a cysteine residue in the active site of sulfatases, required for function	Multiple sulfatase deficiency
	GNPTAB & *GNPTG*	N-acetylglucosamine-1-phosphotransferase	Formation of mannose-6-phosphate residues on lysosomal enzymes in Golgi	Mucolipidosis II/III

Whilst often viewed as orders of excess due to the accumulation of un-degraded substrates, LSDs may also be viewed as deficiency disorders where critical components required for multiple metabolic pathways are reduced in supply as well as a state of over-abundance of un-degraded material[26].

Except for FD, Danon and Hunter's disease which are X-linked, the LSDs are autosomal recessively inherited. Individually rare, their overall estimated live birth prevalence

overall is 1 in 7500[27]. Within each disorder, there is wide-phenotypic variation ranging from a severe, infantile-onset form to more attenuated adult onset phenotype. FD is unusual in that there is no infantile-onset form. GD has traditionally been divided into three sub-types depending on the presence or absence of neurological features. Type 1 GD (GD1) is characterised by a lack of central nervous system involvement and accounts for 95% of GD amongst Caucasians[28]. Type 2 GD is an infantile onset severe neuronopathic form and type 3 GD has more attenuated neurological features; GD1 is the subject of this thesis and the other types of GD are not discussed further.

1.3   Clinical manifestations of GD and FD

GD and FD are both multi-system disorders and are highly heterogeneous in their clinical manifestations, even amongst patients with the same genotype. Both may present at any time from early childhood to old age and, unlike the majority of LSDs, the clinical manifestations are systemic rather than neurodegenerative. Both are rare disorders with an estimated prevalence of 1:50 00 – 1:100 000 for GD and 1: 117 00 in FD[27], although newborn screening studies have suggested a much higher prevalence of *GLA* mutations[29]. Both are pan-ethnic but there is a particularly high carrier rate of GD amongst Ashkenazi Jews in whom the estimated carrier rate is 1:12[30]. There is no known ethnic predisposition for FD.

1.3.1   Gaucher disease

GD primarily affects cells of the reticuloendothelial system resulting in heaptosplenomegaly, bone disease and decreased peripheral blood counts, often associated with a bleeding diathesis. Polyclonal gammopathies are common at presentation and in the longer term there is an increased risk of multiple myeloma[31]. The clinical manifestations and their potential causes are outlined in **Table 1-2**. In symptomatic patients, presenting features range from mild thrombocytopenia to massive splenomegaly and avascular necrosis of the hips[32]. In addition, the diagnosis may be fortuitous following the incidental detection of abnormal blood counts or splenomegaly on investigations performed for other reasons (e.g. insurance medicals, antenatal care).

Whilst GD is a progressive disorder in most patients[33], the disease can be stable over many years particularly amongst Ashkenazi Jewish populations[34]. Differences between

observed and expected numbers of cases in Ashkenazi Jewish populations suggest that in some patients the disease is so mild that it remains asymptomatic and undiagnosed[30].

Table 1-2: Clinical features of GD

Domain	Clinical features	Potential mechanisms
Thrombocytopenia & coagulopathy	Thrombocytopenia (may be incidental finding) Bleeding, especially bruising Increased risk post-operative bleeding or post-partum haemorrhage	*Thrombocytopenia:* Hypersplenism; Bone marrow infiltration; Impaired megakaryopoiesis *Bleeding diathesis:* ↓synthesis/ ↑consumption coagulation factors Defective platelet activation/ adhesion/ membrane function. Increased fibrinolysis
Anaemia	Fatigue Anaemia (may be incidental finding)	Hypersplenism Hepcidin dysregulation Impaired erythropoiesis Haemolysis – membrane abnormality or autoimmune Concomitant B12 deficiency
Splenomegaly	Early satiety Abdominal pain, Distension/ abdominal mass May be incidental finding	Infiltration by Gaucher cells Inflammation
Bone disease	Bone pain, infarcts, avascular necrosis Osteopenia/ osteoporosis Bone remodelling, lytic and sclerotic lesions	Imbalance between osteoclast and osteoblast activity Infiltration by Gaucher cells
Gammopathies & malignancy	Gammopathy: polyclonal and monoclonal Multiple myeloma Other malignancies	Chronic pro-inflammatory microenvironment stimulating clonal evolution Up-regulation of pro-proliferative cell signalling pathways by glycosphingolipids

1.3.2 Fabry disease

The classical presentation of FD is with onset of burning pain, particularly in the hands and feet (acroparasthesia) and the distinctive rash, angiokeratoma, in childhood or adolescence, often accompanied by gastrointestinal upset including pain and altered bowel habit; median age of symptom onset is 9-10 years in males and 13-23 years in females[35;36]. Involvement of critical organ systems including the kidneys, heart and brain ensues over subsequent decades and is life-limiting with a median life expectancy of 50 years in males[37] and 70 years in females[38]. Given its X-linked nature, for many years women were thought to be carriers rather than sufferers of FD. It is now clear that the majority of women with FD develop similar clinical features to those seen in males[38], although often milder and are best considered affected heterozygotes[39]. The mechanisms by which

females can develop clinical manifestations despite sometimes normal enzyme levels remains an enigma[40]. Lyonisation with inadequate cross-correction between cells has been hypothesised but is unlikely to provide a complete explanation[41].

Table 1-3: Clinical features and pathophysiology of FD

Domain	Clinical features	Potential mechanisms
Skin	Angiokeratoma Decreased/ absent sweating	Increased VEGF production Lipid accumulation in dermal microvasculature
Neurological	Neuropathic pain (acroparasthesia) Stroke/TIA/ white matter lesions Tortuous vessels	Microvascular thrombosis and altered cerebral blood flow regulation due to lipid accumulation in endothelium and smooth muscle
Renal	Proteinuria Chronic renal failure	Mesangial widening and glomerular sclerosis resulting in tubular dysfunction Gb3 deposition in podocytes with thickening of basement membrane
Cardiac	Arrhythmias (conduction defects, atrial and ventricular) Cardiac hypertrophy Valvular disease	Energy depletion due to inefficient ATP utilisation by sarcomeres Activation pro-proliferative pathways Ischaemia and fibrosis in conduction system
Gastrointestinal	Abdominal pain Diarrhoea/constipation	Lipid deposition in the ganglions of the mesenteric plexus
Opthalmological	Cornea verticillata (corneal deposits) Vessel tortuosity	Lipid deposition in corneal epithelial basement membrane
Audiovestibular	Tinnitus, vertigo Sensorineural hearing loss	Gb3 deposition in the vestibular and cochlear nerves

The main clinical features and potential underlying mechanisms of FD are shown in **Table 1-3**. They are highly heterogeneous in the time of onset, severity and range of organ involvement both within and between different families. Although the classic phenotype of FD is of multi-organ involvement manifesting as pain and angiokeratoma followed by development of cardiac, renal and cerebrovascular complications in adulthood, cases of FD with predominant renal or cardiac manifestations diagnosed later in life, often with relatively high residual enzyme activity, have been described[42;43]. Whether these comprise a true distinct phenotype or part of an increasingly broad spectrum of manifestations of FD remains unanswered.

1.4 Diagnosing GD and FD

The cornerstone of diagnosis in both GD and males with FD is demonstration of low enzyme activity. In both cases, this biochemical assay can be performed on leukocytes or cultured skin fibroblasts[44]. GLA is secreted at high levels into the plasma and therefore in FD, measurement of enzyme activity can also be performed on plasma, but this is not the case for GD. In females with FD, enzyme activities are usually reduced[45] but can fall within the normal range and genetic analysis is required to confirm or refute the diagnosis.

Demonstration of a pathogenic mutation in the *GLA* gene is required for diagnosis of FD. In FD, mutations are usually private, limited to a single or small number of families and are distributed along the length of the gene. Over 300 mutations have been described[46], most commonly missense mutations, but also nonsense and rearrangements. Deciding whether or not a previously unreported mutation is pathogenic (i.e. disease causing) can be difficult and bioinformatic tools and *in vitro* expression studies are useful tools in this process[47], but may still be inconclusive. Screening studies of newborns or higher-prevalence groups (e.g. cryptogenic stroke) have resulted in the identification of a high number of mutations of uncertain pathogenic significance[48].

In GD, mutational analysis of the *GBA* gene is recommended for patient counselling but is not required for diagnosis. In GD, four mutations in the *GBA* gene, located at 1q22.1, account for >90% of within the Askenazi Jewish population: L444P, N370S, 84GG and IVS2+1G[49]. Amongst non-Ashkenazi patients there is a much wider range of genotypes with almost 300 mutations reported[50], although many patients are compound heterozygotes with the N370S or L444P mutation. Although genotype-phenotype correlations are imperfect they do provide some prognostic information with the presence of at least one N370S allele being considered protective against the neurological manifestations seen in GD1[51].

In both GD and FD, the diagnosis might be suggested by the presence of characteristic storage cells on biopsy specimens. In GD, patients are often diagnosed following the identification of characteristic Gaucher cells – enlarged, lipid laden macrophages with weakly basophilic fibrillar cytoplasmic inclusions - on bone marrow biopsies performed for investigation of splenomegaly and/or low blood counts. In FD, storage material is

visible as "zebra-like" bodies on electron microscopy[52], usually on renal biopsies performed for investigation of proteinuria/ renal failure but occasionally on endomyocardial biopsies. The presence of storage material in either scenario is not sufficient for diagnosis – in GD pseudo-Gaucher cells are found in many haematological disorders including chronic myeloid leukaemia[53] and haemoglobinopathies. Chloroquine use can give rise to histological features identical to FD[54] and therefore for both disorders enzyme activity and/or mutational analysis must be performed.

1.5   Pathophysiology of GD and FD

Although the specific enzyme deficiency and the presence of glycosphingolipid accumulation in GD and FD were identified over half a century ago, relatively little remains known about the mechanisms by which a mutation in the encoding gene results in the clinical manifestations of the disease. Much research has focussed on the consequences of impaired lipid degradation on lysosomal and cellular function. More recently, a potential role for the mutant protein itself in pathogenesis of these disorders has been proposed. Studies of the effects of cellular dysfunction at the wider microenvironment and organ level have been limited in part by difficulties in developing animal models of GD and FD which re-capitulate the disease manifestations seen in humans.

1.5.1   Glycosphingolipid accumulation & cellular metabolism

In both GD and FD, failure of the mutant enzyme to degrade their glycosphingolipid substrate results in accumulation of un-degraded substrate within the lysosomes. Within GD, glucosylceramide is the predominant lipid stored but glucosylsphingosine, also accumulates. In FD, Gb3 and its deacylated derivative accumulate, but in both disorders other abnormalities of glycosphingolipids also occur (see **Table 1-4**). In GD, storage occurs predominantly within macrophages, whilst in FD, storage has been noted in many cell types including endothelial cells, podocytes and cardiomyocytes. Whilst storage of undegraded lipid is the histological hallmark of both disorders, storage lipids comprises <2% of the weight of enlarged spleens in GD or hearts in FD [55], suggesting pathological mechanisms beyond simple storage.

Table 1-4: Lipid abnormalities in GD and FD

Lipid	GD	GD	GD	GD	FD	FD	FD
GluC	↑	↑		↑	↓	→	
GluS		↑	↑	↑			
LacC				↑	→	→	
Gb3	→				↑	↑	
LysoGb3						↑	
GM3	↑			↑			
S1P							↑
Ceramide	→					→	
Reference	56	15	57	16	58	17	59

Determining the effects of storage on cellular function and whether storage per se is a fundamental cause of cellular dysfunction is an important, but largely unanswered question. Potential mechanisms of cellular dysfunction are illustrated in **Figure 1-3**, whereby glycosphingolipid accumulation results in alterations in key cellular functions including signalling pathways, oxidative pathways and intracellular trafficking.

Accumulated lipids in both GD and FD can have direct effects on cellular functioning including calcium homeostasis, cellular proliferation and free radical formation. Excess glucosylsphingosine has been postulated to have a role in the pathogenesis of both bone disease[60] and haematological malignancies[61] in GD. In FD, lysoGb3 has been shown to have a pro-proliferative effect on myocytes[17] and to result in increased production of the cytokine transforming growth factor beta-1 (TGFβ1), which is involved in the control of cell proliferation, by podocytes[62]. In addition, increased S1P in FD has been implicated in the development of cardiac hypertrophy[59].

Altered mitochondrial function and increased production of free radicals has been found in both FD and GD. Impairment of the mitochondrial respiratory chain enzymes has been found in FD[63] and whilst Gb3 accumulation results in oxidative stress and up-regulation of adhesion molecules within the endothelium[64]. In GD, pharmacological inhibition of GBA resulted in mitochondrial dysfunction and increased free radical formation in a dopaminergic cells line[65]. Glucosylceramide has shown to increase mitochondrial mitochondrial respiration in the liver and brain[66].
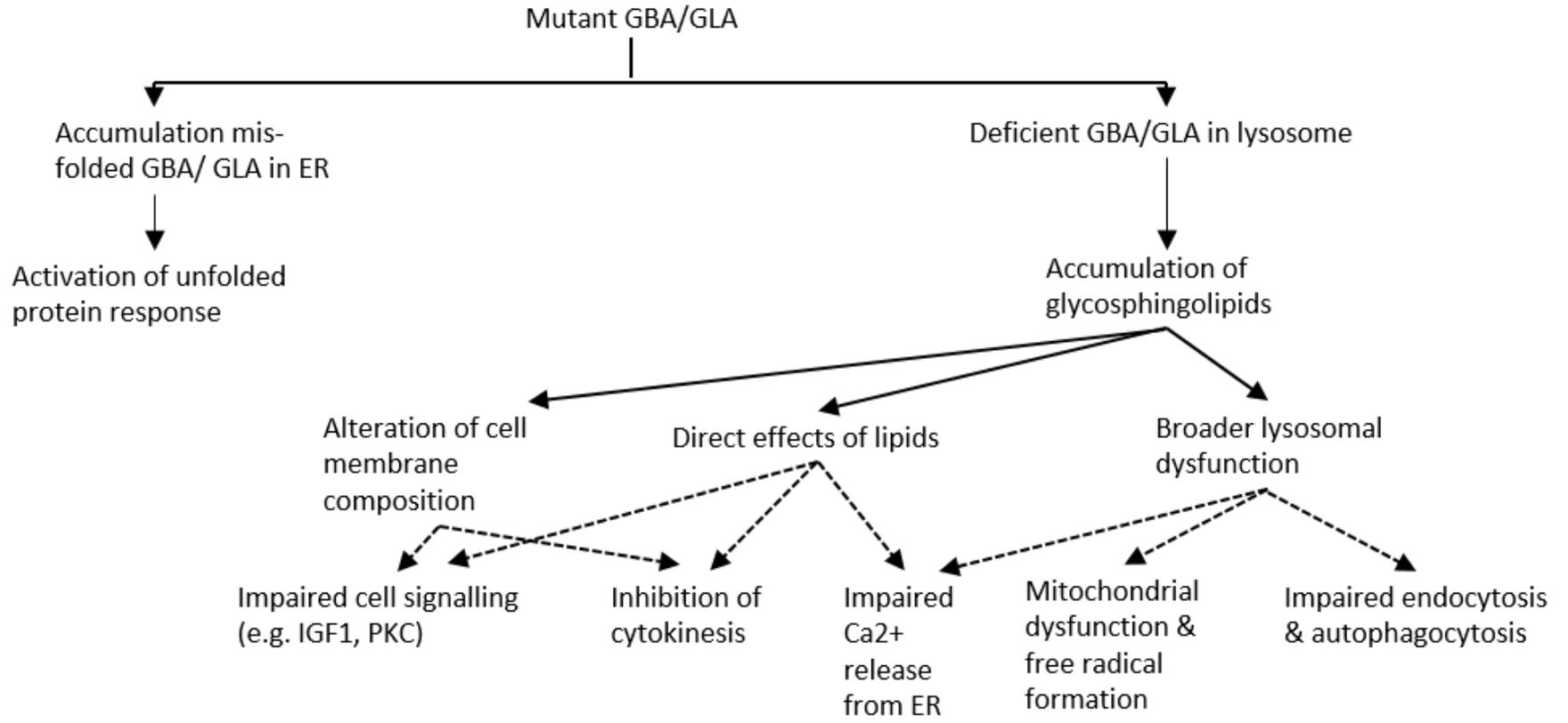
Figure 1-3: Potential mechanisms of cellular dysfunction

1.5.2 Abnormalities in lysosomal trafficking pathways

Enzyme deficiency can result in broader abnormalities of the lysosomal system, particularly affecting trafficking pathways to the lysosome: endocytosis and autophagocytosis. Addition of lactosylceramide to cells with different lipidoses results in accumulation of lactosylceramide in the endosomal system, rather than transport to the Golgi which occurs in normal cells[67]. There is also evidence of accumulation of M6P receptors in the late endosomal system within Neimann-Pick type C[68]. In Neimann-Pick, endocytic dysfunction results in impaired recycling of membrane receptors and altered cell membrane composition[69]; whether this occurs in other glycosphingolipidoses has not been investigated.

Accumulation of autophagosomes has been noted in a number of LSDs[70;71] leading to accumulation of protein aggregates and abnormal mitochondria[72]. Abnormalities of autophagy, with impaired autophagosome maturation have been found in FD[73] and neuronopathic GD[71]. Increased synthesis of the phospholipid phosphatidylcholine, a component of cell membranes[74] and increased levels of other lysosomal enzymes (e.g. cathespins)[75] in GD suggest attempts by the lysosomal system to compensate for the effects of enzyme deficiency.

1.5.3 Missense mutations and the unfolded protein response

Correct folding of both GBA and GLA in the ER is required for onwards transport to the Golgi and then the lysosome. Mis-sense mutations which cause mis-folding of the enzyme result in retention of the mis-folded enzyme in the ER and its subsequent degradation by ERAD. If this pathway is saturated, mis-folded proteins accumulate within the ER, activating the unfolded protein response (UPR) – a signalling pathway that has been implicated in a range of metabolic and neurodegenerative diseases[76]. This process has been suggested to underlie the increased risk of Parkinson's disease in GD[77]; its potential role in other disease manifestations in GD or FD is undetermined.

1.5.4　Altered inflammatory responses and the microenvironment

Histological studies from patient biopsy specimens and of animal models have suggested that altered responses to inflammation play an important role in organ pathology in both GD[60;78] and FD[79]. Cytokines play a key role in intercellular signalling and in the regulation of inflammatory reactions and have been found to be abnormal in both GD and FD. Key cytokines investigated and their functions are outlined in **Table 1-5** and studies revealing abnormalities in their expression in **Table 1-6**.

Table 1-5: Cytokines and their effects. NK (natural killer), IFNγ (interferon gamma), IL (interleukin).

Cytokine	Produced by	Effects on inflammation
TNFα	Macrophages, NK cells, T cells	Promotes inflammation, endothelial activation
IFNγ	T cells, NK cells	Macrophage activation
IL1α	Macrophages, neutrophils, endothelial cells	Pro-inflammatory, stimulates TNFα production
IL1β	Macrophages	Proinflammatory, induces COX2
IL6	T cells, macrophages, osteoblasts	Inhibits production TNFα, IL-1; activates and stimulates production IL1 receptor antagonist and IL10
IL8	Monocytes, macrophages, endothelial cells	Mobilises and activates neutrophils
IL10	Monocytes, T cells	Anti-inflammatory: down-regulates pro-inflammatory cytokine synthesis
IL13	T cells	Anti-inflammatory: down-regulates macrophage activity inhibiting production of pro-inflammatory cytokines

Initial investigations of cytokines in sphingolipidoses showed increased production of IL1 by murine macrophages incubated with GluC[80]. Histological studies of GD spleens have shown that Gaucher cells themselves most closely resemble anti-inflammatory alternatively activated macrophages, but are surrounded by leukocytes strongly expressing pro-inflammatory IL1β[81]. Studies in both GD and FD have yielded sometimes conflicting results but support dysregulation of inflammation with an overall pro-inflammatory tendency. Increased levels of TNFα, IL1β and IL6 have been found in both GD and FD and additionally increased levels of IL8 have been found in some studies of GD.

Table 1-6: Cytokine abnormalities in GD and FD

		TNFα	IFNγ	IL1α	IL-1β	IL-6	IL8	IL-10	IL13	Ref
GD	Plasma	↑								82
GD	Plasma	→			→	↑		→		83
GD	Plasma	→				→	↑			84
GD	PBMC mRNA	↑			↑	→	→			85
GD	Serum	↑			↑	→	→			86
GD	Serum	→	→	→	→	→	↑	→	→	87
FD	Plasma					↑ (F only)				88
FD	Plasma	↑				↑				89
FD	PBMC mRNA	↑	→	→	↑	→				90
FD	PBMC supernatant	→	→		↑	↑			→	90
FD	Dendritic cells	↑			↑	→				90
FD	Monocytes	→			↑	↑				90
FD	Plasma	→				↓				91

1.5.5 Heterogeneity and genetic polymorphisms

It is likely that the clinical sequelae of GD and FD are consequent on the aberrant activation of a number of different cellular signalling pathways and that other modifiers of these pathways, whether genetic or acquired, play a role in determining the severity and organ distribution of clinical features that manifest in individual patients. In the search for disease modifiers, polymorphism studies have been performed on genes encoding cytokines in both GD and FD and additionally on genes involved in coagulation and nitric oxide synthesis in FD.

In FD, the polymorphisms G174C in *IL6* and G894T in endothelial nitric oxide synthase and the FV Leiden mutation (G1691A) have been associated with cerebral white matter lesions[92]. A further study also showed that homozygosity for the *IL6* G174C polymorphism, which is associated with increased IL6 levels, was associated with increased disease severity and cerebrovascular events[93], an association with also exists for stroke in the general population[94]. Conversely, within GD, homozygosity for the IL6 G174C polymorphism has been associated with milder bone disease[95]. A polymorphism in the *IL10* gene has also been associated with neurological disease in FD[96].

1.6   Treatment of GD and FD

Following diagnosis, patients require a comprehensive assessment to establish the extent of organ involvement and determine optimum treatment. In the UK this is undertaken at designated specialist centres which were commissioned in 2005, of which the Royal Free Hospital is one. In GD, radiological assessment of the skeleton, often using magnetic resonance imaging (MRI), assessment of liver and spleen volumes and bone densitometry as well as measurement of the blood count and immunoglobulin levels are undertaken. In FD assessments cover the critical organ systems: renal (quantification of proteinuria, measurement of the glomerular filtration rate (GFR)), cardiac (electrocardiogram and echocardiogram) and neurological (pain assessment and cerebral MRI).

Until the 1990s, treatment of LSDs was limited to supportive care, primarily in the form of blood transfusions and orthopaedic surgery for GD and renal and cardiac therapies for FD. This scenario changed with the introduction of intravenous enzyme replacement therapy (ERT), initially for GD in 1991 and subsequently for FD in 2001. Assisted by the financial incentives of orphan drug legislation, further therapies have been developed for both disorders targeted either at increasing intracellular enzyme levels or reducing glycosphingolipid products (see **Figure 1-4**).
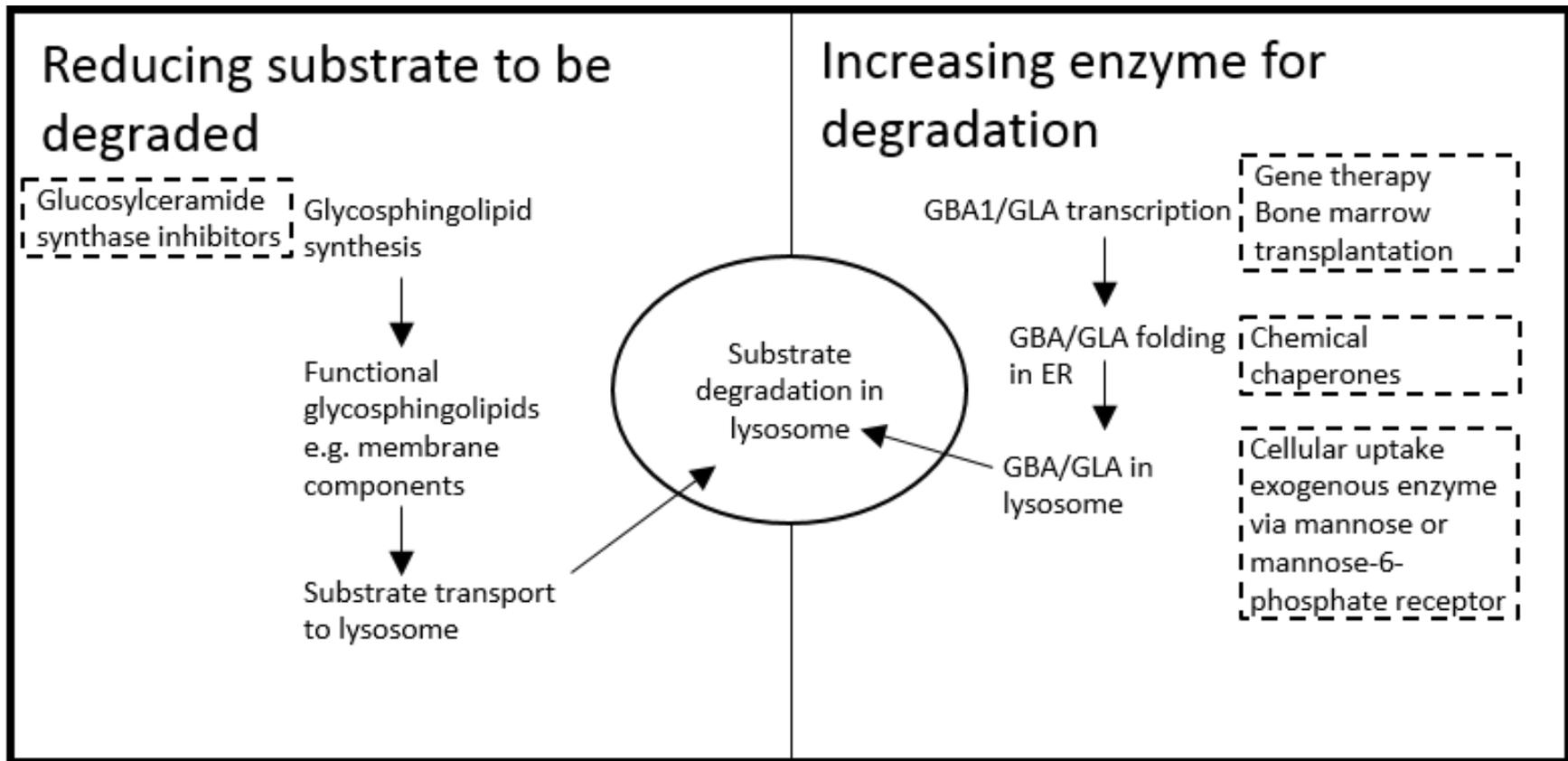
Figure 1-4: Treatment of FD/GD. Treatment strategies involve either increasing intracellular enzyme levels or reducing the amount of substrate requiring degradation.

1.6.1 Enzyme replacement therapy

Although the discovery of the enzymatic defects in the 1960s raised the conceptual possibility of ERT, it was not until the early 1990s that this became a reality for GD, the first LSD for which ERT became available. Initial attempts at infusion of placental-derived enzyme resulted predominant uptake by hepatocytes in the liver, rather than reticuloendothelial cells. Discovery of the macrophage mannose receptor enabled targeting of the enzyme to macrophages, the predominant site of disease, by modifications to oligosaccharide chains[97]. A pivotal study in twelve non-splenectomised patients showed improvement in liver and spleen volumes and blood counts and led to the licensing of the product alglucerase (Ceredase, Genzyme) in 1991[98]. Subsequently three recombinant products have become available: imiglucerase (Cerezyme, Genzyme), velaglucerase alfa (VPRIV, Shire) and taliglucerase (Elyso, Protalix) alfa. All have demonstrated efficacy in haematological and visceral parameters. Improvements in bone disease have been more difficult to demonstrate over the relatively short time course of clinical trials and the effect of ERT on other complications including multiple myeloma is uncertain.

Two recombinant enzyme therapies were developed contemporaneously for FD: agalsidase alfa (Replagal, Shire Human Genetic Therapies) and agalsidase beta (Fabrazyme, Genzyme), which were both licensed in the EU in 2001. Establishing the efficacy of these therapies is challenging – the clinical features of FD develop insidiously over many years and are highly heterogeneous making identification of clinically relevant end points applicable to a broad number of patients and achievable over the relatively short time frame of a clinical trial difficult. Initial trials demonstrated clearance of stored lipids from endothelial cells and improvement in pain over a six month time period[99;100]. Stabilisation of mild-moderate renal impairment[101] and reductions in left ventricular mass [102] have been demonstrated but there is a paucity of data on the effect of ERT on major clinical events including stroke, arrhythmias and death[103]. Patients receiving ERT may still experience clinical events: 5/58 (9%) developed strokes[104] in a 54 month follow up study; progression of white matter lesions on MRI has also been reported[105].

The main side effects of all ERT products is immunogenicity – reported rates of antibody production vary between products, but differences in assay design and sensitivity make direct comparison difficult. Antibodies are predominately IgG and severe allergic reactions or anaphylaxis are rare[106], facilitating home infusion for both GD and FD. The impact of antibody formation on therapeutic responses has not been studied in detail in either disorder.

1.6.2   Substrate reduction therapy

Discovery that certain iminosugars can selectively inhibit the biosynthesis of glycosphingolipids led to the concept of reduction of the amount of substrate to a level at which the patient's own residual enzyme activity is able to fully degrade it. Miglustat was originally developed as an α-glucosidase inhibitor with potential anti-viral activity, but was found to inhibit glucosylceramide synthase at micromolar concentrations[107]. In clinical trials in has been demonstrated to improve haematological and visceral parameters[108] over 24 months, but direct switching of patients from ERT to miglustat may be associated with deterioration in haematological parameters[109]. Diarrhoea is a common side effect and development of paraesthesia has also occurred. A second substrate reduction therapy, eliglustat tartrate (Genz 112638, Genzyme) is a PDMP-based analogue which inhibits glucosylceramide synthase at nanomolar concentrations[110]. Results from phase II trials demonstrate improvement in haematological and visceral parameters comparable to those seen with ERT[111] and phase III trials are expected to report shortly. Phase I trials of SRT for FD have been completed (Genz 682452, NCT01674036) and phase II trials are expected imminently.

1.6.3   Chaperone therapy

Many pathogenic mutations in the *GBA1* and *GLA* genes result in protein mis-folding in the ER and premature degradation. Pharmacological chaperones are small molecules which bind to proteins in the ER stabilising them and helping them to achieve their correct conformation and exit the ER to the Golgi. Initial proof of principle for this approach was demonstrated by administration of galactose, a reversible competitive inhibitor of GLA, to a male patient with FD resulting in increased enzyme activity and improvement of cardiac function over a two year period[112]. 1-deoxygalactonojirimycin (DGJ, migalastat

hydrochloride, Amicus Therapies) is a competitive inhibitor of GLA which increased GLA activity in multiple cell types in patients with mis-folding mutations in a phase II trial[113] and is currently undergoing phase III evaluation.

In GD, isofagomine (Plicera, Amicus Therapeutics) showed good in vitro efficacy[114], but only showed clinically meaningful improvements in 1/18 patients in a phase II trial[115]. High throughput screening studies identified ambroxol, an expectorant, as a potential chaperone[116] and initial clinical data is promising[117] but further evaluation is required.

1.7   Altered blood-endothelial interactions in FD and GD

Abnormalities in interactions between blood constituents and the vessel wall have been implicated in the clinical manifestations of both GD and FD. Two key interactions between the endothelium and blood are haemostasis, the formation and degradation of fibrin clots and their regulation, and leucocyte adhesion, a key part of inflammatory responses involving the adherence of leucocytes to the endothelium and their extravasation into tissues (see **Figure 1-5**). Abnormalities in both these processes have been implicated in the pathogenesis of some of the clinical manifestations of both GD and FD.

1.7.1   Gaucher disease

In GD there is an increased risk of bleeding which may be the presenting feature of GD or give rise to complications during interventions in patients with known GD (e.g. childbirth or orthopaedic surgery).   Whilst this has often been ascribed to thrombocytopenia, bleeding events can occur in patients with relatively normal platelet counts[118] suggest abnormalities in coagulation processes beyond numerical thrombocytopenia.   Identification of concurrent abnormalities in procoagulant, anticoagulant and fibrinolytic pathways has led to the hypothesis of a state of chronic disseminated intravascular coagulation that may contribute to bone infarcts as well as bleeding risk[119].

There is an increased incidence of both polyclonal and monoclonal gammopathies[120;121] with an increased risk of multiple myeloma, estimated to be 5.9[122] to 51.5 times that of the normal population[31]. Immune dysregulation within the bone marrow

microenvironment is thought to underlie these processes with Gaucher cells secreting cytokines which result in chemotaxis of leukocytes to the bone marrow.

1.7.2   Fabry disease

FD has often been considered a vasculopathy, contributing particularly to the development of cerebrovascular complications. Whilst stroke is recognised as a complication of classical FD, it may occur in the absence of cardiac or renal FD complications[123]. It may also be the presenting feature of FD and recent screening studies have identified an incidence of FD of 0.5-3.9% in patients with stroke[124;125]. Multiple mechanisms are thought to contribute to vascular disease including endothelial activation, altered vascular tone and a procoagulant state[126;127], processes which have also been implicated in the development of renal and cardiac complications.

1.7.3   Clinical importance

Better understanding of these clinical manifestations and the pathogenic processes underlying them is important for improving diagnosis, prognosis and enabling better informed management decisions. Both GD and FD are rare disorders, with patients often reporting long delays and circuitous routes between symptom onset and diagnosis. Understanding how patients present and identification of features which could raise suspicion of the correct diagnosis may help improve the diagnostic pathway. Within GD, patients often require operative interventions (e.g. joint replacements) or are potentially at increased risk of bleeding during childbirth. Better understanding of the bleeding risk and the processes underlying it will enable more informed management decisions to be undertaken.

In FD vascular events including cardiac arrhythmias and cerebrovascular events may have life-changing or life-limiting consequences. These complications may occur without warning and currently there is little evidence base on which to be able to predict the risk of occurrence. Understanding the underlying processes and risk factors for vascular events would be useful for predicting prognosis, which may be particularly beneficial when deciding whether to institute therapy or whether genetic testing, particularly in females, should be undertaken. It may also better inform decisions as to whether to institute supportive therapies, (e.g. anti-platelet agents) to reduce risk.

1.7.4   Potential underlying mechanisms

Localised thrombus formation in the vessel lumen and leucocyte adhesion and extravasation are normally tightly controlled and orchestrated, predominately by pro- and anti-inflammatory cytokines (see **Table 1-7**). Abnormal activation or regulation of these processes may contribute to the clinical manifestations GD and FD (see **Figure 1-5**); in addition altered levels of glycosphingolipids may impact on these signalling pathways.

Altered composition of cell membranes as a result of glycosphingolipid accumulation may effect processes that occur on the cell membrane, including those involving coagulation enzyme complexes. Incorporation of Gb3, LacC or GluC into phospholipid vesicles enhances inactivation of FV by the anticoagulant pathway[128], possibly by altering lipid raft formation.

Table 1-7: Effect of cytokines & glycosphingolipids on adhesion & haemostasis

Molecule	Effect on cellular adhesion	Effect on coagulation
TNFα	Up-regulation of adhesion molecules incl. ICAM-1, VCAM-1[129] and E-selectin.	Up-regulation of tissue factor[130]
IFNγ	Up-regulation ICAM1 and VCAM1	Increases TF and PAI expression in mouse models[131]
IL1α	Promotes expression adhesion molecules	Increases EC TF expression and reduces activation of PC by thrombin-thrombomodulin comple[132]. Induces thrombin formation and activates fibrinolysis in baboons[133]
IL1β	Promotes expression adhesion molecules	
IL6	Up-regulation adhesion molecules	Activates coagulation but not fibrinolysis[134] and induces TF expression
IL8	Up-regulation adhesion molecules required for firm adhesion of monocytes to EC[135]	Produced by HUVEC and mononuclear cells in response to FXa[136]
IL10	Down-regulates IFNγ induced expression of adhesion molecules[137]	Inhibits activation of coagulation and fibrinolysis[138]
IL13	Induces expression of VCAM1 but not ICAM1 or E selectin on HUVEC[139]	
LacC	Up-regulation of Mac1 (CR3) on neutrophils[140] and ICAM-1[10] and PECAM-1 on endothelium[141]	Enhanced inactivation of FVa by protein C[128]
S1P	Increases ICAM1, VCAM1 and E selectin expression on HUVEC[142]	Exocytosis of WP bodies, releasing VWF[143]
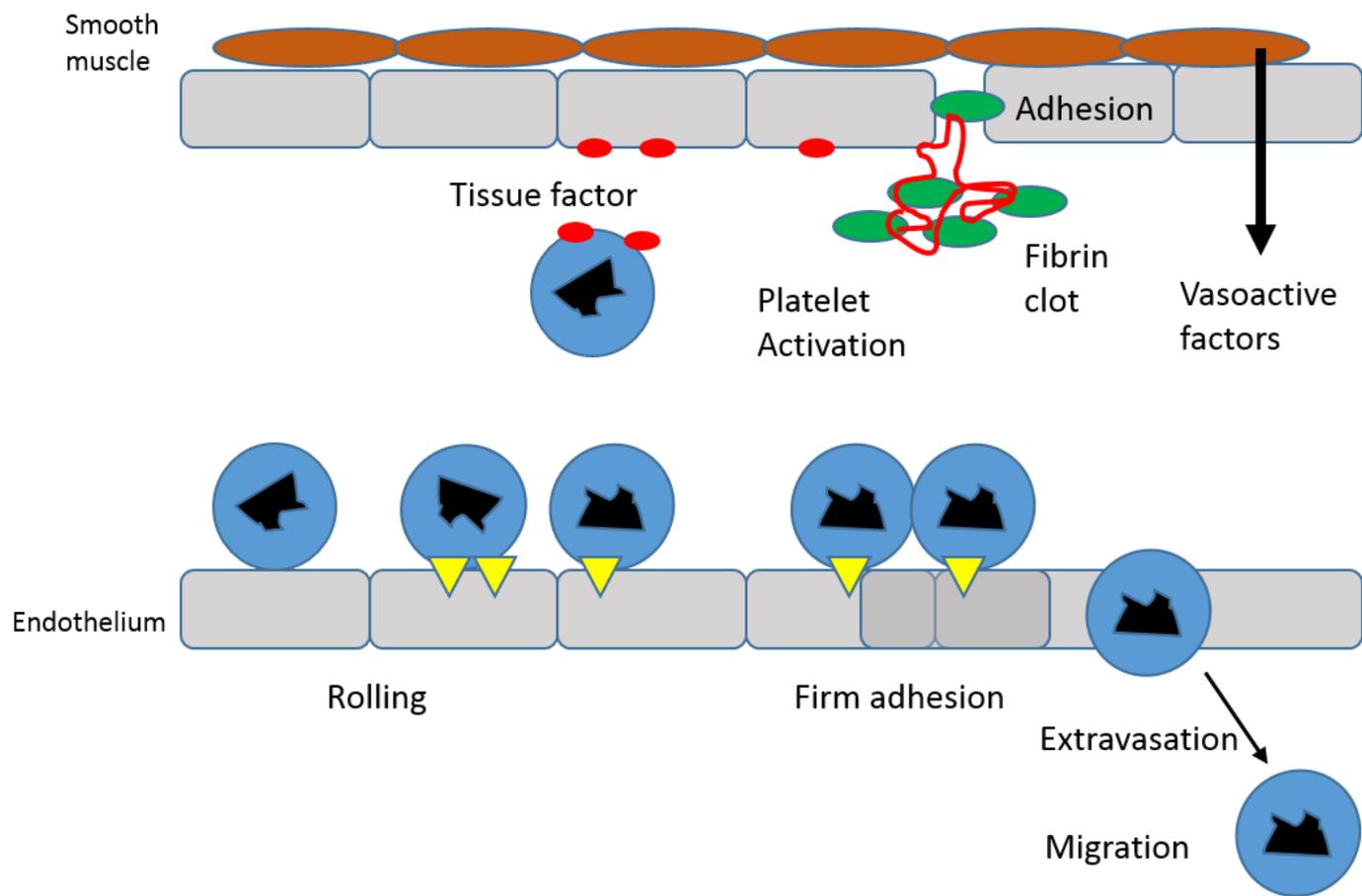
Figure 1-5: Potential vascular mechanisms of disease. Potential sites of abnormalities between the endothelium and blood components resulting in vascular manifestations of GD and FD.

1.8   Leucocyte adhesion and transmigration

The recruitment of activated leucocytes from the bloodstream to sites of inflammation is mediated by cell adhesion molecules expressed on leucocytes and the local endothelium (see **Table 1-8**). There are four key steps in this process: rolling, firm adhesion, extravasation and migration, regulated by processes which control activation or *de novo* synthesis of these receptors.

Table 1-8: Adhesion receptors involved in leucocyte-endothelial adhesion

Family	Role	Name	Tissue distribution	Ligand
Selectins	Bind carbohydrates. Initiate leukocyte-endothelial interaction. Mediate cell margination & slow rolling	P-selectin (PADGEM, CD62P)	Activated endothelium & platelets	PSGL-1, sialyl-Lewis^x
		E-selectin (ELAM-1, CD62E)	Activated endothelium	Sialyl-Lewis^x
		L-selectin (LECAM-1, CD62L)	Lymphocytes, monocytes, NK cells	CD34, GlyCAM
Integrins	Bind to cell-adhesion molecules and extracellular matrix. Strong adhesion. Ligands predominately for immunoglobulin superfamily receptors and complement	CD11a:CD18 (LFA-1, $\alpha_1\beta_2$)	Monocytes, T cells, macrophages, neutrophils, dendritic cells	ICAM-1, ICAM-2, ICAM-3, ICAM-4
		CD11b:CD18 (CR3, Mac-1, $\alpha_M\beta_2$)	Neutrophils, monocytes, macrophages	ICAM-1, iC3b, fibrinogen, FX
		CD11c:CD18 (CR4, p150.95, $\alpha_X\beta_2$)	Dendritic cells, macrophages, neutrophils	iC3b, fibrinogen
		CD49d:CD29 (VLA-4, $\alpha_4\beta_1$)	Monocytes, macrophages	Fibronectin, VCAM-1
Immuno-globulin super-family	Important for firm adhesion and leukocyte transmigration. Ligand for integrins	ICAM-1 (CD54)	Activated endothelium, leukocytes	LFA-1, Mac-1
		ICAM-2 (CD102)	Resting endothelium, dendritic cells, leukocytes	LFA-1
		ICAM-3 (CD50)	Leukocytes	LFA-1, $\alpha_D\beta_2$
		VCAM-1 (CD106)	Activated endothelium	VLA-4
		PECAM (CD31)	Activated leukocytes, endothelial cell-cell junctions	CD31
Scavenger receptors	Bind polyanionic ligands e.g. oxidised or acylated lipids	SRBI (CD36, Platelet GPIV, GPIIIb)	Platelets, monocytes, endothelium.	oxLDL, acLDL, LDL, HDL, collagen

Rolling

Selectins are they key molecules mediating rolling. They are membrane glycoproteins which bind specific carbohydrate moieties and are found on both leucocytes and

endothelial cells as well as platelets. Pre-formed P selectin is stored in Weibel-Palade bodies (WPB) of endothelial cells and appears on the endothelial surface within minutes of exposure to inflammatory mediators (e.g. C5a or histamine)[144]. TNFα and IL-1 also induce WPB exocytosis and additionally induce synthesis of E selectin, which appears on the endothelial surface within 2 hours[145]. Interactions between selectins and carbohydrates allow leucocytes to reversibly adhere to the endothelium, slowly rolling along the surface of the activated endothelium. This slow rolling permits the stronger firm adhesion interactions to occur; should these not occur then the leucocytes detach. Binding to selectins results in leucocyte arrest exposing them to chemokines and other stimuli in the endothelial microenvironment.

Firm adhesion

Firm adhesion involves interaction between integrins on leucocytes and endothelial ICAMs, with ICAM-1 and VCAM-1 thought to be the key mediators on the endothelial surface. Integrins are heterodimers, consisting of an α and β subunit; lymphocyte function-associated antigen 1 (LFA-1), complement receptor 3 (CR3) and complement receptor 4 (CR4) share a common β subunit (β_2, CD18). Normally leukocytes only adhere very weakly to the surface of endothelial cells as integrins exist on the cell surface predominately in an inactive conformation. Cytokines bound to proteoglycans on the surface of endothelial cells bind receptors on leucocytes triggering a calcium-dependent conformational change in the integrins: from a bent, compact form to an extended conformation[146]. Interactions between inflammation, coagulation and leucocyte adhesion are exemplified by CR3 which, in its activated conformation, also serves as a ligand for iC3b, coagulation factor X and fibrinogen. The main ligands of CR4 are C3b and fibrinogen. Leucocyte integrin mediated adhesion is cation dependent.

ICAM-1 is constitutively expressed at low levels on endothelial cells and leukocytes but expression is up-regulated by stimulation (e.g. TNFα or IL-1). ICAM-2 is constitutively expressed at high levels on endothelial cells and does not increase upon cell stimulation. VCAM-1 is not expressed on basal endothelial cells and is synthesised upon stimulation e.g. with TNFα or IL-1.

Extravasation and migration

Leukocyte extravasation also involves LFA-1 and CR3 as well as further adhesive interactions involving PECAM which is expressed both on the leukocyte and at the intercellular junctions of endothelial cells. These interactions enable leucocytes to squeeze between the endothelial cells. They then move through the basement membrane (diapedesis) with the aid of enzymes that break down the extracellular matrix proteins of the basement membrane. Migration through tissues to sites of inflammation occurs along chemokine gradients (e.g. IL-8).

Activators and regulators of leucocyte-endothelial adhesion

Up-regulation of adhesion activity can occur by three mechanisms (1) release of pre-formed stores; (2) conformational change/ increased functional activity and (3) *de novo* synthesis. Inflammatory mediators of this process are released by macrophages and include lipid mediators of inflammation (prostaglandins, leukotrienes and platelet-activating factor (PAF)) – rapidly produced by macrophages through enzymatic pathways that degrade membrane phospholipids. Their actions are followed by those of the chemokines and cytokines that are synthesised and secreted by macrophages in response to pathogens e.g. TNFα and IL1β, which activate endothelial cells. This localises adhesion to sites near inflammation. In unstimulated monocytes and granulocytes about 60% of CD11b and CD11c is located in peroxidase negative granules; stimulation by cytokines or chemotactic stimuli results in mobilisation of these stores, resulting in mRNA synthesis-independent up-regulation of adhesion molecules on the cell surface[147].

Scavenger receptors

Scavenger receptors are structurally heterologous cell surface receptors which are able to bind a wide range of polyanionic ligands, including oxidised and acetylated lipoproteins. CD36, a class B scavenger receptor, is expressed on monocytes, platelets and some endothelial cells. In addition to modified low density lipoproteins, CD36 also binds native lipoproteins: HDL, LDL and VHDL[148] and is thought to play a role in foam cell formation. Scavenger receptors have been implicated in the pathogenesis of atherosclerosis, diabetes and cardiomyopathies[149].

1.8.1 Cellular adhesion in FD & GD

In FD, studies have examined both cellular expression of adhesion molecules and levels of soluble adhesion molecules in the plasma (see **Table 1-9**). Levels of soluble adhesion molecules have been shown to correlate with cell surface expression[150] but do not indicate the source of these – leucocytes, endothelium or both.

Table 1-9: Adhesion abnormalities in FD

Study population	Sample	Intervention	ICAM-1	VCAM-1	P selectin	E selectin	Ref.
Immortalised cells from FD pt	Dermal microvascular endothelial	Gb3 loading	↑	↑		↑	64
		GB3 loading + TNFα	↑	→		→	
		ERT	↓	→		→	
Healthy donor primary cells	Microvascular (MiVEC) & macrovascular (MaVEC) endothelial cells	Gb3 loading	→	↑ (MiVEC) → (MaVEC)			151
		GLA silencing	→	→			
25 male FD, 15 controls	Plasma/serum	Not on ERT	↑	↑	↑	→	152
12 male FD, 15 controls	Plasma/serum	Not on ERT	→	↑	→	→	153

Studies have shown an increase in both VCAM-1 and ICAM-1, the key molecules involved in firm adhesion, as well as on some occasions increases in selectins. Increased expression of adhesion molecules in dermal microvascular cells from a patient with the R112H mutation was also accompanied by increased generation of reactive oxygen species which were implicated in the pathogenesis[64].

Immunophenotyping of monocytes from untreated male FD patients showed increased expression of CD11b and CD18, the two subunits of CR3, but no difference in expression of CD62L or CD49d[152]. Expression of PECAM-1 has also been found to be increased on leukocytes from a mixed population of treated and untreated male patients[154].

Studies of leucocyte adhesion in GD have been more limited. Analysis of chemotaxis of monocytes and neutrophils from GD1 patients has shown reduced neutrophil chemotaxis in 2/7 patients and reduced monocyte chemotaxis in 2/4 patients, associated with impaired phagocytosis[155]. A larger study involving 67 GD patients and 62 controls showed increased leucocyte and erythrocyte adhesion to a glass slide

surface[156]. Erythrocyte but not leukocyte adhesion was correlated with the presence of gammopathies and both were correlated with levels of complement C3. A recent study of erythrocytes[157] from 22 untreated GD patients showed increased erythrocyte adhesion to microvascular endothelial cells associated with increased expression of laminin α5, but without any increased expression of CD36 or CD49d on immunophenotyping. Additionally, a mouse model of neuronopathic GD, with reduced enzyme activity only in neural cells has found increased mRNA levels of VCAM-1 and ICAM-1 in the grey matter of the GD mice compared to controls, associated with up-regulation of TNFα[78].

1.9   Haemostasis

Haemostasis describes the processes whereby blood clot formation occurs at the site of a vessel injury resulting in sealing the break in the vessel wall. It is tightly regulated both in location and timing and encompasses a number of processes including: platelet adhesion and activation; activation of coagulation enzymes to form a stable fibrin clot; termination of clot formation by anti-coagulant pathways and clot dissolution by fibrinolytic pathways as well as alterations in vascular tone. Abnormalities in any of these processes can result in either bleeding or inappropriate thrombus formation.

Platelet plug formation

Platelets play a central role in haemostasis with functions including adhesion to the subendothelial matrix, platelet to platelet adhesion (aggregation), secretion of the contents of platelet granules and by providing a phospholipid surface for the assembly of coagulation factors enzyme complexes (procoagulant response). The initial step in all these processes is platelet activation with activators including thrombin[158] and collagen (potent agonists) and ADP and epinephrine (weak agonists). Tissue factor is a key activator of platelets, acting through the protease-activated receptors PAR-1 and PAR-4[159]. Following activation, platelets undergo conformational changes that facilitate their adhesion to the subendothelial matrix, primarily mediated by von Willebrand factor (VWF) via the GP1b/IX/V receptor, with additional adhesion to collagen. Formation of a stable clot involves cross-linking of platelets with VWF and fibrin via the integrin GPIIb/IIIa. The platelet procoagulant response involves the exposure of procoagulant phospholipids, primarily phosphatidylserine, on the outer

membrane of the platelet surface, on which coagulation factor complexes are then able to assemble.

The coagulation cascade and fibrin clot formation

A firm fibrin clot is formed as the end product of a series of enzymatic reaction catalysed by complexes of coagulation factors on the platelet surface. As with platelets, tissue factor is also the primary activator of thrombin formation *in vivo*[160]. Tissue factor (TF) may arise either due to exposure of the vascular adventitia following vascular damage or as the result of expression in endothelial cells or monocytes following activation by pro-inflammatory cytokines. TF forms a complex with FVIIa and binds FX, activating it to produce a small amount of FXa[161]. This FXa combines with FVa, primarily from platelet alpha granules, to form the prothrombinase complex which activates prothrombin to thrombin (initiation phase). Thrombin has a number of actions one of which is activation of FVIII, FXI and FV, resulting in increased formation of FXa by the tenase complex (FIXa and FVIIIa) and subsequently thrombin (amplification phase). Thrombin also converts fibrinogen to fibrin; fibrin forms polymers which are then cross-linked by FXIII to form a stable clot.

Termination of coagulation

To ensure localised and limited clot formation, anti-coagulant pathways exist to terminate the enzymatic reactions of the coagulation cascade. Anti-thrombin (AT) is a circulating protease inhibitor that inhibits the enzymes of the coagulation cascade, especially thrombin and FXa by forming irreversible complexes with them[162]. Tissue factor pathway inhibitor (TFPI) also circulates in the plasma but at far lower concentrations than AT and inhibits FX activation by binding to FVIIa-TF complexes and FXa[163].

The protein C-protein S pathway is activated by thrombin which binds to thrombomodulin (TM) on the surface of endothelial cells. This binding induces a conformational change in thrombin enabling it to activate protein C (PC). This activation of PC is enhanced by an endothelial receptor for PC (EPCR)[164]. Activated PC associates with protein S on the phospholipid surface and proteolytically activates FVa and FVIIIa. A mis-sense mutation at position 506 in FV, switching arginine to glutamine, termed the FV Leiden mutation, reduces its susceptibility to inactivation by PC[165].

Fibrinolysis

To restore normal vessel flow in the lumen, the clot must be degraded by a process known as fibrinolysis, mediated by the enzyme plasmin which cleaves fibrin polymers resulting in fibrin degradation products, of which D-dimers are a major component. Plasminogen binds to fibrin and is activated by tissue plasminogen activator (tPA), predominantly produced by the endothelium[166]. This process is regulated by plasminogen activator inhibitor 1 (PAI-1) which inhibits tPA and α2-antiplasmin which inhibits plasmin. Additional control of this process is provided by thrombin-activatable fibrinolysis inhibitor (TAFI) which is activated by the thrombin-TM complex and can bind to fibrin protecting it from degradation by plasmin.

1.9.1 Coagulation abnormalities in Fabry and Gaucher

Fabry disease

A number of plasma studies have been performed on FD patients looking for evidence of a procoagulant tendency (see **Table 1-10**). Increased soluble tissue factor has been correlated with disease severity and renal function in male patients[88]. The PC anti-coagulant pathway is the most investigated. Presence of the FV Leiden mutation in addition to a *GLA* mutation has been associated with increased fibrin deposition in a Fabry mouse model[167] and the presence of cerebrovascular disease[92] and poor renal outcomes[168] in FD patients. Additionally, detection of a lupus anticoagulant, which is associated with increased thrombotic events, in 33% of patients in one study[169], suggests an additional procoagulant mechanism.

Table 1-10: Procoagulant abnormalities in FD

No pt	25 M	12 M	17M, 19F	4M, 5F
TF			↑ (M)	(↑)
VWF	→		→	
PC				→
PS				→
TM	↓	→		
TAT			↑ (F)	
TFPI				↓
PAI	↑			
A2AP			→	
tPA	→		→	
Ref	152	153	88	170

Only limited studies have been performed on platelets in FD. Enhanced platelet aggregation to weak agonists has been reported in one study [171] with increased levels of β thromboglobulin, released from platelet α granules, found in this and a further study[88], although platelet factor 4 levels (also released from α granules) were normal. Increased levels of homocysteine, associated with increased risk of vascular events, have also been reported in two studies[153;170]

Coagulation abnormalities in GD:

Abnormalities of all aspects of coagulation have been described in GD but whilst bleeding problems are known to occur in patients, the lack of information regarding bleeding symptoms in most studies makes the clinical significance of these abnormalities difficult to interpret. The presence of bleeding at relatively high platelet counts led to further investigation of platelet function, although the presence of thrombocytopenia at presentation can present technical challenges. Platelet aggregation has been found to be abnormal in 22-50% of patients[172-174], predominately to low dose agonists, with some improvement noted following ERT. The mechanisms underlying the aggregation abnormalities are unknown. Although it has been hypothesised that glucocerebrosides in the plasma adsorb to the platelet membrane impairing function[175], incubation of normal platelets with glucosylceramide for three hours did not result in impaired aggregation[172], suggesting that, at least over this short time period, excess glucosylceramide did not affect aggregation.

Abnormalities of platelet membrane function have also been described. Assessment of platelet adhesion using a cone-plate analyser (Impact-R) revealed reduced adhesion in GD compared to controls, even in patients receiving ERT[118]. Mixing studies suggested that this was due to an intrinsic platelet defect with reduced adhesion being associated with the presence of mucosal bleeding. Abnormal platelet adhesion has also been described in two splenectomised GD patients where increased ristocetin induced platelet aggregation was found despite normal glycoprotein 1b and von Willebrand activity, resulting in a diagnosis of "pseudo-pseudo-Bernard-Soulier" syndrome[175].

Table 1-11: Coagulation abnormalities in GD: percentage of patients with coagulation protein abnormalities

No. patients	30	9	11	15	5	21	10	28
↑PT	42	11	-	47	80	-	100	81
↑APTT	38	55	100	53	100	-	60	-
↓FII	50	11	-	-	20	-	20	-
↓FV	87	22	18	23	100	-	30	27
↓FVII	33	-	9	8	0	-	50	-
↓FVIII	10	11	27	15	60	-	30	27
↓FIX	3	0	73	31	20	-	20	14
↓FX	57	-	9	8	20	-	10	-
↓FXI	27	33	-	0	40	-	0	36
↓FXII	30	-	-	15	25	-	10	27
↓PC	26	-	-	-	-	10	-	-
↓PS	11	-	-	-	-	-	-	-
↓AT	3	-	-	-	-	0	-	-
↑D dimer	68	-	-	-	-	76	-	-
↑TAT	46	-	-	-	-	43	-	-
Reference	119	176	177	174	178	179	180	181

Multiple studies have reported the presence of a prolonged prothrombin time (PT) and/or activated partial thromboplastin time (APTT). Measurement of coagulation factor activity has revealed decreased activity of multiple coagulation factors, with sometimes multiple deficiencies in the same patient, albeit mostly mild (see **Table 1-11**). Two studies which re-measured factor activity following ERT noted improvements in a number of patients [174;180]. Although reduced FXI activity has been hypothesised to be the result of co-inheritance of mutations in the *F11* gene due to the high carrier rate of both disorders in the Ashkenazi Jewish population[182], the presence of multiple factor deficiencies suggests a more global process affecting synthesis, consumption or both.

Variable coagulation factor activity in some patients led to the hypothesis of glycosphingolipid interference with phospholipids in coagulation factor activity assays[176] although measurement of antigen levels showed that these correlate with measured activity[119]. The additional finding of decreased levels of anticoagulant proteins and increased products of fibrinolysis has led to the hypothesis of chronic activation of coagulation resulting in a consumptive coagulopathy [119;179]. Raised D-dimer levels have been correlated with severity of bone disease[183].

1.10   Aims of project

This project explores the disease manifestations of FD and GD which are associated with abnormalities at the blood-endothelial interface, predominately bleeding events in GD and vascular complications in FD. The first half of this project examines these disease manifestations in the presentation and complications of GD and FD within the large patient cohorts of the Lysosomal Storage Disorders Unit at the Royal Free Hospital. It explores the role of these manifestations in the diagnosis of these disorders and offers insights into how the diagnostic process might be improved. It explores the manifestations in relation of other clinical features of these disorders in order to explore potential common underlying mechanisms. The second half of the project details laboratory investigation of cellular abnormalities in leukocyte-endothelial adhesion and cellular support of haemostasis using primary cell isolates from patients and through development of an endothelial cell model of disease to explore the contribution of cellular dysfunction to these processes.

1.10.1 Hypotheses

The central hypothesis of this thesis is that vascular events constitute an important part of the pathology of FD and GD and therefore furtherance of our understanding of these events, from both an epidemiological and pathophysiological stand point, will advance patient care. Specific hypothesis tested are as follows:

Chapter 3: Bleeding features in the presentation of GD

1.   Patients presenting with GD most commonly are diagnosed by haematologists by bone marrow biopsy. *Rationale:* GD is rare and therefore not commonly considered in the differential diagnosis of thrombocytopenia/splenomegaly; consequently it is diagnosed when bone marrow biopsies are performed for other possible diagnoses

2.   Bleeding severity is associated with overall disease severity and the presence of gammopathies and bone infarcts. *Rationale:* the bleeding diathesis has been proposed to arise due to chronic activation of the coagulation system as a result of chronic inflammation

3.   FXI deficiency is a manifestation of GD rather than genetic co-inheritance and improves with ERT *Rationale:* the wide range of factor deficiencies described in prior

studies suggests a disease-related mechanism rather than inherited deficiencies of coagulation factors.

Chapter 4: Vascular features of FD

1.   Patients presenting with FD may experience vascular events (e.g. stroke, arrhythmias) prior to diagnosis and experience long delays between those events and arrival at the correct diagnosis *Rationale:* FD is rare and therefore not commonly considered in the differential diagnosis of vascular events such as stroke or atrial fibrillation

2.   Cerebrovascular events are not predicted by other disease manifestations, enzyme activity or more common cardiovascular risk factors *Rationale:* strokes have been reported in patients without other disease manifestations and in cohort of patients with cryptogenic stroke

3.   Abnormalities of plasma procoagulant and anticoagulant factors are associated with vascular manifestations of FD *Rationale:* von Willebrand factor and anticoagulant pathways are controlled primarily by the endothelium and these disease manifestations have been hypothesised to be due to endothelial dysfunction.

Chapter 5: Cellular adhesion in FD and GD

1. Increased expression of leukocyte adhesion molecules occurs in FD and GD and is associated with vascular manifestations in FD and the presence of bleeding and gammopathies in GD. *Rationale:* FD and GD are associated with a pro-inflammatory state which results in up-regulation of adhesion molecules

2. Lipid loading of endothelial cells, but not reduction in enzyme activity alone results in increased endothelial adhesion activity. *Rationale:* results to date suggest that Gb3 excess rather than GLA deficiency is responsible for endothelial dysfunction. Glycosphingolipids LacC and S1P have been found to up-regulate adhesion molecule expression and other glycosphingolipids may also perform this function.

Chapter 6: Cellular support of haemostasis in FD and GD

1. Monocytes from FD and GD have increased tissue factor expression and are procoagulant. *Rationale:* tissue factor expression is induced by pro-inflammatory

cytokines. FD is associated with a procoagulant state and laboratory studies in GD suggest that chronic activation of coagulation occurs.

2. The platelet membrane in FD and GD has altered procoagulant capacity. *Rationale:* glycosphingolipid accumulation within lipid rafts in the platelet membrane may also membrane dynamics required for assembly of coagulation enzyme complexes (e.g. prothrombinase)

3. The endothelium in FD is procoagulant and supports increased thrombin generation. *Rationale*: microvascular lesions occur in FD and have been hypothesised to be due to increased activation of coagulation.

Chapter 2   Methods

Except where indicated, all experiments were performed by myself. For solutions and buffers, where no supplier is listed these were made in house and full details of their production are listed in Appendix 2. All suppliers of materials were based in the UK.

2.1   Patient and healthy control recruitment

The study received ethical approval from the National Research Ethics Committee (NREC number: 12/LO/0271).   Patients and controls gave written consent for participation in the study. Healthy controls were recruited from departmental members and unaffected relatives/partners accompanying patients to clinic.   The patient information leaflet and ethics approval can be found in Appendix 1.

2.2   General cell culture methods & equipment

All plate readings were performed on a BMG FluoSTAR Galaxy fluorimeter (BMG Lab Tech).

2.2.1   Cell lines

Tissue culture work was performed in a class II biosafety cabinet. Cells were cultured in an incubator at 37°C, 5% CO_2 (HERAcell 150, Kendro)

The RAW 264.7 cell line was obtained from ATCC. RAW264.7 is a semi-adherent murine monocyte-macrophage cell line, established from ascites of a tumour induced by intraperitoneal injection of Abselon Leukaemia Virus into a male mouse. RAW 264.7 were cultured in R10 medium, consisting of RPMI 1640, supplemented with 10% fetal bovine serum (FBS, Life Technologies), 10mM HEPES (Life Technologies), 100 units/ml penicillin, 100µg/ml streptomycin (Life Technologies) and 2mM L-glutamine (Life Technologies). Cells were harvested from tissue culture plastic (TCP) using 0.02% EDTA (Sigma) supplemented with 1mg/ml of lidocaine (Sigma). Cells were passaged every 3-4 days, split 1:3-1:5 and used up to 30 passages.

The EA.hy 926 cell line was obtained from ATCC. It is a human umbilical vein endothelial cell (HUVEC) line established by the fusion of HUVEC with a thioguanine-resistant clone of the A549 adenocarcinoma cell line by exposure to polyethylene glycol[184]. The EA.hy 926 cell line was cultured in high glucose (4.5g/L) Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% FBS, 10mM HEPES, 100 units/ml penicillin, 100µg/ml streptomycin and

2mM L-glutamine. Cells were harvested from TCP with 0.25% trypsin-EDTA solution (Sigma) and were passaged weekly, split 1:10-1:16 and used up to 30 passages.

The HEK293T cell line was obtained from ATCC for use as a packaging cell line for the generation of lentiviral particles. It is a human embryonic kidney (HEK) cell line, derived from the HEK293 cell line into which the simian virus 40 (SV40) T cell antigen has been inserted. The cells were cultured in high glucose DMEM supplemented with 10% FBS, 10mM HEPES, 100 units/ml penicillin, 100μg/ml streptomycin and 2mM L-glutamine. Cells were harvested using 0.25% trypsin-EDTA solution and passaged every 2-3 days, split 1:4.

2.2.2 Cell counts and viability

Cells were enumerated utilising a haematocytometer. Cell viability was measured by Trypan blue (Sigma) dye exclusion and viability of cell lines maintained at >95%.

2.2.3 MTT dye reduction assay

Thiazolyl blue tetrazolium bromide (MTT) is a tetrazolium compound which is reduced by the mitochondrial dehydrogenases NADPH & NADH in metabolically active cells to a water-insoluble but DMSO soluble blue MTT-formazan crystals.

Cells cultured in 96 well plates were exposed to drugs for 24-72 hours prior to the assay being performed. MTT (Sigma Aldrich) was dissolved in appropriate medium DMEM at 5mg/ml. 10μl per well of MTT suspension was added and the plate incubated at 37°C for 2 hours. The medium was carefully aspirated from the wells and 100μl per well of DMSO (Sigma) added. The plate was placed on a plate shaker at 150rpm for 30 minutes, until the crystals were fully dissolved. The resulting absorbance was read at 570nm.

2.3 Sample preparation

2.3.1 Separation of peripheral blood monocyte-enriched mononuclear cells

A monocyte-enriched mononuclear cell fraction was isolated using an iodixanol flotation barrier to produce a monocyte enriched, platelet poor cell fraction. 54% iodixanol was prepared by the addition of 0.6 volumes of 8.5% NaCl solution to 5.4 volumes of 60% iodixanol (Optiprep, Sigma). An iodixanol gradient with a density of

1.074g/ml was prepared by the addition of 6.3 volumes of 60% iodixanol to 23.7 volumes R10 medium.

9ml of blood was collected into a sterile 15ml centrifuge tube containing 1ml of 3.2% sodium citrate solution and inverted to ensure adequate mixing. 2ml of 54% iodixanol was added to the citrated blood to increase its density to approximately 1.105g/ml. 4ml of the increased density blood was added to each fresh 15ml Falcon tube and overlaid with 6ml of the 1.074 iodixanol gradient. The gradient was overlaid with 500µl of R10, to prevent the monocytic layer interfacing with air. The sample was centrifuged at 700g for 30min at 4°C. Following gradient centrifugation, the uppermost band, containing monocyte enriched mononuclear cells was removed with a sterile syringe and quill. The isolated cells were washed once in 8.5% NaCl and re-suspended in 3ml of R10 medium.

2.3.2 Preparation of human serum

5ml of whole blood was collected into a 5ml Gold SST vacutainer (Becton Dickinson - BD) and allowed to clot for 30min at 4°C. The samples were centrifuged at 2000g, 5min, 4°C. 100µl serum was used to make heat inactivated autologous serum by heating at 56°C for 1 hour in a waterbath. The remained was aliquotted and stored at -80°C until use.

900µl of RPMI 1640, supplemented with HEPES, penicillin/streptomycin and L-glutamine was added to 100µl serum to make 10% autologous medium for use in adhesion assays.

2.3.3 Preparation of pooled human serum

Healthy volunteers were bled into 5ml Gold SST vacutainers (BD). The samples were clotted at 4°C for 30min and then centrifuged at 2000g, 5min, 4°C. The serum was aspirated and serum from all samples pooled. The serum was heat inactivated at 56°C in a waterbath for 1 hour. The serum was then aliquoted and stored at -80C prior to use.

2.3.4 Preparation of plasma

Whole blood was collected in 2.7ml citrate vacutainers (BD). For single spun plasma, vacutainers were centrifuged at 2500g, room temperature for 20minutes. The top 2/3 of plasma was aspirated, aliquoted and stored at -80°C until use. For triple spun

plasma, after the initial centrifugation step, the aspirated plasma was centrifuged a further time at 2500g, room temperature for 20 minutes then the top 2/3 plasma aspirated and centrifuged at 14000rpm for 3min using a mini-Eppendorf fixed centrifuge. Triple spun plasma was stored at -80°C until use.

2.3.5 Preparation pooled human plasma

Healthy volunteers were bled into 2.7ml citrate vacutainers and centrifuged at 3500g, room temperature for 20 minutes. The top 2/3 plasma from each sample was collected and the samples were pooled. The plasma was then prepared as for triple spun plasma above, aliquoted and stored at -80°C until use.

2.3.6 Preparation of platelet rich plasma

Blood was collected in 2.7ml citrate vacutainers and centrifuged at 160g, room temperature for 10 minutes. The platelet count was measured using a Sysmex XS-1000i bench top analyser.

2.4   Drugs and lipids stock solutions

2.4.1 Enzyme inhibitors

Amiodarone hydrochloride was obtained from the Royal Free Hospital Pharmacy as a sterile concentrate 50mg/ml in 3ml ampoules. A 50mM stock solution was made by the addition of 1.65ml of sterile water to 3ml of amiodarone concentrate. The solution was aliquotted and stored at -20°C

A 50mM stock solution of chloroquine was made by dissolving 300mg of chloroquine hydrochloride (Sigma) in 11.63ml of sterile water. The solution was sterile filtered, aliquotted and stored at -20°C.

A 100mM stock solution of 1-deoxygalactonijorimycin (DGJ) was made by dissolving 20mg of 1-deoxygalactonijorimycin hydrochloride (Carbosynth) in 1ml of sterile water. The solution was aliquotted and stored at -20°C.

A 100mM stock solution of conduritol β epoxide (CBE) was made by dissolving 5mg of conduritol β epoxide (Sigma) in 308μl of sterile water. The solution was aliquoted and stored at -20°C.

2.4.2 Glycosphingolipids

Stock concentrations of lipids were made by dissolving lipids in methanol. Stocks were aliquoted and stored at -80°C. Stocks were all 1mM, except for sphingosine (10mM). For globotriaosylceramide, hot methanol was used and the sample warmed prior to use to ensure the lipid was re-solubilised. The following lipids were used: globotriaosylceramide (Gb3, Matreya LLC), globotrioasylsphingosine (lysoGb3, Matreya LLC), lactosylceramide (bovine buttermilk, Matreya LLC), glucosphingosine (plant, Santa Cruz Biotechnology), sphingosine (ENZO life sciences) and sphingosine-1-phosphate (Sigma).

2.4.3 Antibiotics

A stock solution of 10mg/ml puromycin dihydrochloride (Sigma) in distilled water was made, aliquotted and stored at -20°C. A stock solution of 100 mg/ml of ampicillin sodium (Sigma) was made in distilled water, aliquotted and stored at -20°C.

2.5 Generation of lenitviral shRNA knockdown endothelial model

An endothelial cell model of Fabry disease was created using small-hairpin RNA targeting the alpha galactosidase A mRNA sequence, delivered to cells using a replication incompetent lentiviral vector. An application was submitted to and approved by the UCL Safety Committee to conduct work involving genetically modified organisms (experiment number 1556 RF – see Appendix 3) and work was conducted in accordance with departmental guidelines. Plasmids were obtained from the UCL Cancer Institute shRNA library; the GIPZ plasmids are also available commercially from the Thermo Scientific Open Biosystems Human GIPZ shRNAmir lentiviral library. The shRNA sequences and vector maps are detailed in 5.4.3.

2.5.1 Isolation of plasmid DNA

Plasmids were supplied as bacterial stabs. A small amount of bacteria was streaked on an Agar plate, supplemented with 100μg/ml of ampicillin, and incubated at 37°C overnight. The following day, a single colony was picked with a pipette tip and transferred to 5ml of LB broth, containing 100μg/ml ampicillin. The broth was cultured overnight at 37°C with 200rpm shaking. Plasmid DNA was isolated using a QIAprep® Spin Miniprep kit (Qiagen), following the manufacturer's instructions.

2.5.2 Quantification of DNA

The DNA was quantified spectrophotometrically using a Nanodrop 1000 (Thermo Scientific). 1.5µl of sample was placed on the pedestal and the DNA quantified based on absorbance of UV light. The purity was ascertained from the ratio of the OD at 260nm to 280nm. Where higher than 250ng/µl, the DNA concentration was adjusted to 250ng/µl using molecular grade distilled water (Sigma). DNA was stored at -80°C until required.

2.5.3 Confirmation of packaging vectors by restriction digest

A restriction digest was performed of the packaging vectors (p8.91 and pMDG) to ensure the complete plasmid was present (**Figure 5-3**). A single digest was also performed to linearise the DNA. 500ng of plasmid DNA was added to an Eppendorf followed by 1µl of the appropriate restriction endonuclease (EcoR1, Bgl II or BamH1, New England Biolabs) and 2µl of the correct buffer supplied with the endonucleases (New England Biolabs). The reaction volume was made up to 20µl with distilled water and the incubated at 37°C for 30 minutes.

A 1% agarose gel was made by dissolving 1g of agarose (Bioline) in 100ml of TBE buffer. Once cooled slightly 5µl of 10mg/ml ethidium bromide (Sigma) was added in a fume hood and the gel poured into a template. The set gel was transferred to the gel tank and the tank filled with 1% TBE buffer. 5µl of loading dye (Thermo Scientific) was added to each reaction mixture. 2.5µl of sample was added per well of the gel. 5µl of Hyperladder I molecular weight marker (BioLine) was added to appropriate wells. The gel was run at 100V for 75 minutes and the bands visualised under UV light (BioRad Gel Doc XR+).

2.5.4 Sequence confirmation of shRNA segment of GIPZ plasmids

Sequencing of the GIPZ plasmids was undertaken to ensure that the correct shRNA insert was present. The pGIPZ primer (Sigma) sequence is (5' to 3'): GCATTAAAGCAGCGTATC. 250ng of plasmid DNA and 1µl of 10µM pGIPZ primer were added to a well of a 96 well sequencing plate (Life Technologies) and the volume made up to 8µl with 10mM Tris/0.01mM EDTA, pH 8.0 (Sigma). The sample was denatured by heating to 98°C for 5 minutes on a thermal cycler (BioRad C1000). The sample was cooled on ice for 7 minutes prior to the addition of 2µl of BigDye v1.1 (Life Technologies). The DNA was the amplified by polymerase chain reaction (PCR)

on the thermal cycler at 96°C for 20 seconds, 50°C for 5 seconds, 60°C for 120 seconds, for 40 cycles. The 10μl of reaction mixture was then transferred to a DyeEx clean-up plate (Qiagen) and centrifuged at 1000g for 3 minutes to remove the excess dye terminators. The samples were then air dried at 70°C for 1 hour and then re-suspended in 10μl of HiDi-formamide (Life Technologies).

The DNA was sequenced on an ABI 3500 automated sequencer (Life Technologies) using the standard run module. The sequences analysed using Mutation Surveyor Software v3.3 (SoftGenetics) and compared to the sequences supplied by the manufacturer.

2.5.5 Lentiviral vector production

Replication incompetent lentiviral particles containing the shRNA sequence of interest were generated using a third generation packaging system in HEK293T cells. The three plasmids used comprised:

1. P8.91: the packaging vector containing a minimal set of genes required to generate the virion structural proteins
2. pMDG: vesicular stomatitis virus G protein envelope vector
3. pGIPZ: shRNA vector containing the shRNA sequence of interest as well as puromycin resistance gene and GFP gene.

On the day prior to transfection (D-1), HEK293T cells were seeded at sub-confluence on 10cm tissue culture treated plates and cultured overnight at 37°C.

The following day (D0), 1μg of p8.91 DNA, 1μg of pMDG DNA and 1.5μg of pGIPZ DNA were mixed together in an Eppendorf. In a separate Eppendorf 10μl of Fugene 6 (Promega) was added to 200μl of Optimem (Invitrogen). The DNA mixture was added to the Fugene/Optimem mix and left to stand at room temperature for 15 minutes. The medium on the HEK293T cells was changed with fresh pre-warmed medium and the DNA/Fugene/Optimem mix added to the plate. The plate was incubated overnight.

On D+1, the medium on the HEK293T plates was changed. On D+2, the medium contain the virus particles was collected and passed through a 0.2μm sterile filter (Sartorius). Fresh medium was added to the HEK293T plates and further virus

collected on D+3 and D+4. Virus was stored overnight at 4°C prior to use and surplus virus aliquoted and stored at -80°C.

2.5.6 Infection of EA.hy 926 cells with lenitviral vector

On D-1, EA.hy cells were seeded at 100 000 per well in 6 well plates (Corning) and cultured overnight. On D0, the medium was changed to antibiotic and serum free DMEM. 1.5ml of virus was added per well. To increase the infection rate, 8µg/ml of hexadimethrine bromide (polybrene, Sigma) was added and the plate centrifuged for 1 hour at 500g, room temperature prior to incubation overnight at 37°C. On D+1, medium was replaced with fresh DMEM containing serum and antibiotics. On D+2, successful viral infection was confirmed by visualisation of GFP expression under a fluorescent microscope (Nikon Eclipse TS100). Puromycin selection of infected cells was commenced using a dose of puromycin pre-determined by MTT assay to result in death of uninfected cells over 3-6 days. shRNA knockdown was confirmed by measurement of GLA enzyme activity and protein levels. Cells cultures were maintained in puromycin containing medium.

2.6 Flow cytometry

2.6.1 General flow cytometry method

Monoclonal antibodies were used as direct conjugates with fluorescein isothiocyanate (FITC), AlexaFluor® 488, phycoerythrin (PE), peridinin-chlorophyll-protein complex (PerCP) and allophycocyanin (APC). $1-10 \times 10^5$ cells per aliquot were incubated in the dark with the designated panel of antibodies for 30mins before being washed and re-suspended in PBS. Samples were acquired on a FACS Calibur (BD), with 5000-10 000 events being recorded and analysed with CellQuest (BD) or FlowJo (Tree Star).

Table 2-1: Antibodies used in flow cytometry

Target	Species	Ig class	Product code	Company	Clone	Conjugate	Dilution
CD11a	Mouse	IgG2a κ	555379	BD	G43-25B	FITC	1:10
CD11b	Mouse	IgG1κ	301306	Biolegend	ICRF44	PE	1:10
CD11c	Mouse	IgG1κ	301614	Biolegend	3.9	APC	1:10
CD50	Mouse	IgG2b κ	555958	BD	TU41	FITC	1:10
CD54	Mouse	IgG1κ	353112	Biolegend	HA58	APC	1:10
CD102	Mouse	IgG1κ	328506	Biolegend	CBR-IC2/2	PE	1:10
CD62L	Mouse	IgG1κ	304816	Biolegend	DREG-56	AlexaFluor 488	1:20
CD49d	Mouse	IgG1κ	304304	Biolegend	9F10	PE	1:10
CD142	Mouse	IgG1	FAB23391G	R&D systems	323519	AlexaFluor 488	1:20
CD36	Mouse	IgG2aκ	336208	Biolegend	5-271	APC	1:10
CD204 (SR-AI)	Mouse	IgG2b	FAB2708P	R&D systems	351615	PE	1:10
CD14	Mouse	IgG2b κ	345786	BD	MφP9	PerCP	1:10
CD14	Mouse	IgG2b κ	345785	BD	MφP9	PE	1:10
CD3	Mouse	IgG1κ	345766	BD	SK7	PerCP	1:10

2.6.2 Quantification of monocyte percentage by flow cytometry

50μl of monocytic fraction isolated over an iodixanol gradient was placed in a round bottom tube (BD). 5μl of CD14 PE and 5μl of CD3 PerCP was added and the sample incubated in the dark for 30 minutes. The % of monocytic cells was ascertained from the percentage of CD14 positive cells within a gate set to include monocytes and lymphocytes based on their forward and side scatter properties, as detailed in section **5.4.2**.

2.6.3 Flow cytometry of D0 monocytes for adhesion receptors and tissue factor

50μl of the monocytic fraction was placed in each of 4 round bottomed tubes (BD) and the panels of antibodies added (see Table 2-2). Further analysis was undertaken after data collection using FlowJo to determine the mean fluorescent intensity of the adhesion receptor in different mononuclear cell populations.

Table 2-2 Antibody panels for D0 monocyte adhesion

	Tube 1	Tube 2	Tube 3	Tube 4
FITC/AlexaFluor®488	CD11a	CD50	CD62L	CD142
PE	CD11b	CD54		CD36
APC	CD11c	CD102	CD49d	CD204
PerCP	CD14	CD14	CD14	CD14

2.7   Adhesion assays

2.7.1   Monocyte adhesion to tissue culture plastic

The monocyte fraction isolated over an iodixanol gradient was adjusted to a concentration of $1x10^6$ monocytes/ml. Cells were re-suspended in either 10% heat inactivated autologous serum 10% heat inactivated pooled human serum. 10^5 cells were added per well of a 96 well TCP plate (Corning) with or without addition of 5mM EDTA. All conditions were performed in triplicate. The plate was incubated at 37°C for 1 hour to allow the monocytes to adhere.

The plate was washed three times with PBS to remove non-adherent cells. 100µl of 100% methanol was added per well and the cells fixed overnight at 4°C. The plate was washed once with PBS and 100µl per well of 20% Giemsa stain (Sigma) added. The plate was left one hour to stain, then washed three times with tap water. The Giemsa was solubilised by adding 100µl per well of 100% methanol and placing the plate on a plate shaker for 30 minutes. Absorbance was read at 570nm. The adherence of monocytes was reported as the ratio of the mean absorbance of the test sample to the mean absorbance of the pooled human serum.

2.7.2   Adhesion of RAW cells to EAhy cells

EA.hy cells were seeded at 40 000 per well (for 3 day cultures) or 20 000 per well (for 7 day cultures) with additional drugs (lipids/ enzyme inhibitors) added where indicated, and cultured at 37°C. For 7 day cultures, the medium was changed after 3 days. On the day of the adhesion assay, where indicated, EA.hy were stimulated with recombinant human tumour necrosis factor alpha (TNFα, Life Technologies) at the doses and durations indicated.

RAW cells were harvested from tissue culture plastic using 0.02% EDTA supplemented with 1mg/ml of lidocaine the day prior to the adhesion assay. They were then cultured overnight on 10cm bacteriological plastic petri-dishes to allow recovery of adhesion receptors. For the adhesion assay, RAW were harvested using 5mM EDTA and re-suspended at $1x10^6$/ml in serum free medium. 5µl per ml of Vybrant ® DiI cell labelling solution (Life Technologies) was added and the cells incubated at 37°C for 5 minutes followed by 15 minutes at 4°C. DiI is a fluorescent

lipophilic carbocyanine dye. The cells were then washed three times in serum-containing DMEM and re-suspended at 2×10^6/ml.

Medium was removed from the EA.hy plate and replaced with 100μl per well of DiI labelled RAW. Under some conditions, 5μl per well of 100mM EDTA was also added. The plate was incubated at 37°C for 1 hour and then washed three times with PBS to remove non-adherent cells. Fluorescence was measured at excitation 530nm, emission 590nm.

2.8 Cell staining techniques

EA.hy cells for cell staining were grown on 13mm No1 glass coverslips (Scientific Laboratory Supplies). Coverslips were dipped in ethanol and flame sterilised over an electric Bunsen burner. EA.hy cells were seeded 100 000 per well of a 24 well plate (Corning) and cultured for 7 days. Prior to staining, coverslips were washed three times in PBS prior to fixation in methanol or 4% paraformaldehyde as indicated below.

2.8.1 Giemsa staining

The cells were fixed for 20 minutes in methanol then stained with 5% filtered Giemsa for 45 minutes. The coverslips were rinsed in tap water and dried before being mounted face down in DPX Mountant (Sigma) on glass microscope slides (VWR International).

2.8.2 Fluorescent staining for von Willebrand factor

Cells were fixed in 4% paraformaldehyde for 20 minutes then washed twice with PBS. The cells were permeabilised with 0.1% Triton in PBS for 10 minutes, washed and blocked by incubating with 3% BSA in PBS for 1 hour at room temperature. Primary antibody staining was performed with a 1:200 dilution of polyclonal rabbit anti-human VWF (A0082, Dako) and incubated for 1 hour at room temperature. The coverslips were washed three times with PBS and secondary antibody staining performed with Texas Red conjugated goat anti-rabbit antibody (1:200 dilution, GeneTex) for 1 hour at room temperature. The coverslips were washed three time in PBS then nuclear staining performed with 1:1000 dilution of 4',6'-diamidino-2-phenylindole (DAPI, Life Technologies), 1 minute incubation. The coverslip was washed and mounted face down on a glass slide in Prolong Gold mountant (Life Technologies).

2.8.3   Cellular uptake of DiI labelled acetylated LDL

EA.hy cells were grown to confluence on glass coverslips in a 24 well plate. On the day of the assay, human DiI labelled acetylated LDL (Intracell) was made up in medium at a concentration of 10µg/ml. The medium was aspirated from the wells and replaced with 300µl of DiI acLDL containing medium. The cells were incubated at 37°C for 4 hours. The coverslips were removed from the 24 well plate, washed and fixed in 4% paraformaldehyde before being mounted in Prolong Gold mountant.

2.8.4   Imaging

Slides were imaged using a Nikon Eclipse TS100 microscope and Nikon DsFi1 camera. Imaging of fluorescently stained slides was performed on a Leica TCS SP5 confocal microscope with assistance of David Westmoreland, MRC Laboratory of Molecular Cell Biology, UCL.

2.9   Enzyme assays

2.9.1   Cell lysate preparation

For cell lines, the medium was aspirated from confluent monolayers of cells in 96/24 well plates 50/150µl ProtoJet Mammalian Cell lysis solution (Fermentas) added. The plate was placed on a plate shaker, 150rpm for 15 minutes, following which the cells were disrupted with a pipette tip to ensure fully lysed and the lysate collected into an Eppendorf. The sample was centrifuged at 13 000rpm for 15 min and the supernatant used in assays or stored at -80°C until required.

For patient samples, 10ml blood was collected into a vaccutainer containing EDTA (BD). 5ml of Lymphoprep (Axis Shield) was added to the bottom of a 15ml centrifuge tube (Falcon) and overlaid with 10ml of whole blood. The sample was centrifuged at 650g, 4°C for 25 minutes, brake off. The mononuclear layer was aspirated into a 1.7ml Eppendorf and centrifuged at 8000rpm, 2 minutes. The supernatant was aspirated. The cell pellet was re-suspended in 1ml of red cell lysis solution and left for 5 minutes at room temperature. The sample was centrifuged at 8000rpm, 2 minutes, the supernatant discarded and the pellet re-suspended in 1ml PBS. After further centrifugation 8000rpm, 2 minutes, the PBS was discarded and the pellet stored at -80°C. On the day of enzyme assays/protein quantification the pellet was thawed and re-suspended in 800µl of Mammalian cell lysis solution. The sample was placed on a

plate shaker at 150rpm for 15 minutes and then centrifuged at 13 000rpm for 15 minutes. The supernatant was used for protein quantification or enzyme assays, usually after a 1:3 dilution in distilled water.

2.9.2 Protein quantification

Protein quantification was performed using a Bicinchoninic Acid (BCA) Protein Assay kit (Sigma). This is based on the method of Lowry [185] and relies on the formation of a Cu^{2+}-protein complex under alkaline conditions, with subsequent reduction of Cu^{2+} to Cu^{1+}. The amount of reduction is proportional to the amount of protein present. BCA forms a purple-blue complex with Cu^{1+} under alkaline conditions with an absorbance of 560nm.

The assay was performed in a 96 well plate. A bovine serum albumin (BSA) standard supplied in the kit was used to construct a standard curve across the linear range of the assay (0-1000μg/ml). 25μl of test or standard samples were added to wells in duplicate. BCA working reagent was made up by mixing 1 part of 4% copper (II) sulphate pentahydrate solution with 50 parts of bicinchionic acid solution (bichionic acid, sodium carbonate, sodium tartrate and sodium bicarbonate in 0.1N NaOH, pH 11.25). 200μl of working solution was added to each sample well and the plate incubated at 37°C for 30 minutes. Absorbance was read at 560nm and the test sample protein concentrations read off the standard curve.

2.9.3 General method for enzyme activity measurement

Assays were performed in 24 well plates (PlateMax 24, 4titude), laid out as summarised in **Table 2-3**. 150μl of substrate-inhibitor working solution was added to wells 3-5 and 15μl of sample of known protein concentration to wells 4 & 5. The plate was incubated at 37°C for 1 hour. After the incubation, 1000μl of 1M glycine stopping solution was added to wells 3-5. 200μl of 4-methylumbeliferone standard working solution was added to well 1 and 200μl of distilled water to well 2. 950μl of 1M glycine was added to wells 1&2 and 15μl of sample to wells 1-3. The fluorescence was then measured at excitation 365nm, emission 450nm

.**Table 2-3: Layout of enzyme assays**

Additions	1	2	3	4	5
	Standard	*Standard blank*	*Substrate blank*	*Sample reaction*	*Sample reaction*
Pre-incubation	Nil	Nil	150µl substrate	150µl substrate 15µl sample	150µl substrate 15µl sample
After 1hr incubation			1000µl Glycine	1000µl glycine	1000µl glycine
After glycine	15µl sample 200µl standard 950µl glycine	15µl sample 200µl ddH₂O 950µl glycine	15µl sample		

The enzyme activity (nmol/hr/mg protein) was then calculated by the equation:

$$Enzyme\ activity\ = \frac{Fluorescence\ of\ sample}{Fluorescence\ of\ standard} \ x \ \frac{1000}{15} \ x \ \frac{1}{protein\ conc\ (\frac{mg}{ml})}$$

Where the fluorescence of the sample equals the average test reading of the sample (4 & 5) minus the substrate blank (3) and the fluorescence of the standard = the standard reading (1) minus the standard blank (2).

2.9.4 Preparation of substrate-inhibitor solutions

At acid pH, alpha galactosidase A hydrolyses the substrate 4-methylumbelliferyl-α-galactopyranoside to 4-methylumbelliferone and galactose. Addition of an excess of α-N-acetyl galactosamine inhibits the activity of alpha galactosidase B. A 8mM stock substrate solution was prepared by dissolving 250mg of 4-methylumbelliferone-α-D-galactopyranoside (Sigma) in 74ml of Mcilvaine's buffer. This was aliquoted and stored at -20°C. An inhibitor-substrate working solution was made by adding 500mg of N-acetyl-D-galactosamine (Sigma) to 11.3ml of stock substrate solution.

At acid pH, alpha galactosidase B (N-acetyl-galactosaminidase) hydrolyses the substrate 4-methylumbelliferyl-N-acetyl-β-D-galactosaminidine to 4-methylumbelliferone and N-acetyl-galactosamine. Substrate solution was made by dissolving 100mg of 4-methylumbelliferyl-N-acetyl-β-D-galactosaminidine (Sigma) in 264 ml of Mcilvaine's buffer, pH 4.7.

At acid pH, beta glucocerebrosidase hydrolyses the substrate 4-methylumbellifryl-β-D-glucopyranoside to 4-methylumbelliferone and glucose. Sodium taurocholate added to the substrate solution inhibits the activity of unrelated β-glucosidases. The

substrate stock solution consists of 4.8mM 4-methylumbelliferyl-β-D-glucopyranoside made by dissolving 250mg of 4-methylumbelliferyl-β-D-glucopyranoside (Sigma) in 96ml of Mcilvaine's buffer pH5.9. Sodium taurochlorate is then added to give a final solution of 4.8mM 4.8mM 4-methylumbelliferyl-β-D-glucopyranoside, 1.5% sodium taurocholate (Sigma), pH 5.1.

2.10 Western blot analysis

Western blots were performed using the NuPage ® system (Life Technologies). The primary antibodies used are listed in Table 2-4.

2.10.1 Cell lysate preparation

EA.hy cells for western blot analysis were seeded at 2×10^5 per well in 24 well plates and cultured for 3 days or 1×10^5 per well and cultured for 7 days. For cell lysis, medium was aspirated from the wells and the cells washed in 1ml of warmed Hanks buffer. Cell lysis solution was made by supplementing 1ml of whole cell lysis solution with 10μl of protease cocktail inhibitor (Sigma) and 10μl of 100mM phenylmethylsulfonyl fluoride (17.4mg/ml in DMSO). Hanks buffer was aspirated from the wells and 100μl of cell lysis solution added per well. The plate was left on ice for 10 minutes and then the cells disrupted with a pipette tip and the lysate transferred to an Eppendorf. The samples were centrifuged at 14 000rpm, 10 minutes at 4°C. The lysate was collected and stored at -80°C until analysis.

2.10.2 Preparation of samples for loading onto gel

Loading dye mix was made by adding 2.5 volumes of 1M DL-dithiothreitol to 7.5 volumes of loading dye (NuPage, Invitrogen). 10μl of loading dye mix was added to 20μl of sample. Molecular weight markers for loading were made by adding 3μl of Novex ® Sharp Pre-stained Protein Standard (Life Technologies) to 17μl distilled water and adding 10μl of loading dye mix. All samples for loading were then heated at 80°C for 10 minutes.

2.10.3 Gel electrophoresis

Running buffer was made by diluting 50ml of 20X NuPage ® MOPS SDS running buffer (Life Technologies) in 950ml of distilled water. A 12% Bis-Tris NuPage ® gel (Life Technologies) was washed with running buffer and then inserted into the gel tank. 500μl of NuPage ® antioxidant (Life Technologies) was added to the centre well of

the tank and both the inner and outer wells filled with running buffer. Samples were loading using a Hamilton syringe. The gel was run at 200V for 50 minutes.

2.10.4 Transfer of proteins from gel to membrane

Transfer buffer was made from 12.5ml of 20X transfer buffer, 25ml methanol, 200μl anti-oxidant and made up to 250ml with distilled water. The gel was removed from the tank and cracked open using a gel spatula. Buffer-soaked filter paper was applied to the back of the gel. Hybond membrane (GE Healthcare) was applied to the front of the gel and covered with filter paper. The gel-membrane sandwich was placed in the transfer cassette, encased with buffer-soaked sponges and placed in the gel tank. The inner tank was filled with transfer buffer and the outer tank with tap water. The transfer was run at 25V for 90 minutes.

The gel-membrane sandwich was removed from the transfer cassette and the filter paper and gel discarded. The membrane was stained for 30 seconds with Ponceau S (Sigma) to ensure transfer of the protein bands.

2.10.5 Blocking and antibody staining

The membrane was washed for 30-45 minutes in TBS-Tween on a plate shaker at 80rpm, with 3-5 changes of wash solution. The membrane was then placed in a roller bottle and 10ml of blocking solution supplemented with 500μl of FBS and 100μl of 10% azide added. The bottle was placed in a rotating oven at 21°C and blocked for 1 hour. After 1 hour, the primary antibodies were added at the appropriate dilution and the incubated overnight in the rotating oven.

The following day, the membrane was removed and washed 5 times in TBS-Tween. The membrane was placed in a clean roller bottle and 10ml of blocking solution, supplemented with 500μl FBS added. The appropriate HRP-conjugated secondary antibody was added and the membrane incubated for at least 2 hours in the rotating oven, prior to being washed 5 times in TBS-Tween.

Table 2-4: Antibodies used for Western blotting

Antibody	Species	Target band size	Manufacturer	Dilution (µl per 10ml)
Alpha galactosidase A	Rabbit	50kDa	Santa Cruz (H-104)	10µl
VCAM-1	Sheep	85kDa	R&D systems (AF809)	10µl of 100µg/ml
ICAM-1	Sheep	90kDa	R&D systems (AF720)	5µl of 200µg/ml
Hsp60	Mouse	60kDa	Stressgen	3µl of 1:10 dilution
PARP	Mouse	113kDa (plus 89kDa on cleavage)	BD	3µl
Aldolase	Sheep	39kDa	Abcam (ab182915)	3µl
HRP-conjugated anti-rabbit	Goat	N/A	Dako	3µl
HRP-conjugated anti-sheep	Goat	N/A	Dako	3µl
HRP-conjugated anti-mouse	Rabbit	N/A	Dako	3µl

2.10.6 Enhanced chemiluminescene visualisation of bands

Pierce^{TM} enhanced chemiluminescent substrate (Thermo Scientific) was used for detection of horse radish peroxidase enzyme activity. A 1:1 solution of reagent A and reagent B was made. The membrane was placed on a Perspex board and 1ml of ECL mixture added to membrane and incubated for 1 minute. The membrane was wrapped in Saran wrap and exposure to X ray film (Fuji) for 2, 6, 20, 60, 180 and 600 seconds. The film was then developed manually by placing in developer for 1 minute followed by fixer for 1 minute and washing in tap water before drying.

2.10.7 Staining with a house keeping antibody for normalisation

Normalisation for protein content was performed using the housekeeping protein heat shock protein 60 (Hsp60). After initial antibody staining, the membrane was washed 5 times in TBS-Tween. Further antibody staining was then performed by incubating the membrane overnight in blocking solution containing mouse anti-human Hsp60 antibody. The membrane was then washed and incubated for 2 hours with HRP conjugated rabbit anti-mouse secondary antibody. ECL was performed as above. For a small number of later blots, aldolase was used for normalisation due to the unavailability of Hsp60.

2.10.8 Densitometry

The density of the bands was measured using Quantity One 4.6.9 (BioRad). The density of each band of protein of interest was then normalised to the density of the Hsp60 band for that sample.

2.11 Coagulation assays

2.11.1 Tissue factor procoagulant assay

Monocytes isolated over an iodixanol gradient were re-suspended in TrisHCl-1% BSA buffer at 1×10^6 monocytes/ml. Pooled citrated plasma and 25mM calcium chloride were warmed to 37°C. 100µl of monocyte suspension and 100µl of pooled plasma were added per macrocuvette (Merlin Medical) and placed in a KC4 coagulometer (Amelung). The plasma and monocytes were incubated together at 37°C for 120 seconds. 100µl of 25mM calcium chloride was added and the time to clot formation measured. For the construction of a standard curve, the monocytes were replaced by dilutions of Recombiplastin (DiaPharma). The assays were performed in triplicate and the tissue factor activity in arbitary units read off the standard curve.

2.11.2 Platelet rich plasma thrombin generation

Thrombin generation was performed using the Calibrated Automated Thrombogram (Thrombinoscope BV). Use of a slow-reacting fluorogenic substrate for thrombin allows repeated measurement of thrombin generated over a prolonged period of time. A mathematical program in the CAT calculates the free thrombin generated from the raw thrombin generation data. Measurements were performed in duplicate.

1pM tissue factor is made by diluting Innovin (Sysmex, UK) which is thought to have a tissue factor concentration of 6nM in HEPES buffer. 80µl of platelet rich plasma was added to 3 wells in an Immunolon 2HB round bottomed 96 well plate. 20µl of 1pM tissue factor was added to the two test wells and 20µl of thrombin calibrator (Stago Diagnostica) added to the calibrator well. The reaction mixture was incubated at 37°C for 10 minutes. The reaction was started by the addition of 20µl per well of a calcium solution containing the fluorogenic substrate (FluCa, Stago Diagnostica) and the measurements commenced. Readings were taken automatically every 20 seconds for 60 minutes.

2.11.3 Preparation of washed platelets

Platelet rich plasma (PRP) was diluted 2:1 with Krebs Ringer buffer, pH 5.0 and centrifuged at 500g, room temperature for 10 minutes. The platelet pellet was re-suspended in Krebs Ringer buffer supplemented with glucose and was twice further. The platelet pellet was re-suspended in Tris-HCl-1% BSA buffer and the platelet count measured.

2.11.4 Prothrombinase assay

The conversion of prothrombin to thrombin is catalysed by the prothrombinase complex (FVa-FXa) in the presence of calcium ions, occurring on a phospholipid surface. In this assay FVa and FXa are present in excess so that the phospholipid surface provided by the platelets is the rate limiting factor. A schematic overview of the assay method is shown in Figure 2-1.

A 300nM stock of FXa was made by diluting FXa (Haematologic Technologies) to a total volume of 7.25ml in TrisHCl-1%BSA. A 600nM stock of FVa was made by diluting FVa (Haematologic Technologies) to a total volume of 496μl in TrisHCl-1%BSA. A 16μM stock of prothrombin was made by diluting prothrombin in a total volume of 1736μl in TrisHCl-1%BSA. All coagulation factors were aliquoted and stored at -20°C. A thrombin-specific chromogenic substrate, S2238 (Chromogenix), was re-constituted at 3mM in distilled water.

Working solutions of FVa and FXa were made just before performing the assay by diluting the stock solution 1:10 in TrisHCl-1%BSA. Washed platelets were re-suspended at 100×10^9/L and 50×10^9/L in Tris-Hcl-1%BSA. The assay reaction was performed in a water bath at 37°C. 158μl of platelet suspension (or dilutions of phospholipid standard, Bell and Alton Platelet Substitute, Diagnostic Reagents Ltd.) was placed in a cuvette in a waterbath and incubated for 3 minutes. 5μl of 0.11M calcium chloride was added and after a further minute 25μl of FVa and 25μl of FXa were added. After a further minute, the reaction was started by the addition of 60μl of prothrombin. 5μl sub-sampling of the reaction mixture into 200μl of stopping buffer in a 96 well plate was performed at specified time points (0, 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 240, 360, 480 and 600 seconds).

On completion of the prothrombinase reaction, 10µl of chromogenic substrate was added to each well of the 96 well plate. After 3 minutes the reaction was stopped by the addition of 50µl of 50% acetic acid. The absorbance was read on the plate reader at 405nM.
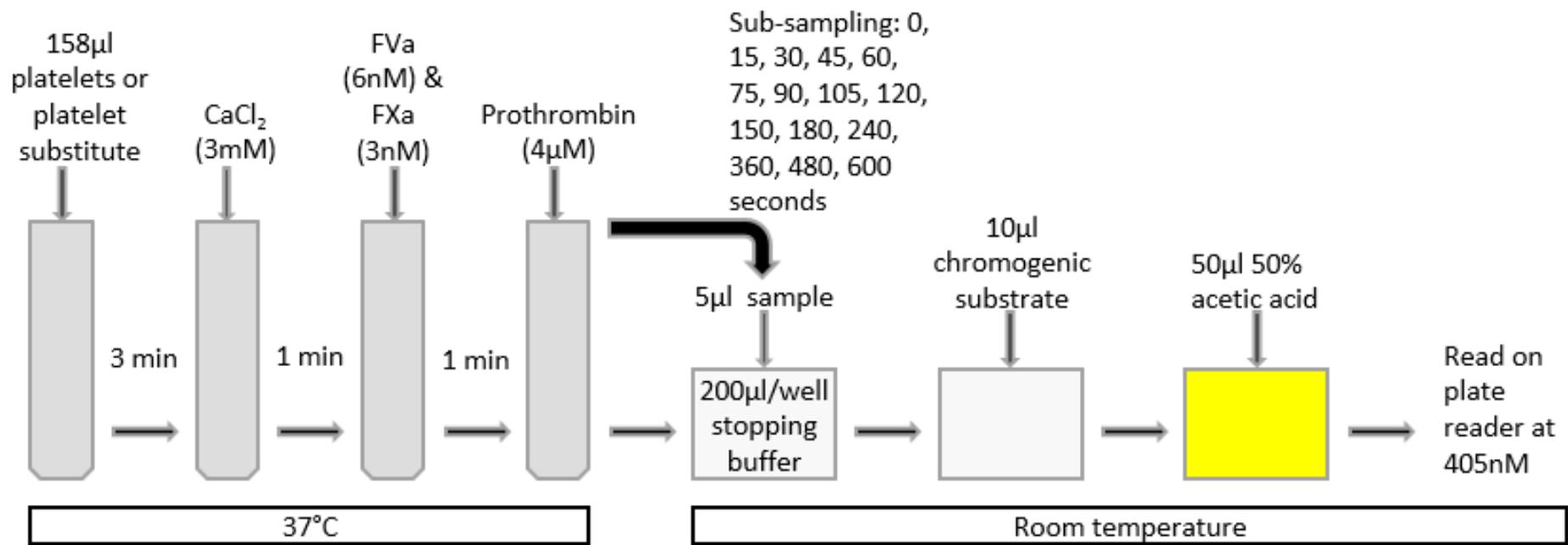
Figure 2-1: Prothrombinase assay

2.11.5 Measurement of plasma von Willebrand factor antigen level by ELISA

Plasma von Willebrand factor (VWF) was measured by an in house enzyme-linked immunosorbant assay in the Royal Free Haemophilia Centre. A 96 well ELISA plate (Immunolon 1, Dynatech Laboratories) was coated with the primary antibody the day before the assay. A 1:1000 dilution of rabbit anti-human von Willebrand antibody (A0082, Dako) in coating buffer was made and 100µl added per well. The plate was stored overnight at 4°C.

CRYOcheck normal reference plasma, with a known concentration of VWF (Precision Biologic) was used to construct a standard curve. CRYOcheck was diluted 1:80 in dilution buffer and then a further range of dilutions made. Test samples were diluted 1:200 in dilution buffer. All samples were tested in duplicate. The coated plate was washed times with wash buffer and the 100µl standard/sample added per well. The plate was incubated for 1 hour on a plate shaker. The plate was washed three times with wash buffer. A 1:8000 dilution of tag antibody (HRP-conjugated rabbit anti-human VWF, P0226, Dako) was made in dilution buffer. 100µl of tag antibody was added per well. The plate was incubated for 1 hour on a plate shaker. The plate was washed three times with wash buffer. Substrate solution was made by adding 6mg of O-phenylene dihydrochloride (OPD, Sigma) and 7µl of 30% hydrogen peroxide (Sigma) to 15ml of substrate buffer. 100µl of substrate solution was added to each well and the plate incubated on the bench for 8-10 minutes. The reaction was stopped by addition of 100µl of 0.5M sulphuric acid per well. The absorbance was read on a plate reader at 492nm. The VWF concentrations were read off the standard curve and the results multiplied by two, to account for the 1:200 dilution of the test samples.

2.11.6 Coagulation assays performed by the haemophilia centre laboratory

The following assays performed by the haemophilia centre laboratory biomedical science staff following the standard SOPs of the diagnostic laboratory and the methods employed are discussed in the relevant chapters:

- Thrombophilic protein coagulation activity: FVIII, protein C, protein S, anti-thrombin and dilute Russell Viper venom test (see Chapter 4)

- Mutation analysis for FV Leiden (G1691A) and prothrombin gene mutation (G20210A) (see Chapter 3)
- FXI activity and *F11* gene sequencing (see Chapter 3)

2.12   Statistics

Statistical analyses were performed using Excel 2010 (Microscoft) and GraphPad Prism version 6.02 (GraphPad). For comparisons between two groups, t-tests were used for normally distributed data and Mann Whitney U if the data was not normally distributed. Linear regression analyses were performed to establish the relationship between two variables. Except where otherwise stated a p value of <0.05 was considered significant.

Chapter 3   Bleeding features in the presentation of Gaucher disease

3.1   Introduction

Whilst the initial description of GD was of a patient with splenomegaly, GD affects the entire reticulo-endothelial system with disease manifestations including bone infarcts, peripheral blood cytopenias and a bleeding diathesis. Whilst bleeding has been reported in patients with GD, the pattern and severity of bleeding symptoms is not well characterised.

Both abnormalities of platelet function and coagulation factor deficiencies have been described and various hypotheses proposed as to the underlying mechanisms (see Table 3-1).   In addition interference of glycosphingolipids with the assays has also been proposed at as a cause for *in vitro* abnormalities[119]. Improvements following ERT have been reported in both platelet aggregation abnormalities and coagulation factor activity.

Table 3-1: Potential mechanisms of coagulopathy in coagulopathy in GD

Platelets	Coagulation factors
Decreased production due to bone marrow infiltration	Decreased production due to:
	Genetic abnormalities (e.g. in *F11* gene).
Splenic sequestration	Hepatic impairment
Increased consumption	Increased consumption
	Adsorption by glucocerebrosides

It is not known how prominent bleeding manifestations are in the presentation of GD. With the advent of effective specific therapy for GD and the suggestion that early initiation of therapy may reduce long term complications[186], there is clear impetus to make a timely diagnosis.   Better understanding of the clinical features with which patients present may help with earlier identification and investigation of patients with possible GD. The relationship between bleeding and other manifestations of GD has not been examined – it is not known whether bleeding symptoms are a marker of disease severity or whether they impact on the development of other disease features, in particular bone disease.

3.2 Purpose of this chapter

This chapter explores the presenting features of GD in the Royal Free cohort of patients in order to identify specific patterns of clinical and laboratory abnormalities which may serve to raise suspicion of the diagnosis. The severity and nature of bleeding symptoms is examined and correlated with other disease manifestations to identify any common mechanisms. Finally, the changes in deficiency of a specific coagulation factor in response to enzyme replacement therapy are described. FXI is specifically discussed as this is the coagulation factor that was frequently found to be reduced in our cohort. The chapter consists of three sections:

1. Description of the presenting features of patients in the cohort:

   a) Presenting features

   b) Diagnosing speciality and diagnostic test

   c) Identification of clinical and laboratory abnormalities commonly found at baseline

2. Bleeding manifestations within the cohort and their relation to:

   a) Baseline platelet counts and coagulation abnormalities

   b) Overall disease severity, bone disease and gammopathies

3. In patients with FXI deficiency at baseline:

   a) Effect of FXI on bleeding score/complications

   b) Changes in FXI levels following initiation of ERT

3.3 Hypotheses

1. Patients presenting with GD most commonly are diagnosed by haematologists by bone marrow biopsy. *Rationale:* GD is rare and therefore not commonly considered in the differential diagnosis of thrombocytopenia/splenomegaly; consequently it is diagnosed when bone marrow biopsies are performed for other possible diagnoses

2. Bleeding severity is associated with overall disease severity and the presence of gammopathies and bone infarcts. *Rationale:* the bleeding diathesis has been

proposed to arise due to chronic activation of the coagulation system as a result of chronic inflammation

3.   FXI deficiency arises as a manifestation of GD rather than genetic co-inheritance and improves with ERT *Rationale:* the wide range of factor deficiencies described in prior studies suggests a disease-related mechanism rather than inherited deficiencies of coagulation factors.

3.4   Methods

3.4.1   Historical case notes review

Patients with GD have been seen at the Royal Free since 1974, with the centre being designated a national specialist centre in 1997. A retrospective case note review was undertaken to establish key features regarding the time course from symptom onset to diagnosis, the diagnosing speciality and diagnostic tests. The primary presenting feature was defined as the main clinical feature which, on investigation, led to the diagnosis of GD. Bleeding manifestations and baseline laboratory abnormalities were recorded. Laboratory abnormalities were defined as being outwith the normal range for the laboratory at the time the test was performed. The cohort included patients managed at the Royal Free GD clinic up until May 2012.

3.4.2   Disease severity scoring

The severity of GD was scored using the Zimran severity score index (SSI) based on patients' baseline characteristics [34], see **Table 3-2**.

Table 3-2: Zimran severity score

Domain	Feature	Score
Cytopenia	Unsplenectomised	1
	If splenectomised:	
	Leukopenia	1
	Anaemia	1
	Thrombocytopenia	1
Splenomegaly	None	0
	Mild	1
	Moderate	2
	Massive	3
	Splenectomised	3
Hepatomegaly	None	0
	Mild	1
	Moderate	2
	Massive	3
Liver function tests	Normal	0

	Some abnormal	1
	All abnormal	2
Clinical signs liver disease		4
CNS involvement		20
Other organ involvement (e.g. lung)		4
Bone – objective	No signs/symptoms	0
	Xray or scan signs	1
Bone – subjective	No pain	0
	Mild/occasional pain	2
	Chronic pain	3
Bone – fractures	None	0
	Post traumatic	2
	Avascular necrosis or pathological	5

3.4.3 Assessment of bone disease

Historical assessment of bone disease was with plain film radiographs, which are able to identify many abnormalities including avascular necrosis, infarcts and bone remodelling. Over the last two decades, the use of MRI imaging to assess bone disease has been employed. This is more sensitive in detecting bone abnormalities and also enables semi-quantitative assessment of the degree and site of bone involvement, using the bone marrow burden (BMB) scoring system to be undertaken[187], with a higher score signifying more severe bone involvement (see **Table 3-3**). All MRIs were reviewed by a radiologist specialising in GD and the BMB scores reported as part of routine clinical care.

Table 3-3: Bone marrow burden score

Domain	Abnormality	Score
MRI signal intensity: T2 images (spine and femora) relative to subcutaneous fat	Hyperintense	2
	Slightly hyperintense	1
	Isotense	0
	Slightly hypointense	1
	Hypointense	2
	Mixed type	3
MRI signal intensity: T1 images (spine and femora). Relative to subcutaneous fate	Slightly hyperintense or isotense	0
	Slightly hypointense	1
	Hypointense	2
Sites of involvement (femora)	Diaphysis	1
	Proximal epiphysis/apophysis	2
	Distal epiphysis	3
Sites of involvement (spine)	Patchy	1
	Diffuse	2
	Absence of fat in basivertebral vein region	1

3.4.4 Bleeding severity score

The condensed MCMDM-1 VWD bleeding states questionnaire[188] was used to obtain a standardised bleeding history in an interview between the patient and the investigator (see **Table 3-4**). It was originally developed to aid in the diagnosis of von Willebrand disease (VWD); 100 healthy controls had a mean bleeding score of 0.16 ($\pm$2SD -3.2-+3.6) and therefore an abnormal bleeding score was defined as ≥ 4[188].

Table 3-4: Condensed MCMDM-1 VWD bleeding score

Domain	-1	0	1	2	3	4
Epistaxis		No or trivial (<5 min)	>5-<10 minutes	Consultation only	Packing/ cauterisation/anti-fibrinolytics	Blood transfusion/ factor replacement/ desmopressin
Cutaneous		No or trivial (<1cm)	>1cm and no trauma	Consultation only		
Bleeding from minor wounds		No or trivial (<5min)	>5 min	Consultation only	Surgical haemostasis	Transfusion / factors/ DDAVP
Oral cavity		No	Reported at least once	Consultation only	Surgical haemostasis/ antifibrinolytics	Transfusion / factors/ DDAVP
GI bleeding		No	Associated with ulcer/ portal hypertension/ haemarrhoids	Spontaneous	Surgical haemostasis/ transfusion/ DDAVP/ factors/ antifibrinolytics	
Tooth extraction	No bleeding in at least 2 extractions	Not done or nil in 1 extraction	In <25% procedures	In >25% procedures, no intervention	Resuturing or packing	Blood transfusion/ factors/ DDAVP
Surgery	No bleeding in at least 2 surgeries	Not done or nil in 1 surgery	In <25% surgery	In >25% surgery, no intervention	Surgical haemostasis or antifibrinolytics	Transfusion / factors/ DDAVP
Menorrhagia		No	Consultation only	Antifibrinolytics or pill use	Curretage or iron therapy	Transfusion / factors/ DDAVP/ hysterectomy
Post partum haemorrhage	No bleeding in at least 2	No deliveries or no	Consultation only	Curettage/ iron therapy/ anti-fibrinolytics	Transfusion/ factors/ DDAVP	Hysterectomy

	deliveries	bleeding in 1				
Muscle haematoma		Never	Post trauma, no therapy	Spontaneous, no therapy	Requiring DDAVP or factors	Requiring transfusion or surgery
Haemarthrosis		Never	Post trauma, no therapy	Spontaneous, no therapy	Requiring DDAVP or factors	Requiring transfusion or surgery
CNS bleeding		Ever			Subdural, any intervention	Intracerebral, any intervention

3.4.5 FXI activity and mutation analysis

Patients who had been previously identified as FXI deficient, either due to investigation of a prolonged APTT at baseline or investigation of bleeding symptoms were identified and baseline FXI levels recorded. A follow up sample was collected and FXI activity measured by automated one-stage ATPP-based activity assay using an ACL TOP analyser (Instrumentation Laboratories) by the biomedical staff of the haemophilia centre laboratory (Royal Free Hospital). In patients found to have a FXI level below the normal range on follow up, sequencing of the 15 exons of the *F11* gene was performed (Ms G Mellars, chief BMS, Genetic Laboratory, Haemophilia Centre, Royal Free Hospital).

3.5   Results

3.5.1   Overview of GD cohort

The cohort consisted of 86 patients: 49 males (57%) and 37 females (43%). 45 (52%) of these patients were referred at the time of diagnosis, 29 (34%) had a known diagnosis of GD but were not receiving ERT at the time of referral and 12 (14%) were already receiving ERT and transferred care either due to geographical reasons or following the designation of specialist centres in 1997 (see **Figure 3-1**). Although ERT has been available since 1991, there are still patients diagnosed with GD in the pre-ERT era being referred for specialist review. Sixty-eight patients (79%) had at least on N370S mutation with compound heterozygosity for the N370S with another (non-N370S or L444P) mutation being the commonest mutation type.

A Referral patterns to Royal Free LSD Unit

B Genotype
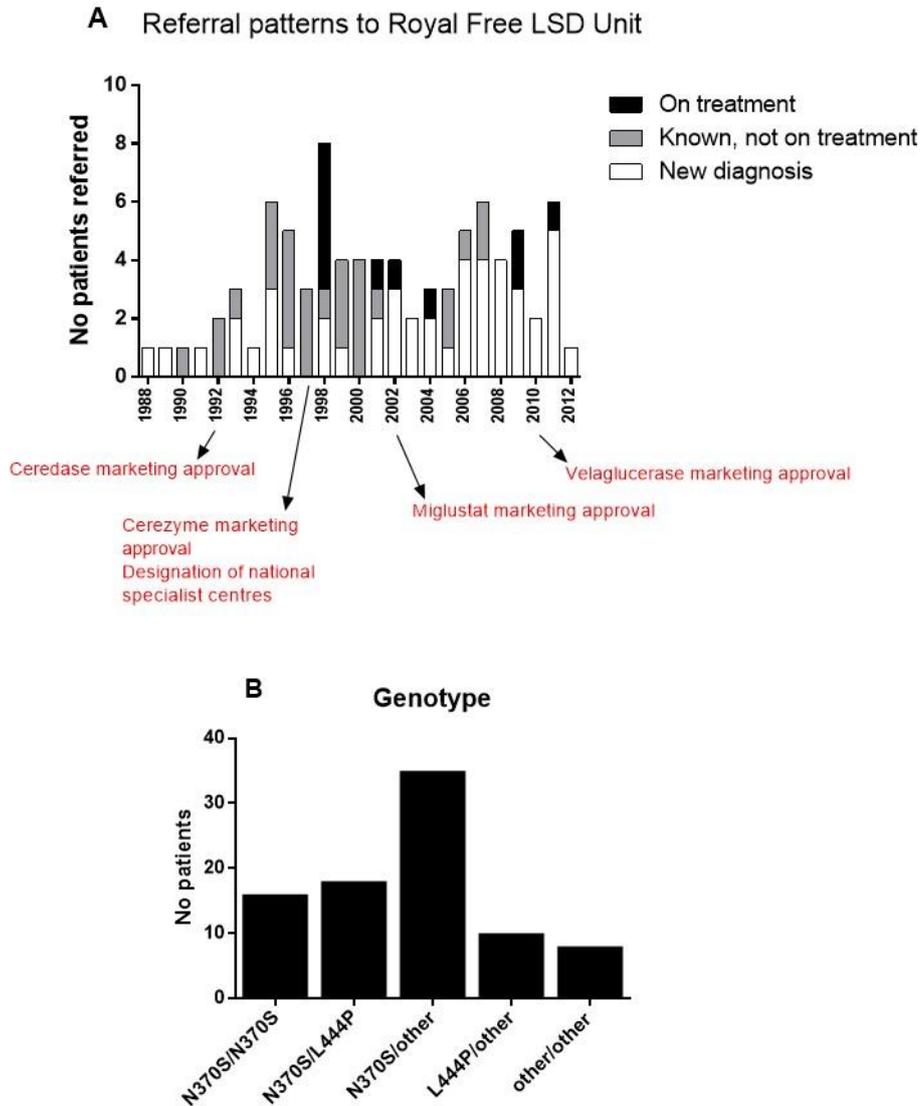
Figure 3-1: RFH cohort overview. A: Referrals to RFH: 2-4 new diagnoses are seen each year; despite the availability of ERT since 1991 there are still patients with known diagnoses being referred on for treatment, suggesting that awareness of therapy is incomplete. B: genotypes of the cohort.

3.5.2 Presentation of Gaucher disease

The key symptom, investigation of which led to the diagnosis of GD, was considered the presenting feature. Symptoms related to splenomegaly and bleeding were the commonest presenting features (see **Table 3-5** and **Figure 3-2**). Bleeding most commonly consisted of excessive bruising, but two patients presented with post-operative bleeding (following a hip replacement and gingivectomy). Symptoms related to splenomegaly included abdominal pain, early satiety and distension or a palpable mass. Bone pain was the third commonest presentation, primarily with hip pain; this was associated with avascular necrosis in two cases and one patient presented with a pathological fracture and was found to have a concurrent diagnosis of multiple myeloma. In 16 patients (18.6%) the diagnosis arose following investigation of abnormalities (predominately thrombocytopenia and splenomegaly) detected opportunistically during routine antenatal care, routine examinations (e.g. insurance medicals) or unrelated medical problems (e.g. road traffic accidents).

GD has an increased prevalence in the Ashkenazi Jewish population and carrier screening for this and other high prevalence disorders (e.g. Tay-Sachs) is offered to this community by some charities (e.g. Jewish Genetic Disorders UK). Two patients were diagnosed with GD by this method. An additional eight were diagnosed following the diagnosis in an affected sibling or after being born to two known carrier/affected parents.
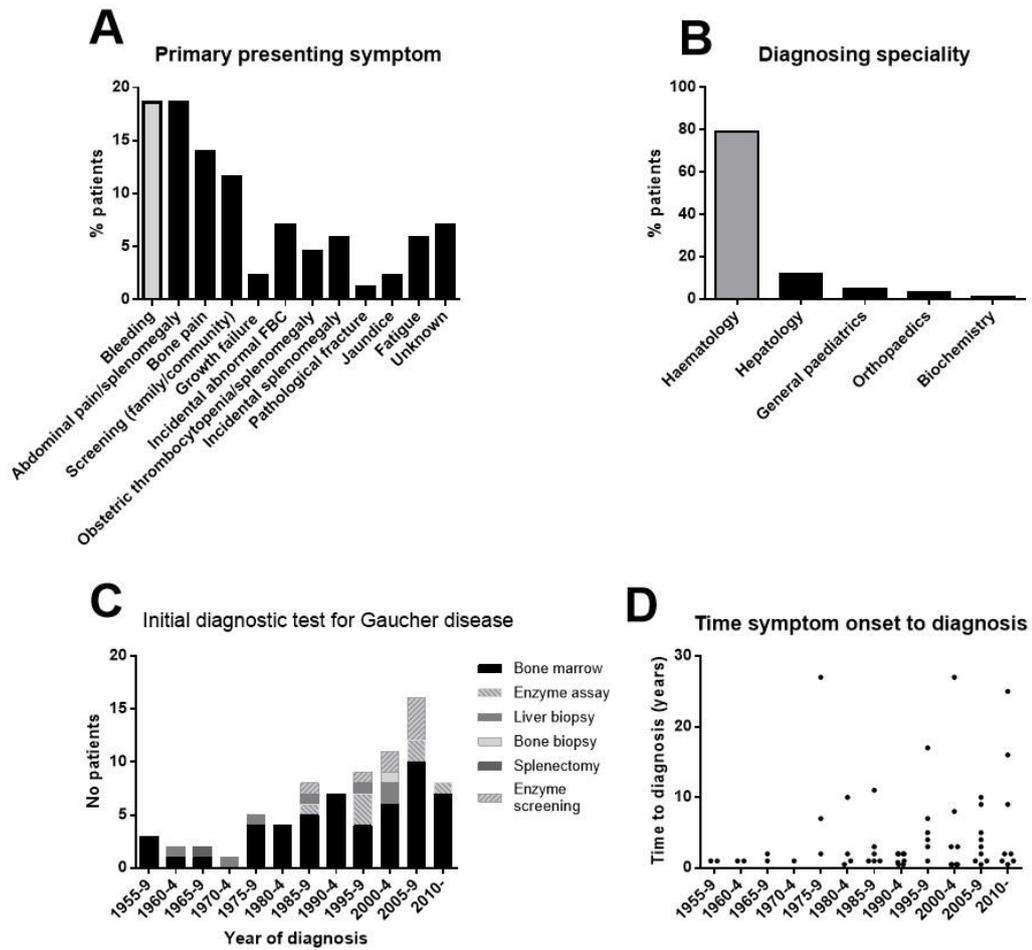
Figure 3-2: Presentation of GD. A: Primary presenting symptom. B: Diagnosing speciality. C; Initial diagnostic test. D: Time from symptom onset to diagnosis.

Table 3-5: Primary presenting features of GD

Category	Feature	No. (%)
Symptomatic	Abdominal pain/distension	16 (18.6)
	Bruising/ bleeding	16 (18.6)
	Bone pain	12 (14)
	Fatigue	5 (5.8)
	Growth failure	2 (2.3)
	Jaundice	2 (2.3)
	Pathological fracture	1 (1.2)
Incidental	Thrombocytopenia/splenomegaly in pregnancy	4 (4.6)
	Incidental abnormal FBC	6 (7)
	Incidental splenomegaly	5 (5.8)
	Incidental abnormal liver function tests	1 (1.2)
Screening	Affected sibling/ parents	8 (9.3)
	High-risk community	2 (2.3)
Unknown		6 (7)

Diagnosing speciality and diagnostic test

Consistent with the common presentations of bruising and splenomegaly, 79% of patients were diagnosed by haematologists (including haemostasis specialists in 4%) (see **Figure 3-2**). Despite bone pain being a common primary presenting feature, patients were rarely diagnosed by orthopaedic surgeons or rheumatologists. One patient was diagnosed by a clinical biochemist following investigation of marked hypertriglyceridaemia, having presented to their GP with debilitating fatigue.

Bone marrow biopsy was the primary diagnostic test and was performed in 58 (68%) of patients with the diagnosis being made less commonly following other tissue biopsies: liver 8 (9%), splenectomy 1 (1%) and bone (1%). Despite the availability of peripheral blood leucocyte enzyme assays since 1970[189], enzyme assays were only used as the primary test in 8 patients (9%) outside the context of family or Jewish community screening.

Time from symptom onset to diagnosis

The median age at diagnosis was 26 years (range 0-76). The median time from onset of symptoms attributable to GD to diagnosis was 2 years (range <0.5-27 years). The time from symptom onset to diagnosis has not changed significantly with the advent of specialist services and ERT: prior to 1997, the median time to diagnosis was 1.5 years (range 0.5-27) and after 1997 3.0 years (range 0.5-27), p = 0.27. The shorter times from symptom onset to diagnosis in those diagnosed prior to 1975 is likely to

due to the fact that those with long diagnostic delays during this period would have been diagnosed at an older age and may be already deceased.

Most delays were due to non-investigation rather than mis-diagnosis. In the eight patients with delays of 10 or more years, three had a long history of hip pain with one being diagnosed with Perthes disease age 13 and the other two were diagnosed with avascular necrosis of the hip in their 30s. Two had a history of significant bleeding requiring surgical intervention post dental extraction and tonsillectomy, both aged 7 and another was had been diagnosed with "chronic immune thrombocytopenia purpura" on the basis of chronic thrombocytopenia. The other two patients had long histories of persistent abdominal pain and splenomegaly dating back to childhood.

3.5.3 Clinical and laboratory abnormalities at the time of presentation

Full blood counts were analysed in non-splenectomised patients referred either at the time of diagnosis or prior to enzyme replacement therapy (N = 64), see **Figure 3-3**. Mean haemoglobin was 12.3g/dl (range 6.6-16.3), mean white cell count 4.84×10^9/L (range 1.97-11.28) and mean platelet count 90×10^9/L (range 30-255). Mean neutrophil count was 3.0×10^9/L (range 1.2-8.41) and mean lymphocyte count 1.48×10^9/L (range 0.6-4.56).

The forty five patients who were referred for assessment at the time of initial diagnosis had a more complete clinical and laboratory assessment performed and these were analysed in order to identify key features, which if present in combination, should raise suspicion of GD amongst clinicians (see **Figure 3-3**). Splenomegaly, either palpable or on ultrasound assessment, (87%), hepatomegaly (44%) and bruising (40%) were the commonest clinical features. Bone pain was reported by 36% with 11% already having avascular necrosis of the hip at the time of diagnosis. Blood tests performed at diagnosis included: full blood count, coagulation screen, liver function tests, immunoglobulins, serum protein electrophoresis, ferritin and serum angiotensin converting enzyme (ACE). A raised serum ACE (97%), hyperferritinaemia (87%), thrombocytopenia (82%) and low HDL cholesterol (75%) were the commonest laboratory abnormalities. 43% had a prolonged APTT at baseline.
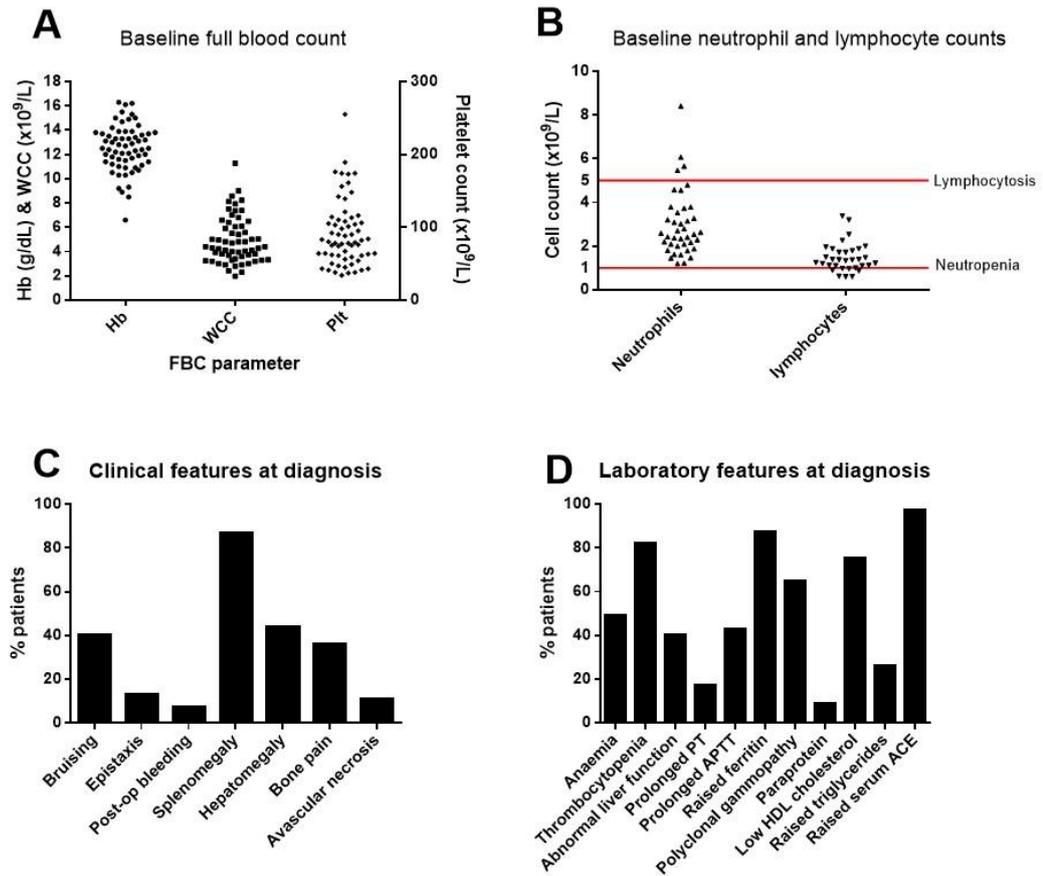
Figure 3-3: Discriminating GD from other diagnoses.

3.5.4 Bleeding symptoms in Gaucher disease

54% of patients had documented bleeding symptoms at presentation. These were predominately bruising (45%) but post-operative bleeding (including post-dental extraction) was reported in 8%. Bleeding scores were obtained in 40 GD patients (15 females, 25 males) and 47 (33 females, 15 males) healthy controls. The median bleeding score in healthy controls was 0 whilst the median bleeding score in GD patients was 3. 19 (47.5%) of GD patients had a score ≥4, whilst only 1 control (2.1%) did.

No controls or GD patients reported any gastrointestinal, central nervous system, joint or muscle bleeds. Bleeding symptoms in controls were confined to epistaxis, menorrhagia, post partum haemorrhages and, in one control, a bleeding wound (see **Figure 3-4**), consistent with findings from previous studies[188]. In contrast, in GD patients bleeding symptoms occurred across the spectrum of mucocutaenous bleeding symptoms and were more severe than in controls. Dental extraction and post-surgical bleeding often required surgical intervention or, in one case, transfusion. One patient had a hysterectomy to control post-partum haemorrhage and had also had other severe bleeding events, including post dental extraction resulting in a total score of 15.

Bleeding symptoms and platelet count

The relationship between the baseline platelet count and bleeding symptoms, both historical recorded symptoms and bleeding scores was examined (see **Figure 3-5**). The median platelet count in patients with bleeding symptoms was not significantly lower than in those without (77 $\times 10^9$/L (range 34-225) vs. 103 $\times 10^9$/L (range 37-189), p=0.13. The median platelet count in those with post-operative bleeding was 61×10^9/L (range 41-92). Only two patients with epistaxis and one patient with post-operative bleeding had a platelet count <50 $\times 10^9$/L.

Similarly, there was no relationship between bleeding score and platelet count. The median platelet count in those with a bleeding score <4 was 87×10^9/L (range 37-241) compared to 64×10^9/L (range 38-159) in those with a score ≥4 (p = 0.33). Three patients were excluded from this analysis as there was no pre-splenectomy platelet count available.
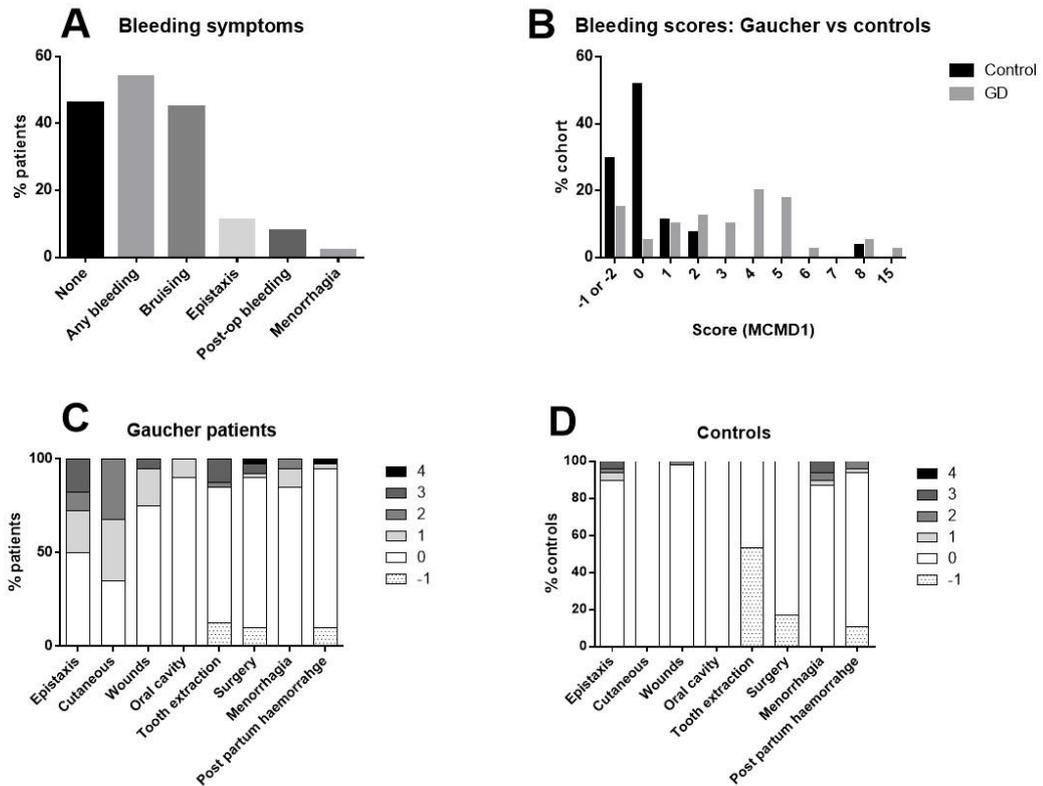
Figure 3-4: Bleeding symptoms and severity. A: bleeding symptoms established from historical case notes review. B: MCMD1 bleeding scores in GD patients and controls. C: bleeding score subsets Gaucher patients. D: bleeding score subsets controls.
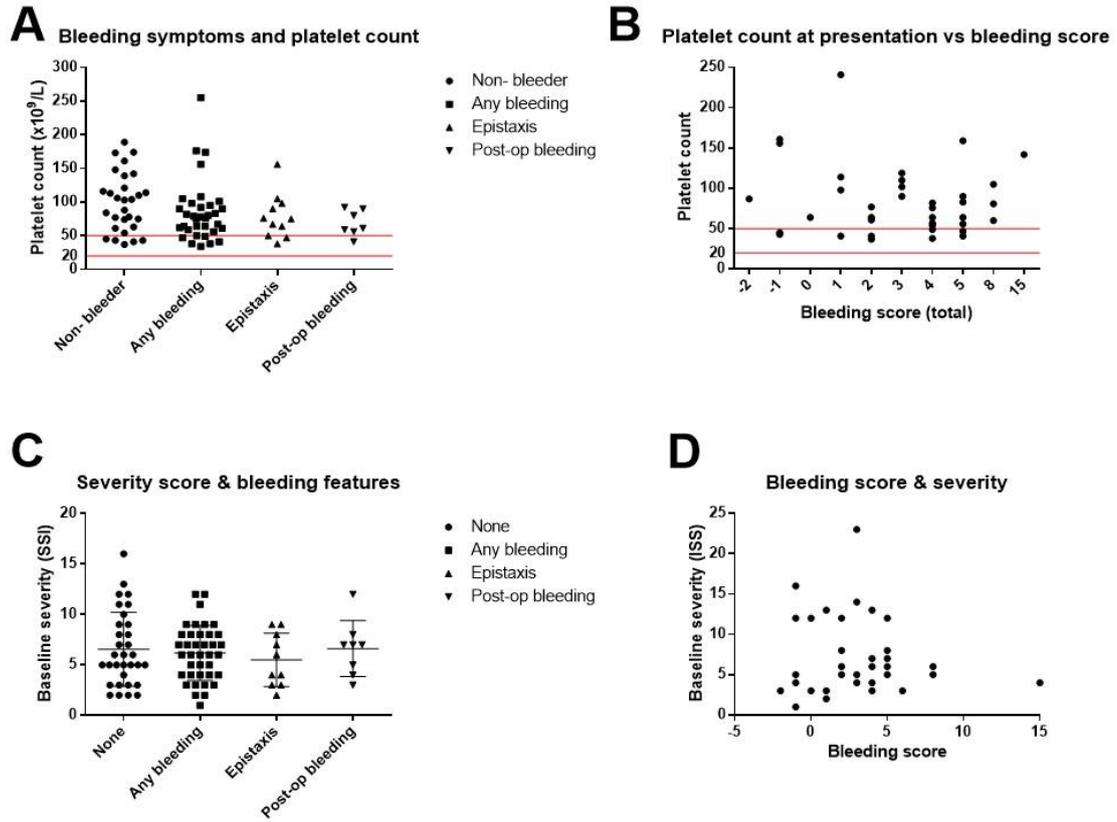
Figure 3-5: Bleeding features in relation to platelet count and disease severity (ISS). A: Baseline platelet count and bleeding symptoms – historical cohort. B: baseline platelet count and bleeding score; C: baseline severity and bleeding symptoms – historical cohort; D: baseline severity and bleeding score.

Bleeding symptoms and disease severity

There was no difference in the baseline SSI severity score between GD patients with and without bleeding symptoms (median 6, range 1-12 vs. median 5.5, range 2-16; p = 0.62), see **Figure 3-5**. The median score in those with post-operative bleeding was 7 (range 3-12).

Amongst those patients with bleeding scores, there was similarly no relationship between baseline severity and bleeding score. The median SSI of those with a bleeding score <4 was 5 (range 1-23), whilst the median SSI of those with a bleeding score ≥4 was 6 (range 3-13), p = 0.92.

There was no difference in bleeding scores between patients who had a gammopathy, either a diagnosis or long term follow up (see **Figure 3-6**). Median bleeding score was higher in those with bone infarcts at baseline (4 in infarcts, vs. 2 without infarcts) and follow up (4 vs. 2.5) but these differences were not statistically significant (p = 0.74 and 0.54 respectively).
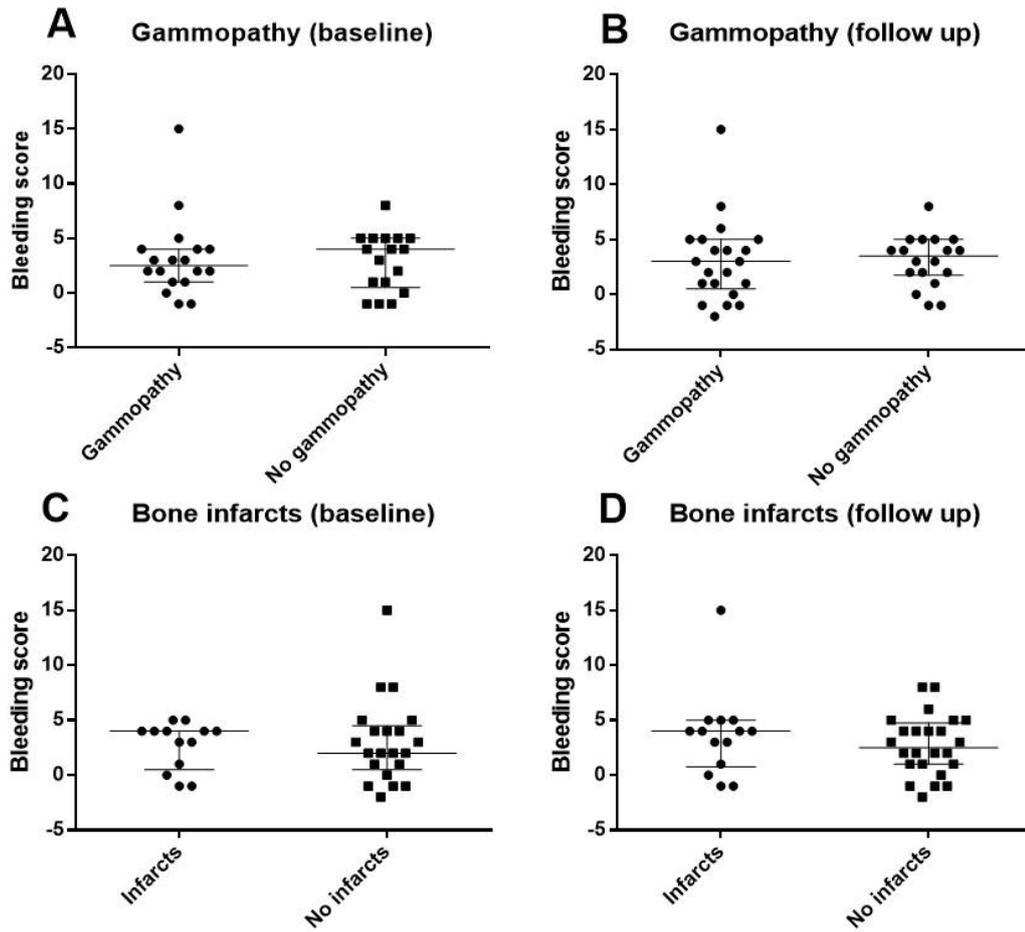
Figure 3-6: Relationship between bleeding scores and gammopathies at baseline (A) and follow up (B) and bone infarcts at baseline (C) and follow up (D).

3.5.5 Coagulation abnormalities and FXI deficiency in Gaucher disease

As indicated above, an abnormal APTT was found in 43% of patients from the historical cohort. The APTT was considered prolonged if the coagulation time was longer than that of the normal range of the laboratory at the time of testing. The normal ranges varied over time due to changes in methodology, particularly reagents. Of the patients with a prolonged baseline APTT, the APTT has normalised in all except two patients, one of whom had recently commenced ERT. 17% of patients had a prolonged PT, in conjunction with a raised APTT, all of which normalised with ERT (see **Figure 3-7**). The bleeding scores of patients with and without a prolonged APTT at baseline are shown in **Figure 3-7**. The median bleeding score of patients with a prolonged APTT was 4 compared to 2 for those without; this is of borderline significance (p = 0.064). 10/18 (55.5%) of patients with a prolonged APTT at baseline had a bleeding score ≥ 4 compared to 9/22 (40.9%) of patients with a normal baseline coagulation screen.

Despite the prevalence of bleeding symptoms and abnormal coagulation screens at presentation in patients with GD, systematic evaluation for a bleeding disorder had not been undertaken in patients. Thus, identification of more specific haemostatic defect was, to a degree opportunistic. Amongst the cohort, 17 patients who had been found to have FXI deficiency (reduced FXI coagulation factor activity) were identified from the case notes. Five of these patients were seen at a haemophilia centre prior to the diagnosis, where they were found to have FXI deficiency. Of these three were also found to have abnormal platelet aggregometry. Two were diagnosed with a "weak agonist receptor defect" and their thrombocytopenia ascribed to chronic immune thrombobocytopenic purpura. The other was further investigated and the diagnosis of GD made.
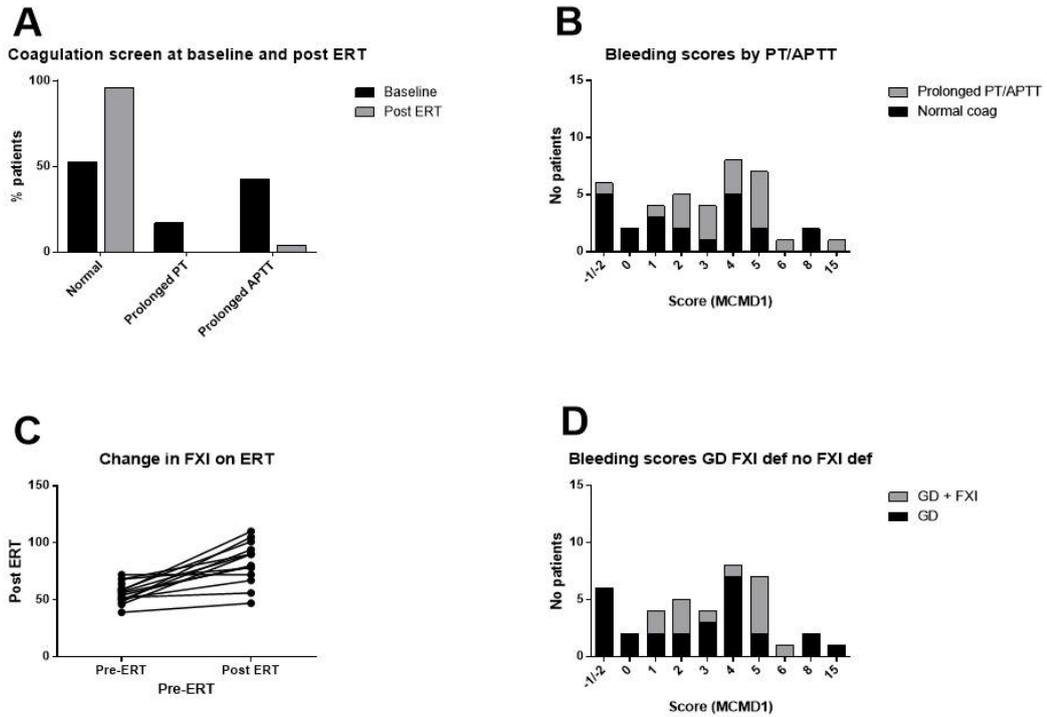
Figure 3-7: Coagulation screen & FXI activity in relation to bleeding scores & response to therapy. A: normalisation of PT and APTT following at least 1 year ERT. B: relationship between bleeding scores and abnormal coagulation screens at baseline. C: Changes in FXI levels in those with FXI deficiency at baseline. D: relationship between bleeding scores and FXI deficiency at baseline.

FXI deficiency

Of the seventeen patients with FXI deficiency at baseline, only two had no bleeding symptoms, one of whom was diagnosed on Jewish community screening and has not required therapy for their GD. Of the other fifteen patients, four reported only bruising, three had additional menorrhagia, two epistaxis and six post-operative bleeding (including dental extraction). Thirteen of these patients had a bleeding score ascertained. The median bleeding score in those with FXI deficiency was 4 compared to 3 in those without (see **Figure 3-7**). Baseline and follow up FXI levels were performed in 13 patients who had received ERT for a prolonged period (median 14.9 years, range 9.2-19.6). The median FXI level at baseline was 55 IU/dL (range 39-68; normal range for laboratory >70IU/dl) and the median FXI level at follow up was 90IU/dL (range 47-110, $p<0.0001$). Three patients still had low FXI levels. Mutational analysis of the *F11* gene in two of these patients failed to reveal evidence of a genetic abnormality. The third was unavailable for mutational analysis. Of the four patients without follow-up FXI levels, three had recently commenced on ERT (within 1 year). Mutational analysis of the *F11* gene in these patients found no evidence of a *F11* mutation. The fourth patient is untreated and was not available for further evaluation.

3.6 Discussion

3.6.1 Presentation of GD:

Patients most commonly presented with symptoms related to visceral or haematological involvement consistent with the findings of a previous study of patients from the USA and Australasia[190]. Although bone pain was a common presenting complaint, the high incidence of additional splenomegaly or full blood count abnormalities, most likely results in ultimate diagnosis of these patients by haematologists. Despite the availability of ERT and the development of national specialist services for GD in the UK, some patients continue to experience long delays between symptom onset and diagnosis, with little change over the past decades. This is likely to be attributable to a number of factors including a lack of awareness of GD, the non-specific nature of presenting symptoms and the relative wellbeing of many patients leading to less impetus to make a concrete diagnosis.

GD often has an insidious onset and patients may develop extensive organ involvement with minimal overt symptomatology. Investigation of 37 N370S homozygotes identified through a community carrier screening program found that although 65% were asymptomatic, 97% had splenomegaly, 14% had bone infarcts of MRI imaging, 52% had osteopenia and 9% established osteoporosis[191]. The presence of established avascular necrosis of the hip in 11% of patients in this study at diagnosis highlights the need for earlier diagnosis. Once bone infarction has occurred it is irreversible and avascular necrosis of the hip ultimately results in joint replacement in most patients and therefore the aim should be to diagnose and institute therapy prior to the development of such irreversible complications.

3.6.2 Diagnosing GD in the haematology clinic

Given the rarity of GD, making the diagnosis requires a high index of suspicion in the consulted physician. Thrombocytopenia and splenomegaly, whether symptomatic or incidental findings, are common reasons for referral to haematologists and both have wide differential diagnoses. Amongst haematologists asked to give a differential diagnosis of a 42 year old with anaemia, thrombocytopenia, hepatosplenomegaly and bone pain, all classic features of GD, 65% selected leukaemia, 36% lymphoma and 20% GD[190] as part of their differential diagnosis. A study of over 2000 patients with splenomegaly found a haematological cause in 57%, mostly commonly leukaemia or lymphoma; amongst those with a haematological diagnosis 87% had generalised lymphadenopathy and 85% a raised cell count (haemoglobin, platelets or white cells)[192]. This is in contrast to our GD cohort where generalised lymphadenopathy not was documented and, in the absence of splenectomy, patients had reduced rather than raised cell counts. The chronicity of the symptom onset and the only modest reductions in cell counts, without neutropenia should make differentiation from acute leukaemia relatively straightforward. Chronic myeloid leukaemia may cause marked splenomegaly but a review of over 400 cases revealed only 4.5% of patients had a white cell count $<4.5 \times 10^9/L$ and 4.5% a platelet count $<150 \times 10^9/L$[193]. Whilst lymphoproliferative disorders may present with splenomegaly, the vast majority have an associated lymphocytosis[194] rather than the lymphopenia seen in our GD cohort.

3.6.3   Bleeding symptoms in the diagnosis of GD

Bleeding symptoms, most commonly bruising, are reported by over half of patients at diagnosis, a similar incidence to that found in an Israeli cohort[118]. In addition to bruising, more significant bleeding can occur, with post dental and/or post-operative bleeding occurring in eight patients pre-diagnosis. The condensed MCMDM-1 score was initially developed to assist in the diagnosis of VWD but had also been evaluated for its utility in the diagnosis of mild bleeding disorders[195] and FXI deficiency[196]. The commonest diagnoses in this study were mild platelet function defect, FXI deficiency and VWD – abnormalities which have all been reported in GD. 47.5% of GD in our study had a bleeding score ≥4, which has been shown to have a positive predictive value of 71% for the diagnosis of a mild bleeding disorder[195]. It is therefore not surprising that some patients were reviewed by haemostasis specialists and diagnosed with mild factor deficiencies or platelet function defects without the true underlying diagnosis being discerned.

Whilst GD is rare, it should be considered in patients presenting with bleeding symptoms, particularly if thrombocytopenia (even mild) or splenomegaly are also present, or if bleeding is disproportionate to the coagulation abnormalities present. Whilst bleeding symptoms have often been attributed to thrombocytopenia, within this cohort bleeding symptoms were out of proportion to the platelet count and there was no definite relationship between platelet count and bleeding symptoms. Whilst a platelet count of 50×10^9/L is often deemed acceptable for major surgical procedures[197], only one out of seven patients with post-operative bleeding had a platelet count $<50 \times 10^9$/L, supporting the presence of defects in platelet function and/ or the coagulation proteins.

FXI is of particular interest within haemostasis. Unlike other coagulation factor deficiencies, the level of FXI does not predict clinical bleeding risk[198] and the reasons for this are yet to be understood. Both GD and hereditary F11 deficiency are common within the Ashkenazi Jewish population with carrier rates of 1 in 12 and 1 in 8 respectively. With the increasing availability of mutational analysis techniques, it should be possible to clarify patients who have inherited FXI deficiency and the diagnosis of GD should be considered in patients in whom no mutation is identified.

3.6.4   Role of laboratory abnormalities in the differential diagnosis of GD

A history of bruising or bone pain or detection of splenomegaly on clinical examination should raise suspicion of the diagnosis of GD. In addition, in this study, four key laboratory parameters, beyond the full blood count, were identified which are abnormal at presentation in the majority of GD patients: raised serum ACE, polyclonal gammopathy, raised ferritin and low HDL cholesterol. These assays are readily available in most routine hospital laboratories.

ACE is a zinc-metallopeptidase which has a wide cellular distribution and is also secreted into plasma[199]. Increased serum ACE is found in disorders involving activation of cells of the monocyte/macrophage lineage, particularly sarcoidosis[200] and GD[201]. In contrast, ACE levels are normal or decreased in haematological malignancies[202] and thus may be a useful discriminatory test.

Low HDL cholesterol levels in GD have been correlated with disease severity[203;204] and response to ERT. Whilst hypocholesterolaemia has also been found in myeloproliferative disorders, these should be readily distinguishable from GD by the presence of increased cell count and the blood film appearances. Hypergammaglobulinaemia is most commonly found in inflammatory disorders, particularly liver disease and connective tissue disease[205] and it is notable that a number of patients were diagnosed by hepatologists, with a few presenting with abnormal liver function tests or jaundice secondary to hepatic involvement. Hyperferritinaemia, outside the context of iron overload, is also a feature of connective tissue disorders including Still's disease and haemophagocytic lymphohistiocytosis (HLH). HLH is characterised by dysregulated activation of the immune system and haemophagocytosis by macrophages. It shares many laboratory features with GD including cytopenias, hyperferritinaemia, hypertriglyceridaemia and a coagulopathy[206] but usually presents with an acute severe febrile illness[207]. One could hypothesise that these similarities arise due to overload of glucocerebrosidase following the increased delivery of glycosphingolipids to the lysosome that accompanies haemophagocytosis[208].

3.6.5   Pathophysiology of bleeding manifestation of GD

The presence of bleeding symptoms in GD has long been recognised, but the mechanisms underlying them and their relationship to other disease manifestations is less clear. Whilst a number of case reports and studies have investigation platelet and/or coagulation abnormalities in GD (see **Table 1-11**) the results have been conflicting. In addition the lack of characterisation of the bleeding phenotype beyond presence/absence of bleeding symptoms in these studies makes the clinical significance of any abnormalities difficult to interpret. Our study confirms that reduced levels of FXI are a disease manifestation of GD, rather than due to co-inheritance of *F11* mutations and improve significantly with ERT.

In our study we found no correlation between overall disease severity and bleeding symptoms or bone disease. The findings of multiple abnormalities in the coagulation system in previous studies, with reductions in coagulation factors and increased products of fibrinolysis has led to the hypothesis that coagulation abnormalities are the result of chronic activation of the coagulation system[119], with inflammation being the driver of that process. Increased levels of pro-inflammatory cytokines have been reported in GD[82-84], albeit, similarly to haemostatic assays, with conflicting results. Inflammatory processes have been implicated in many of the manifestations of GD, particularly gammopathies. We, however, could find no difference in bleeding symptoms between patients with or without a gammopathy suggesting other factors are involved in this process.

Coagulation factors are synthesised and, in many cases post-translationally modified in the liver and therefore infiltration of the liver by Gaucher cells may impair these functions even in the absence of overt hepatic dysfunction. Similarly to GD, liver disease is associated with a raised serum ACE[209], low HDL cholesterol, hyperferritinaemia and polyclonal gammopathy. Indeed, liver dysfunction as well systemic macrophage activation may contribute to these abnormalities in GD. It is therefore possible that low levels of coagulation factors as in part due to liver dysfunction, although why the abnormalities are so variable between studies is unclear. The liver is also involved in the synthesis of anticoagulant proteins (AT, PC and PS); these have been less well studies in GD. In one study of 30 patients, AT was only low in one[119], so it is likely that other mechanisms are involved.

Investigating the mechanisms underlying the bleeding diathesis is challenging, both due to the disorder itself and the nature and limitations of haemostatic assays. GD is a rare disorder and therefore most centres will only see a handful of new cases per year, meaning that recruitment to studies needs to run over a prolonged period to achieve even double-digit recruitment. As evidenced by the results for FXI levels in previous studies of coagulation factors and platelets[174;178], abnormalities often normalise with enzyme replacement therapy meaning that studies ideally need to be done on patients at diagnosis. In the absence of baseline pre-treatment data, studies on treated patients are likely to be difficult to interpret.

The studies performed to date examine plasma coagulation factors and platelet function – predominately aggregometry, but adhesion function has also been measured using a cone plate analyser in one centre. The conflicting results from these studies suggest that further investigations that purely examine these parameters may not be sufficient for gaining further mechanistic insights. Further work should include more novel assays of both platelet function and haemostasis control, ideally using whole blood assays which more closely reflect the complex interactions that occur between circulating cells, plasma proteins and the vessel lining in haemostatic processes. These assays would require fresh patient samples, an additional hurdle particularly for collaborative studies.

3.7   Conclusions

GD most commonly presents with symptoms related to splenomegaly, bleeding or bone pain. Ultimately the diagnosis is made by haematologists in almost 80% of patients, usually after performing a bone marrow rather than an enzyme assay. This, coupled with the oft long delays from initial symptoms suggest that increased awareness regarding GD is still required amongst these clinicians.

Whilst bleeding symptoms are most commonly bruising, more severe bleeding episodes do occur including epistaxis and post-procedural bleeds (including surgical and dental) and may lead to patients' presentation to specialist haemostasis clinics. The diagnosis should be considered especially in those with bleeding out of proportion to the laboratory abnormalities. The mechanisms underlying the bleeding diathesis remain unclear and bleeding does not clearly correlate with other disease

manifestations. Looking beyond plasma abnormalities, subsequent chapters examine the role of leucocytes and platelets in GD.

Chapter 4   Vascular features of Fabry disease

4.1   Introduction

The classic triad of clinical features of Fabry disease comprises pain (acroparasthesia), a rash (angiokeratoma) and sweating abnormalities beginning in childhood-adolescence with subsequent development of cardiac, renal and cerebrovascular complications resulting in a shortened life expectancy. As an X-linked disorder, the historical focus of clinical studies (both mechanistic and therapeutic trials) has been on male patients; however, in recent years it has been demonstrated that many females with a *GLA* mutation develop clinical features of FD[210]. Since the advent of ERT a large number of screening studies have been undertaken aiming to identify undiagnosed patients with FD who have renal, cardiac or cerebrovascular complications[48]. This has resulted in the increasing recognition of a broader phenotypic spectrum of FD than that classically described.

Evidence from cohort and registry studies of delays between symptom onset and diagnosis led to the suspicion that Fabry disease is under-diagnosed[35]. This has led to both newborn screening studies [29] and targeted screening studies of patient populations with evidence of organ dysfunction which can occur in FD (e.g. LVH[42;211], dialysis[43;212], stroke[125;213]). A key driver behind these studies is that with the advent of ERT, the natural history of FD might potentially be altered. Whilst these studies have demonstrated that undiagnosed cases of FD can be found in these populations, they have also raised new questions as to the natural history of FD in this broader phenotypic population in whom disease manifestations may be milder or later in onset than classically described.

A key aim of ERT is to prevent, or at least modify the progression of, critical organ dysfunction: renal, cardiovascular and cerebrovascular disease. This chapter examines the importance of vascular disease manifestations in a large, single-centre contemporary cohort of FD patients. The diagnostic pathways of index cases are examined and ways to improve this process discussed. Use of baseline assessments of the cohort allows a cross-sectional view of the disease manifestations of FD at different ages in both males and females. The prevalence of vascular manifestations and their relation to mutation status and age is explored. Particular attention is paid to cerebrovascular disease in FD. Unlike

other disease manifestations, stroke occurs almost as frequently in females as males[123] and has been described as occurring in the absence of other disease manifestations. Whilst declines in renal function or risk for ventricular arrhythmias can, to a degree be predicted, there is currently no method for predicting stroke risk in FD and stroke can often have an instantaneous, life-changing impact. Better understanding of the natural history of stroke in FD and potential risk factors may allow preventative intervention to reduce risk.

4.1.1 Purpose of this chapter

Whilst registry studies have proved valuable in advancing our knowledge of FD, they have limitations. Datasets are often incomplete and there is a potential registration bias towards more severely affected patients. The presence of a large specialist centre enables the study of a relatively large single cohort of patients. This chapter describes the patient cohort of the RFH Fabry clinic with particular focus on vascular features affecting critical organ function (renal, cardiac and cerebrovascular) and comprises three sections:

1. A cross-sectional description of the cohort:

> a) Prevalence of vascular disease in the presentation of index cases and incidence of vascular complications during their follow-up

> b) Prevalence of vascular complications at baseline and follow up in patients diagnosed on family screening.

2. Incidence of stroke/TIA and its relationship to:

> a) Disease factors: mutation, enzyme activity, disease severity, other disease manifestations

> b) Cardiovascular risk factors

> c) Prognostic scores

3. Abnormalities of plasma pro-coagulant factors and their relation to disease manifestations.

4.2   Hypotheses

1. Patients presenting with FD may experience vascular events (e.g. stroke, arrhythmias) prior to diagnosis and experience long delays between those events and arrival at the correct diagnosis. *Rationale:* FD is rare and therefore not commonly considered in the differential diagnosis of vascular events such as stroke.

2. Cerebrovascular events are not predicted by other disease manifestations, enzyme activity or more common cardiovascular risk factors *Rationale:* strokes have been reported in patients without other disease manifestations and FD diagnosed in cohorts of patients with cryptogenic stroke

3. Abnormalities of plasma procoagulant and anticoagulant factors are associated with vascular manifestations of FD *Rationale:* von Willebrand factor and anticoagulant pathways are controlled primarily by the endothelium and these disease manifestations have been hypothesised to be due to endothelial dysfunction.

4.3   Methods

Patients have been reviewed at the Royal Free since the late 1990s, when a number of males were enrolled in clinical trials of agalsidase alfa (Replagal), commencing in 1999. Replagal and Fabrazyme enzyme replacement therapies were both licensed by the European Medicines Agency on 3/8/2001 and services for the management of lysosomal storage disorders were nationally commissioned in 2005. Decision making regarding institution of enzyme replacement therapy was undertaken according to national guidelines current at that time.

4.3.1   Historical clinical record review

The case records of families attending the Lysosomal Storage Disorders Unit since its inception until the end of August 2013 were reviewed.

4.3.2   Baseline clinical assessment

At baseline, patients had a full medical and family history taken. A comprehensive family tree was constructed to include all known family members with FD. Assessment of organ function included quantification of urinary albumin and protein, measurement of glomerular filtration rate (GFR) using radio-isotopes, brain magnetic resonance imaging (MRI) and review by a cardiologist including electrocardiogram (ECG) and

echocardiogram. Left ventricular mass was calculated using the Devereux formula[214] and adjusted for height; increased left ventricular mass was defined as $\geq47g/m^{2.7}$ in females and $\geq50g/m^{2.7}$ in males. Dermatological review for angiokeratoma was undertaken by a dermatologist with a specialist interest in FD.

The baseline assessment for the purposes of this study is the date of initial comprehensive assessment of disease status undertaken at a specialist centre. Prior to the start of clinical trials of ERT in the UK few patients had had a comprehensive assessment of their disease status (e.g. cardiac, renal and cerebral) and therefore for patients diagnosed pre-1999 there is a lapse in time between date of diagnosis and baseline assessment. All baseline assessments were undertaken prior to institution of therapy.

All patients had measurements of enzyme activity and mutational analysis undertaken. The majority of enzyme activities were measured on plasma at the Royal Free or the Institute of Child Health (ICH), University College London using identical methodology (see Methods chapter) and reference ranges (4-21 nmol/ml/hour). A small number of patients had enzyme activity assays performed by other laboratories with different methodologies and reference ranges; where no enzyme activity results from RFH/ICH are available, these patients are therefore excluded from analyses relating to enzyme activity. Mutations have been classified as nonsense, missense and deletions. Patients with missense mutations have been divided into three groups: the N215S mutation, mutations described as being associated with a mild/late onset phenotype (A143T, R301Q, R112C and R112H) and other missense mutations.

4.3.3 Severity and prognostic scores

All severity and prognostic scores were calculated by the researcher from the historical baseline data for the purpose of this study to ensure internal consistency across the data set. Disease severity scores were calculated using the FOS-MSSI (severe disease manifestations.

Table 4-1), which scores disease features across four domains with the score weighted towards more severe disease manifestations.

Table 4-1: FOS-MSSI Severity Score for FD

General (max = 15.5)	Neurological (max = 15)	Cardiovascular (max = 18)	Renal (max = 18)
Angiokeratoma (1.5) Oedema (1) Musculoskeletal pain (1) Cornea verticillata (1) Hypo/hyperhidrosis (1) *or* anydrosis (2) Abdominal pain (1) Diarrhoea/constipation (1) Haemarrhoids (1) Pulmonary involvement (2) NYHA classs (1/2/3/4)	Tinnitus (1) Vertigo (1) Pain attacks (4) *or* chronic pain (6) TIA/PRIND (3) *or* stroke (6) *or* radiographic lesions only (1) Depression (1)	ECG abnormalities (2) LVH (6) *or* cardiomyopathy (10) Pacemaker (4) Hypertension (1) Valve insufficiency (1)	Proteinuria (4) *or* low GFR (8) *or* end stage renal failure (12) *or* dialysis (18)

As severity increases with age, the score was adjusted for age by subtracting the calculated score from the predicted score for the patients age[215]:

Males: predicted score = $[2.29 + (0.05 * \text{Age})]^2$

Females: predicted score = $[0.96 + (0.05 * \text{Age})]^2$

Prognostic scores were calculated using the Fabry International Prognostic Index (FIPI) (see **Table 4-2**) [216], a score developed to try to predict risk of developing clinically severe events: renal (ESRF, transplant or dialysis), neurological (stroke, TIA or PRIND), cardiac (stage III/IV heart failure, MI surgery or pacemaker/ICD insertion) or death.

Table 4-2: Fabry International Prognostic Index (FIPI)

Domain	Score	Risk
Cardiac	eGFR<60ml/min/m$^{2.7}$) = 1.5 Hearing impairment = 1 LVMI ≥50g/m$^{2.7}$ = 1 Proteinuria (>300g/24hr) = 0.5 Vertigo = 0.5 Angiokeratoma/telangiectasia = 2	Low 0-2 Medium 3-4 High 5-7
Renal	eGFR<60ml/min/m$^{2.7}$) = 2 Male gender = 1.5 Proteinuria (>300g/24hr) = 1.5 Angiokeratoma/telangiectasia = 2	Low 0-2 Medium 3-4 High 5-7
Neurological	Hearing impairment = 2.5 eGFR<60ml/min/m$^{2.7}$) = 2 Vertigo = 1.5	Low 0-2 Medium 3-4 High 5-7

	Anhydrosis/hypohydrosis = 1	
Death	eGFR<60ml/min/m$^{2.7}$) = 4 Abnormal ECG =2	
Composite	eGFR<60ml/min/m$^{2.7}$) = 2 Hearing impairment = 2 Microalbuminuria = 1 LVMI ≥50g/m$^{2.7}$ = 1 Anhydrosis/hypohydrosis = 1	Low 0-3 High 4-7

4.3.4 Thrombophilia screening

The thrombophilia assays were performed by the biomedical scientist staff of the haemophilia centre (Royal Free Hospital).

Protein C (PC) activity: PC was activated by venom of *Agkistodon coutartrix*. Activated protein C activity was measured using chromogenic substrate S2366 (HaemosIL).

Protein S (PS) free activity: was measured using a latex agglutination assay. Purified C4BP adsorbed onto latex beads reacts with free PS in the plasma. A monoclonal antibody against PS is bound to a second latex reagent, resulting in agglutination by the adsorbed free PS.

Anti-thrombin (AT) activity: plasma was incubated with an excess of bovine thrombin, in the presence of heparin, resulting in inhibition of the thrombin. Residual thrombin was then measured using a chromogenic substrate, Tos-Gly-Pro-Arg-ANBA-IPA (HaemosIL). The residual thrombin is inversely proportional to the AT activity.

FVL and prothrombin gene mutation: PCR-based mutational analysis was performed using the GeneXPERT ® system

Lupus anticoagulant dilute Russel Viper Venom Test (DRVVT): the coagulation time following the activation of FX with Russel Viper Venom (American Diagnostica) was measured and ratio to normal plasma calculated. If the ratio was >1.10, a neutralisation test performed by addition of a high concentration of phospholipid to demonstrate phospholipid dependence. The test was positive if >10% correction occurred with addition of phospholipid. If positive, the test was repeated after an interval of at least 12 weeks to ascertain persistence.

FVIII activity: FVIII activity was measured using a standard APTT-based one stage assay, performed on an ACL TOP coagulometer (Instrumentation Laboratory).

4.4   Results

4.4.1   Overview of the RFH FD cohort

233 patients, from 91 different family pedigrees were included in the cohort study, of whom 92 (39.5%) are male and 141 (60.5%) female. 65 patients (27.9% cohort, 44 male, 21 female) were index cases and 168 patients (72.1%, 48 male, 120 female) were diagnosed on family screening. In two pedigrees, two index cases were diagnosed independently within the same family due to lack of awareness of the diagnosis in different branches of the family. Of the index cases 36 males and 14 females were diagnosed following investigation of symptoms whilst 8 males and 6 females were diagnosed following further investigation of incidentally detected abnormalities (e.g. abnormal ECG). For both index cases and those diagnosed on family screening, most cases were diagnosed within the last decade.

Across 87 families the median number of affected family members was 3, range 1-31. Two index cases were adopted and one was estranged from the rest of their family. One index case was from overseas and the rest of the family lived in a country where testing and treatment were not available.

53 different mutations were found in 91 family pedigrees. The recurrent mutations are shown in **Table 4-3**. 44 mutations were point mutations with 36 (67.9%) being missense mutations and 8 (15.1%) being nonsense mutations. There were 8 short deletions and one pedigree had a deletion of exon 1. The full list of different mutations is shown in **Figure 4-1**; mutations were found in all exons.

Table 4-3: Recurrent mutations in FD

Mutation	Mutation type	Exon	No pedigrees	No pt in cohort
C52G	Missense	1	2	2
A143T	Missense	3	3	10
P205T	Missense	4	3	13
c.717del2	Deletion	4	2	2
N215S	Missense	5	29	84
A257P	Missense	5	2	2
R301Q	Missense	6	2	9
I317T	Missense	6	2	9
R342Q	Missense	7	2	3

EXON 1
A13P
A31V
N33D
M42V
R49L
C52G
Exon1 del

EXON 2
S78X
I91T
D93Y
Q107X
R112C/H
c.359_364del5
c.402delT

EXON 4
Y184X
P205T
W209X

EXON 5
N215S
R220X
Q221P
R227X
A257P
G261V
c.700_702del3
c.717-8del2

EXON 7
E338K
R342Q
G361A/R
L372P
T401I/L
P409T

EXON 3
S126G
A143T
L166P
c.466delG
c.520delT

EXON5/ INTRON5
boundary
c.748_801+8del62
c.802-3_802-2del2

EXON 6
V269A
W277X
Q279H
Q280H
P293H
R301P/Q/X
I303N
A309P
V316E
I317T

Figure 4-1: Location of GLA mutations within the cohort. GLA homodimer showing missense mutation sites (red) and nonsense mutation sites (pink); drawn using PyMol, based on crystal structure 1r46.

4.4.2 Causes of death

Eleven patients in the cohort, all male, died during follow up. Ages and causes of death are shown in **Table 4-4**. The median age at death was 53 years (range 34-81); cardiac complications were the most frequent cause of death. All seven males who were diagnosed following family screening had significant manifestations of Fabry disease – either angiokeratoma/acroparasthesia in the three diagnosed in childhood/adolescence or cardiac/renal events for those diagnosed in the later decades.

Table 4-4: Age and cause of death

Age diagnosis	Age death	Cause of death	Presenting feature	Mutation	ERT
6	34	Cardiac	Family screening – subsequent acroparasthesia & angiokeratoma	A143T	Age 21-34
13	45	Cardiac	Pain	c.520delT	Pre-ERT
14	53	Stroke	Family screen – acroparasthesia	R227X	Age 44-53
15	43	Subarachnoid haemorrhage	Family screen – acroparasthesia & angiokeratoma	G361R	Age 41-43
29	58	Stroke & cardiac failure	Stroke	G361R	Age 50-58
38	41	Cardiac	Rash	c.466delG	Age 38-41
48	52	Cardiac	Cardiac failure & syncope	E338K	Age 48-52
65	66	Mesothelioma	Family screen but stroke aged 57	A13P	Age 65-66
69	71	Cardiac	Family screen but MI and AVR aged 59	N215S	Age 71
72	76	Cardiac	Family screen – ICD for VT aged 70	N215S	Age 72-76
73	81	Cardiac	Family screen but diagnosed "HOCM" age 60	N215S	Age 73

4.4.3 Vascular features in the presentation of index cases

Presenting features

Presenting features were defined as the primary clinical features, investigation of which led to the diagnosis of FD, whilst initial symptoms are the first symptoms related to FD which the patient experienced and in some cases differ from the presenting feature.

There were 44 index males, with a median age of diagnosis of 49.5 years (range 10-74) and 21 index females with a median age of diagnosis of 44 years (range 25-75). The presenting features and median age of diagnosis for each presentation are shown in **Table 4-5**. Cardiac manifestations were the commonest presentation, being the presenting feature in 66% of males and 33% females. There was no difference in age at diagnosis between males and females presenting with cardiac features (58yr vs 54 yr, p =0.99). Symptoms related to arrhythmias (palpitations, syncope) were commonest with arrhythmias ranging from atrial fibrillation to ventricular fibrillation.

Twenty five percent of males presented with a rash and/or acroparasthesia; these men presented at a significantly younger age than those with cardiac symptoms (median age 19 yr vs 58yr, p<0.0001). This was a rare presentation in females with only one female presenting with acroparasthesia. In contrast, stroke was a common presentation in females, but was the presenting feature in only one male. Four females were diagnosed following the finding of cornea verticillata on slit lamp examination of the eyes, but this presentation was not seen in men.

Table 4-5: Presenting symptoms and age at diagnosis of index cases

Organ system	Symptom	No. (%) patients		Age at diagnosis: median (range, years)	
		Male (N = 44)	Female (N = 21)	Male	Female
Cardiac	*All cardiac*	*29 (65.9)*	*7 (33.3)*	*58 (33-74)*	*54 (45-75)*
	Abnormal ECG/ LVH on imaging	6 (13.6)	-	49 (42-56)	-
	Syncope	7 (15.9)	1 (4.7)	59 (41-74)	46
	Palpitations/ arrhythmia	5 (11.4)	2 (9.5)	59 (40-74)	54 (48-60)
	Chest pain	5 (11.4)	1 (4.7)	55 (51-72)	45
	Shortness of breath	4 (9.1)	2 (9.5)	56 (33-71)	71.5 (68-75)
	Heart murmur	2 (4.5)	-	57 (54-60)	-
	FH of sudden death	-	1 (4.7)	-	54
Skin & pain	*All rash/ acroparsthesia*	*11 (25)*	*1 (4.7)*	*19 (10-33)*	*23*
	Rash	10 (22.7)	-	21 (13-33)	-
	Acroparasthesia	1 (2.3)	1 (4.7)	16	23
Neurological	**Stroke/TIA**	**1 (2.3)**	**5 (14.3)**	**29**	**43 (25-49)**
Renal	*All renal*	*3 (6.8)*	*3 (14.3)*	*25 (25-38)*	*27 (25-35)*
	Proteinuria	1 (2.3)	3 (14.3)	25	27 (25-35)
	Haematuria	1 (2.3)	-	25	-
	Hypertension secondary to renal failure	1 (2.3)	-	38	-
Opthalmological	**Corneal deposits on eye exam**	**-**	**4 (19.0)**	**-**	**33.5 (30-45)**
Gastrointestinal	**Abdominal pain/ diarrhoea**	**-**	**1 (4.7)**	**-**	**29**

Table 4-6: Initial symptoms/ disease manifestations in index cases

| Organ system	Symptom	No. patients (%)		Age at onset (years): median & range		Time onset to diagnosis (years): median & range	
		Male (N=44)	Female (N=21)	Male	Female	Male	Female
Cardiac	*All cardiac*	*21 (47.7))*	*5 (23.8)*	*54 (32-73)*	*59 (42-69)*	*2 (<1-18)*	*3 (<1-9)*
	Syncope	6 (13.6)	-	59 (45-73)		6.5 (1-15)	
	Palpitations	3 (6.8)	1 (4.8)	59 (57-61)	48	1 (1-1)	<1
	Incidental LVH	4 (9.1)	-	50.5 (41-57)		10 (<1-18)	
	Abnormal ECG	4 (9.1)	1 (4.8)	49 (44-54)	59	<1 (<1-4)	9
	Chest pain	2 (4.5)	2 (9.5)	44.5 (39-50)	51 (42-60)	8 (4-12)	1.5 (<1-3)
	SOB	2 (4.5)	1 (4.8)	47 (32-62)	69	1.5 (1-2)	6
Skin & pain	*All rash/acroparasthesia*	*18 (40.9)*	*5 (23.8)*	*9 (5-16)*	*20 (7-26)*	*17 (1-53)*	*20 (2-29)*
	Acroparasthesia	16 (36.4)	4 (19.0)	9 (5-16)	17 (7-21)	19 (6-53)	15 (2-29)
	Rash	2 (4.5)	1 (4.8)	12 (9-15)	26	1.5 (1-2)	20
Neurological	*All neurological*	*0*	*5 (23.8)*	*-*	*40 (24-46)*	*-*	*6 (1-13)*
	Stroke/TIA	-	4 (19.0)	-	39.5 (24-46)	-	4 (1-13)
	Migraine with aura	-	1 (4.8)	-	40	-	9
Renal	*All renal*	*3 (6.8)*	*2 (9.5)*	*29 (25-38)*	*27(25-29)*	*1 (1-27)*	*3 (<1-6)*
	Proteinuria	2 (4.5)	2 (9.5)	27 (25-29)	27 (25-29)	14 (1-27)	3 (<1-6)
	Hypertension	1 (2.3)	-	38	-	1	-
Gastrointestinal	*Abdo pain/ IBS*	*2 (4.5)*	*1 (4.8)*	*45 (41-49)*	*18*	*18 (6-30)*	*11*
Eyes	*Asymptomatic cornea verticillata*	*-*	*3 (14.3)*	*-*	*37 (30-45)*	*-*	*-*
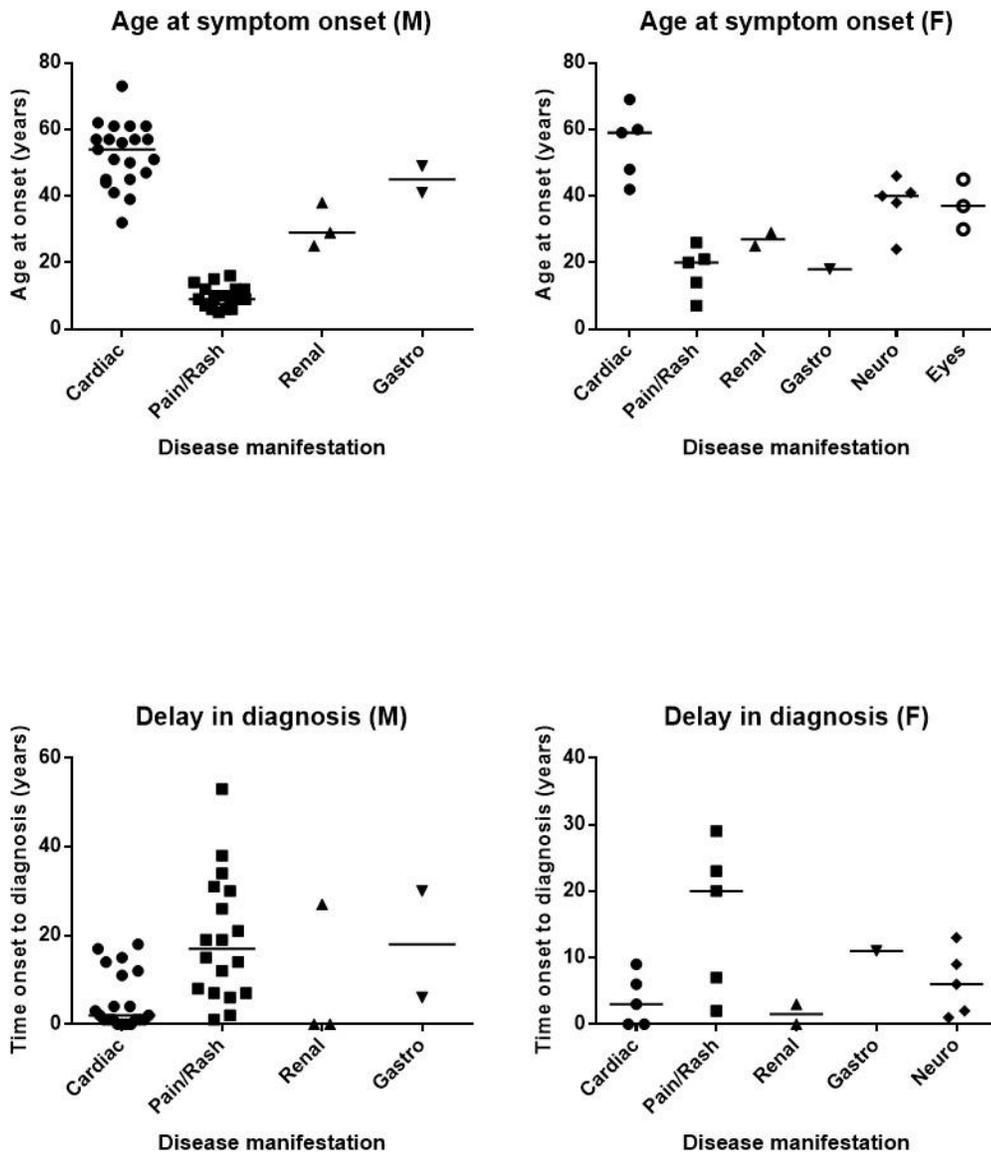
Figure 4-2: Age at symptom onset and delay from symptom onset to diagnosis in male and female index cases.

Initial symptoms and diagnostic delays

Acroparasthesia was the commonest single initial symptom in males, being the initial symptom in 41% of index males, see **Table 4-6**. Males whose initial symptom was acroparasthesia or a rash had a significantly younger age of symptom onset than males whose initial symptom was cardiac-related (9yr vs 54yr, p<0.0001) and experienced significantly longer diagnostic delays (17yr vs 2 yr, p = 0.0001), see **Figure 4-2**. In eight males and one female, the first awareness of disease manifestations was the finding of an abnormality on an ECG or chest radiograph performed for other reasons (e.g. pre-operatively or as part of an insurance medical). In four cases, further investigation of these findings yielded the correct diagnosis but in the other five patients, the correct diagnosis was not reached until development of overt cardiac symptoms a number of years later.

Whilst acroparasthesia and cardiac symptoms were also common initial symptoms in females, cerebrovascular events, occurring in the absence of preceding FD symptoms were the initial disease manifestations in five females. This contrasts to males where cerebrovascular events did not feature as the initial symptom in any index case.

Most diagnostic delays appeared to be due to non-diagnosis rather than mis-diagnosis. In some cases patients were labelled as having a clinical feature/ syndrome (e.g. hypertrophic cardiomyopathy or an arrhythmia) but the correct underlying diagnosis was not reached for a considerable period of time. In other cases an erroneous diagnosis was assigned, but usually a diagnosis of exclusion, for which there is no concrete diagnostic test (e.g. irritable bowel syndrome). These were most commonly rheumatological or cardiac (see Table **4-7**).

Table 4-7: Alternative diagnoses given prior to diagnosis of Fabry disease

	Diagnosis	Index cases	Family screening
Rheumatological	*All*	*3*	*7*
	Rheumatic fever	1	2
	Rheumatoid arthritis	1	0
	Growing pains	1	5
Cardiac	*All*	*5*	*6*
	HOCM	2	4
	Sick sinus syndrome	1	1
	Wolf-Parkinson-White syndrome	1	1
	Long QT syndrome	1	0
Other	*All*	*5*	*5*
	Multiple sclerosis	1	0
	Irritable bowel syndrome	3	3
	Pre-eclampsia	1	0
	Hereditary haemorrhagic telangestcaisia	0	1
	Chronic fatigue syndrome	0	1

In all patients the diagnosis of FD was confirmed by enzyme assay and mutational analysis but in all six patients presenting with renal manifestations, the diagnosis was originally suspected by the presence of storage material on renal biopsy. In one male presenting with cardiac hypertrophy, an endomyocardial biopsy was performed pre-diagnosis which again showed storage material and led to confirmatory tests for FD.

Change pattern of presentation of FD

Over the past decade there has been a rapid increase in the number of both male and female index cases. Amongst males this is attributable to a large number of males with the N215S mutation who are primarily presenting at an older age with cardiac manifestations, see **Figure 4-3**. Almost all female index cases have been diagnosed within the last decade, presenting with a wider variety of clinical features and mutation types.
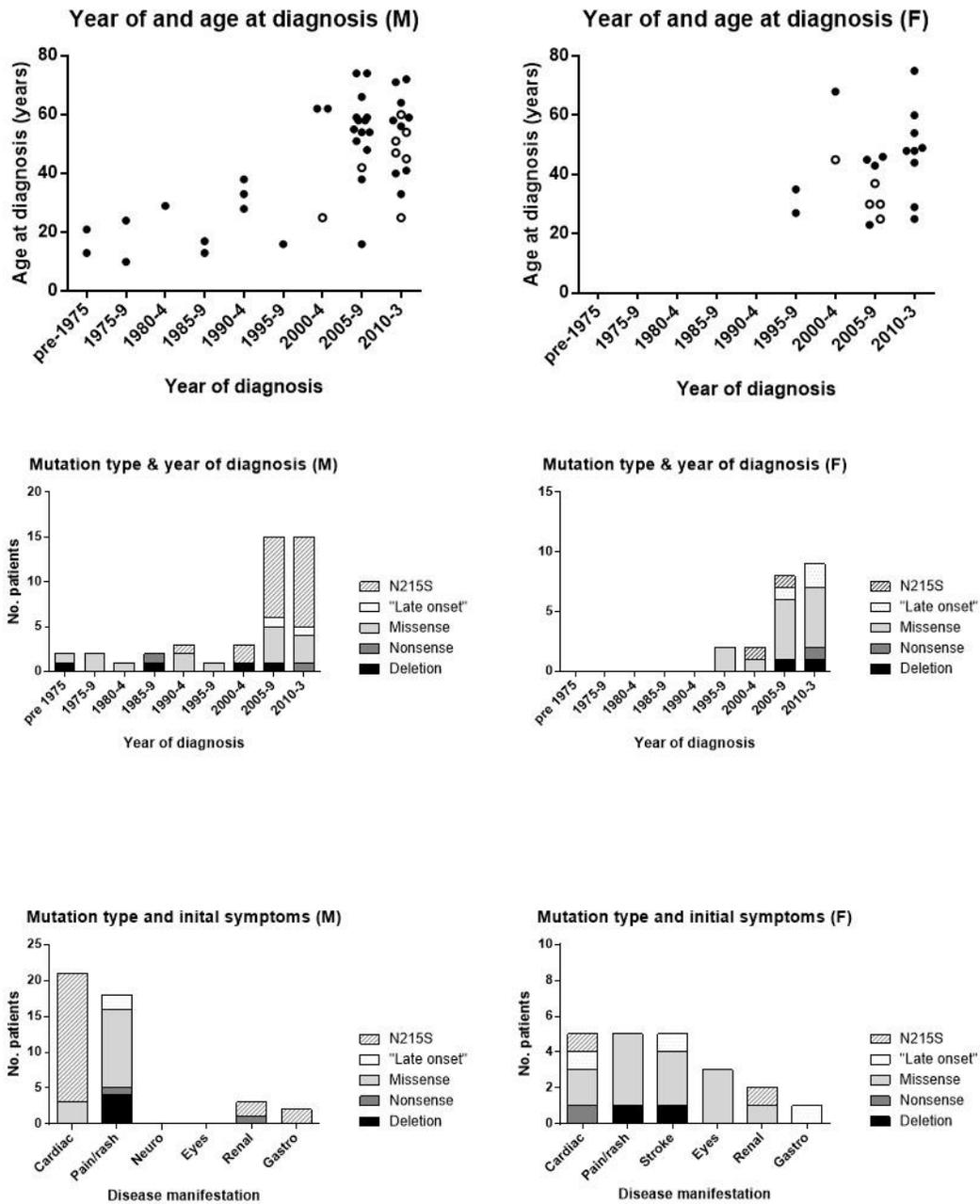
Figure 4-3: Changing pattern presentation of FD index cases: age of diagnosis by year of diagnosis; mutation type and year of diagnosis; and mutation type and initial symptoms. Open circles = incidental cases.

4.4.4   Vascular manifestations in index cases

Vascular disease manifestations at both baseline and follow-up were analysed by mode of presentation, with a focus on cerebrovascular disease. Data on PPM/ICD insertion and development of ESRF (GFR<15ml/min/m$^{2.7}$) were also analysed both as an indication of severe disease in other organ systems and because these complications, similarly to stroke, can be life changing or limiting.

Male index cases:

The vascular complications experienced by male index cases are detailed in **Table 4-8**. One male had a stroke pre-diagnosis but five additional males suffered a stroke during follow up, two of whom are now deceased. Strokes were commonest in those whose initial symptoms were "classical" features of acroparasthesia/angiokeratoma, despite the younger age of this group of patients. One third of male index cases have developed WML on MRI in the absence of a clinical stroke/TIA. PPM and ICD insertions occurred predominately in those with cardiac presentations, who were also older, and ESRF only occurred in the three males whose initial disease manifestations were renal.

Table 4-8: Vascular complications in male index cases

Initial symptom	All	Pain/ rash	Cardiac	Renal	Other
No.	44	18	21	3	2
Median age at baseline	51 (16-74)	40 (16-59)	58 (33-74)	47 (25-56)	62.5 (55-70)
Median age latest FU/death	58 (22-77)	43.5 (22-64)	61 (35-77)	58 (27-59)	67 (61-73)
TIA/Stroke pre-diagnosis	1 (2.3%)	1	0	0	0
TIA/Stroke at latest FU	6 (13.6%)	4	2	0	0
WML w/o stroke at baseline	17 (38.6%)	6	7	2	2

WML w/o stroke on latest follow up	17 (38.6%)	8	5	2	2
PPM/ICD at baseline	9 (20.4%)	0	8	0	1
PPM/ICD at latest FU	11 (25%)	1	9	0	1
ESRF	3 (6.8%)	0	0	3	0
Death	4 (9.1%)	3	1	0	0

Female index cases:

The vascular manifestations in female index cases are detailed in **Table 4-9**. Six females had suffered a stroke/TIA pre-diagnosis, in five of whom stroke was the initial symptom of FD. No further strokes/TIA have occurred during follow up. One third of females have WML on MRI in the absence of a clinical event. The incidence of stroke was higher in female index cases compared to males (28.5% vs. 13.6%) with a similar incidence of WML in both groups. In contrast to males, no female index cases have developed end stage renal failure or required a cardiac defibrillator or pacemaker and there have been no deaths amongst females.

Table 4-9: Vascular complications in female index cases

Initial symptom	All	Pain/ rash	Cardiac	Stroke	Renal	Other
No.	21	5	5	5	2	4
Median age at baseline	44 (24-75)	30 (24-46)	60 (46-75)	48 (25-53)	31.5 (28-35)	35.5 (29-49)
Median age latest FU	48 (26-77)	38 (28-51)	62 (48-77)	49 (26-54)	39 (29-49)	39.5 (30-57)
TIA/Stroke pre-diagnosis	6 (28.5%)	1	0	5	0	0
TIA/Stroke at latest FU	6 (28.5%)	1	0	5	0	0
WML w/o stroke at baseline	6 (28.5%)	1	3	N/A	1	1
WML w/o stroke on	7 (33.3%)	1	3	N/A	2	1

latest follow up						
PPM/ICD at baseline	0	0	0	0	0	0
PPM/ICD at latest FU	0	0	0	0	0	0

4.4.5 Vascular manifestations in males diagnosed on family screening

168 patients were diagnosed as part of family screening, comprising 48 males (median age diagnosis 24 years; range 6-72) and 120 females (median age diagnosis 34 years; range 7-78). Thirty two males (66.6%) had overt symptoms of FD at the time of diagnosis. The critical organ vascular complications encountered in males, both at baseline and subsequently are summarised in **Table 4-10** and discussed in detail below.

Table 4-10: Vascular complications in males diagnosed on family screening

Symptoms at diagnosis	All	Pain/ rash	Cardiac/ cerebrovasc.	Other	Asymp. organ involvement	Features during follow up	Asymptomatic, untreated
No. (%)	48	21 (43.8%)	8 (16.6)	3 (6.3)	8 (18.2)	4 (8.3)	4 (8.3)
Age at diagnosis: median (range)	24 (6-72)	20 (6-39)	65.5 (53-73)	28 (7-29)	39 (6-61)	10 (5-24)	14.5 (14-70)
TIA/Stroke pre-diagnosis	2 (4.2%)	0	2	0	0	0	0
TIA/Stroke at any time	9 (18.7%)	3	3	2	0	1	0
WML at baseline	16 (33.3%)	9	6	0	0	1	0
WML on latest follow up	22 (45.8%)	12	6	2	0	2	0
PPM/ICD at baseline	2 (4.3%)	0	2	0	0	0	0
PPM/ICD at any time	4 (8.3%)	1	3	0	0	0	0
ESRF	2 (4.3%)	1	1	0	0	0	0
Death	7 (14.6%)	2	4	0	1	0	0

Males with acroparasthesia/ angiokeratoma pre-diagnosis

Twenty one males reported pain and/or angiokeratoma as their first symptom with a median age of onset of 7 years (range 5-12) and a median age of diagnosis of 20 years (range 6-39); the median delay from symptom onset to diagnosis was 11.5 years (range <1-33 years). In three patients the symptoms had been attributed to growing pains and one patient had been given an erroneous diagnosis of hereditary haemorrhagic telengectasia. None of these patients had suffered a stroke or required dialysis or a pacemaker/defibrillator prior to diagnosis. Full baseline assessments occurred at a median age of 35 years (range 16-51) by which time seven had LVH, seven proteinuria, four a GFR <60ml/min/m$^{2.7}$and nine radiological evidence of white matter lesions. Three have subsequently had strokes, one has required a pacemaker and one has developed end stage renal failure; two have died from complications of FD. Five of these men had deletions, seven nonsense mutations and nine missense mutations.

Males with cardiac or cerebrovascular manifestations pre-diagnosis

Six males had overt cardiac manifestations, for which they were under the care of cardiologists, prior to the diagnosis of FD being made on family screening. The median age of symptom onset in these men was 58 years (range 52-64). One man had had an aortic valve replacement and pacemaker insertion prior to diagnosis and both he and another male had been given an insufficient diagnosis of hypertrophic obstructive cardiomyopathy (HOCM). Two further males had suffered a TIA as their initial FD related manifestation at the ages of 57 & 69 years. Both had also developed cardiac manifestations prior to FD diagnosis with one requiring an ICD insertion for presyncopal VT and the other being investigated for possible cardiac amyloid after an abnormal echocardiogram. The median time from symptom onset to diagnosis in these eight men was 3 years (range 1-14). Baseline assessments in these eight men were performed at a median age of 65 years (range 52-73) at which point 7/8 had left ventricular hypertrophy, with the one patient without LVH having experienced VT; all had radiological evidence of cerebral WML and two had a GFR <60ml/min/m$^{2.7}$, although one of these was diabetic and required dialysis. Proteinuria was present in 3/8, all with concomitant diabetes.

Subsequently, two men have suffered strokes, one of whom had initially had a TIA. Four are deceased, three from FD complications and one from mesothelioma. The mutations found in these eight men were N215S (6) and other missense (2).

Males with other symptoms pre-diagnosis

Three other males were symptomatic at the time of diagnosis by family screening. One was being investigated for growth failure at the age of seven, when his mother was diagnosed with FD, with a short deletion. He subsequently developed acroparasthesia and at baseline assessment at the age of 17 he had ECG evidence of LVH and has subsequently suffered a TIA and declining renal function. Another had developed symptoms of Raynaud phenomenon at the age of 24 and was diagnosed at the age of 29 (P205T mutation) by which time he had ECG evidence of LVH; he has not developed renal or cerebrovascular disease manifestations. The third man had developed gastrointestinal symptoms and vertigo in his 20s and on baseline assessment at the age of 27 had LVH and proteinuria and has subsequently suffered a TIA with declining renal function.

Males asymptomatic at diagnosis

Sixteen males were asymptomatic at the time of diagnosis, with a median age at diagnosis of 15 years (range 5-70). Two males with nonsense mutations, both diagnosed age 5 years developed acroparasthesia at the ages of 7 and 12 years and have subsequently developed LVH, proteinuria and one has had a stroke. By the time of baseline assessment at a median age of 27 years (range 15-50), nine of the remainder had developed disease manifestations (2 acroparasthesia, 4 LVH and 3 LVH plus proteinuria) of whom one also had radiological evidence of WML but none have developed a stroke. One died suddenly from a presumed cardiac arrhythmia aged 34 years. Of the other five males one has proteinuria and renal impairment but has declined ERT and the other four are pauci-symptomatic and have also declined ERT presently. Other than the two patients with nonsense mutations, the mutations found in these patients were N215S (9) and those associated with a later onset phenotype (5).

Mutation type and changing pattern of presentation

Similar to index cases, over the last decade there has been an emergence of male patients diagnosed on family screening in the later decades of life who predominately have the N215S mutation and cardiac disease (see **Figure 4-4**). The number of deletions, nonsense and missense mutations has remained relatively stable and the majority of males with these mutations already had "classical" FD symptoms of acroparasthesia/ angiokeratoma pre-diagnosis. Only four males remain untreated, three of whom are relatively young with the N215S mutation and one of whom has the A143T mutation but with a considerably higher residual enzyme activity than would be expected for an affected male.
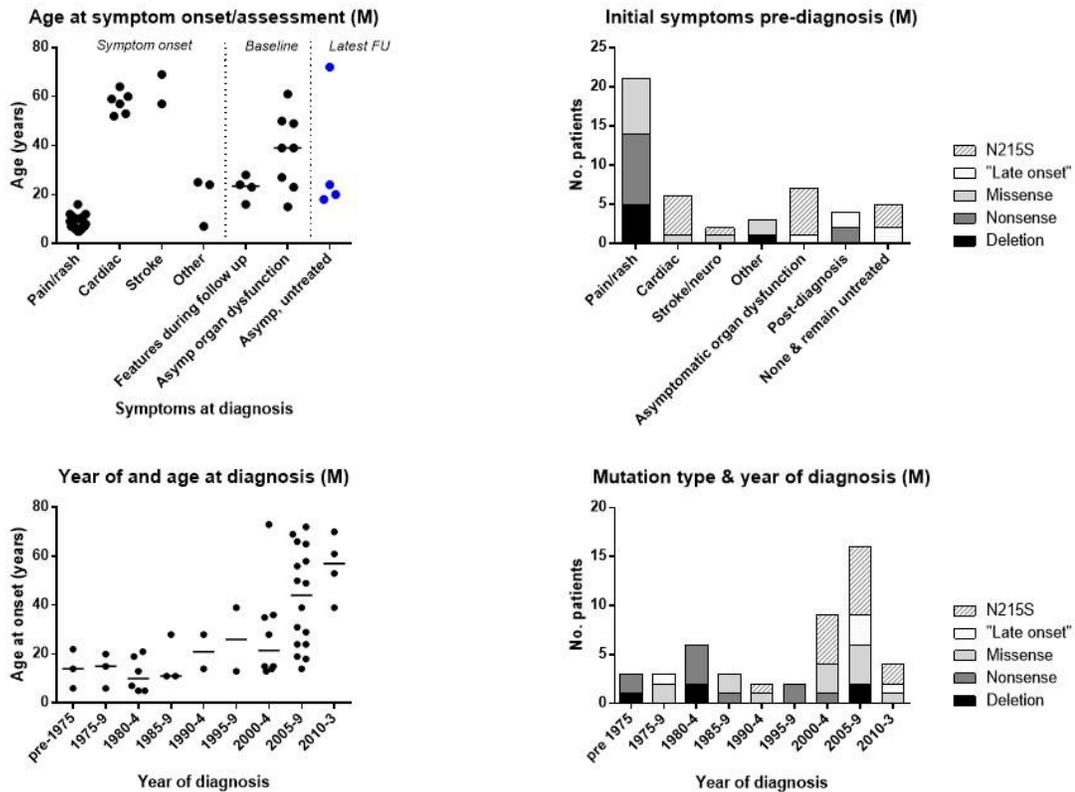

Figure 4-4: Disease manifestations at presentation of males diagnosed on family screening: age (blue = not receiving ERT) and mutation by initial symptoms. Increasing number of diagnoses with increased age at diagnosis and increased prevalence of N215S mutation in past decade.

4.4.6 Vascular manifestations in females diagnosed on family screening

Forty (33.3%) of females had overt symptoms of FD at the time of diagnosis by family screening with another six (5%) developing symptoms post-diagnosis and 21 (17.5%) having evidence of FD-related organ dysfunction on baseline assessment. Critical organ dysfunction is much less prevalent than in male with 53 females remaining asymptomatic and untreated and five of those with GI symptoms also not requiring ERT. The main vascular complications experienced by these females are shown in **Table 4-11**. Eleven females (9.2%) have suffered from a stroke/TIA, of whom four had the event pre-diagnosis. Over one third had WML on brain MRI without suffering a clinical stroke. Only 4 females (3.3%) required a PPM/ICD insertion and none developed ESRF.

Table 4-11: Vascular complications in females diagnosed on family screening

Symptoms at diagnosis	All	Pain/rash	Cardiac/ cerebrovasc	Other	Asymp organ involvement	Features during FU	Asymptomatic, untreated
No. (%)	120	19 (15.8%)	7 (5.8)	14 (11.7)	21 (17.5)	6 (5)	53 (44.2)
Age at diagnosis: median (range)	34 (7-78)	27 (8-53)	62.5 (34-78)	25 (14-50)	45 (10-72)	38.5 (7-49)	33 (8-65)
Age at BL: median (range)	37 (11-78)	39 (13-2)	67 (51-78)	26 (14-50)	55 (32-72)	46.5 (35-66)	33 (11-68)
TIA/Stroke pre-diagnosis	3 (2.5)	2	1	0	0	0	0
TIA/Stroke at any time	11 (9.2)	4	2	0	4	1	0
WML at baseline	37 (30.8)	4	5	2	14	4	8
WML on latest follow up	41 (34.2)	7	5	2	15	4	8
PPM/ICD at baseline	1 (0.8)	0	1	0	0	0	0
PPM/ICD at any time	4 (3.3)	1	2	0	1	0	0

Females with acroparasthesia pre-diagnosis

Nineteen females had developed acroparasthesia prior to the diagnosis of FD, at a median age of 13 years (range 5-30). The median delay from symptom onset to diagnosis was 12 years (range 3-39). In two females the symptoms had been attributed to growing pains and in another to rheumatic fever. Prior to diagnosis two of these females had also suffered a TIA. Median age at baseline assessment was 41 years (range 13-62) by which time 6/19 had LVH, 3/19 proteinuria and 2/19 had WML on MRI in the absence of a clinical event (stroke/TIA). During follow up two further patients suffered a stroke (one of whom had WML at baseline) and one patient required an ICD insertion. None have developed a GFR <60ml/min/m$^{2.7}$. Mutations identified in these patients were: deletions (5), nonsense (4), missense (7).
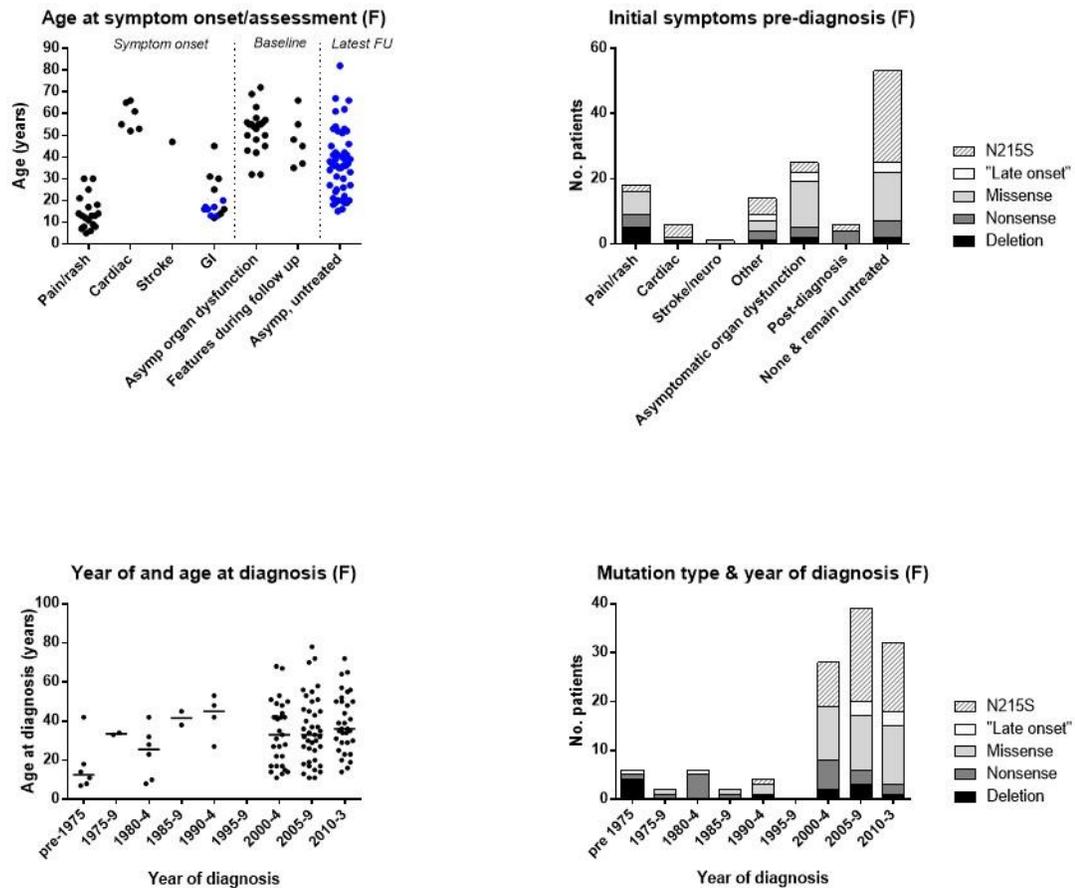
Figure 4-5: Presentation of females diagnosed on family screening. Age of onset and mutation type by initial symptoms. There is an increasing rate of diagnosis of cases by family screening over the past decade.

Females with cardiac or cerebrovascular manifestations pre-diagnosis

Seven females had symptomatic cardiac disease prior to being diagnosed with FD, one of whom had been found to have hypertrophic cardiomyopathy on screening after her sister's death but the underlying cause had not been identified. The median age of onset was 64 years (range 34-78) and the median time from symptom onset to diagnosis was 6 years (range 2-15). One patient had a PPM for bradycardia prior to diagnosis and one patient had suffered a stroke pre-diagnosis. At baseline assessment all except the patient with a PPM had LVH, one had proteinuria and 4/6 had WML. One patient required an ICD during follow up but there have been no further cerebrovascular manifestations.

134

Additionally, one female with a missense mutation (L372P) had suffered a TIA pre-diagnosis at the age of 47; on baseline assessment she also had LVH but no proteinuria.

Females with other symptoms

Thirteen females had IBS-type symptoms pre-diagnosis with a median age of onset of 16 years (range 12-31), with three being given a formal diagnosis of IBS by gastroenterology. Of these 13 females, one had LVH at baseline and two had WML on MRI but there were no stroke/TIAs experienced during follow up and no evidence of renal disease. Additionally one female had been noted to have cornea verticillata on a routine eye examination five years pre-diagnosis but had not been investigated for an underlying cause. She is otherwise asymptomatic.

Females asymptomatic at diagnosis

Eighty females were asymptomatic at the time of diagnosis, of whom fifty three have remained asymptomatic and have not received ERT. Eight of these have cerebral WML on MRI but, in the absence of other disease manifestations, have not been commenced on ERT. Twenty one females had evidence of organ dysfunction of baseline assessment, performed at a median age of 55 years, four of whom have subsequently suffered a stroke. Six developed FD manifestations during follow up, one of whom has suffered a stroke.

Changing pattern of diagnosis

There has been a rapid increase in the number of females diagnosed with FD over the past decade. Only twenty females were diagnosed prior to 2000, but since 2000 one hundred females have been diagnosed, averaging 7.7 new diagnoses per year. Sixty (60%) of these have not required enzyme replacement therapy, 32 of whom have the N215S mutation.

4.4.7 Cerebrovascular disease in FD

Overall 15 males (16.3% male patients) and 17 females (12.1% female patients) suffered a stroke or TIA, with the event occurring prior to the diagnosis of FD in three males and nine females. This almost equal incidence of cerebrovascular events between male and females with FD is in contrast to renal and cardiac events which are much commoner in

males than females (see **Figure 4-6**). Asymptomatic WML on brain MRI were also almost as common in females as males. Whether these arise due to the same pathological mechanism as stroke in FD and hence might be predictive of stroke in unknown. Of those who had a brain MRI prior to their stroke/TIA, 8/12 (66.6%) of males and 6/8 (75%) females had WML; however, 32/76 (42.1%) of males and 33/119 (27.7%) of females had WML on brain imaging at baseline and have not suffered a stroke/TIA.
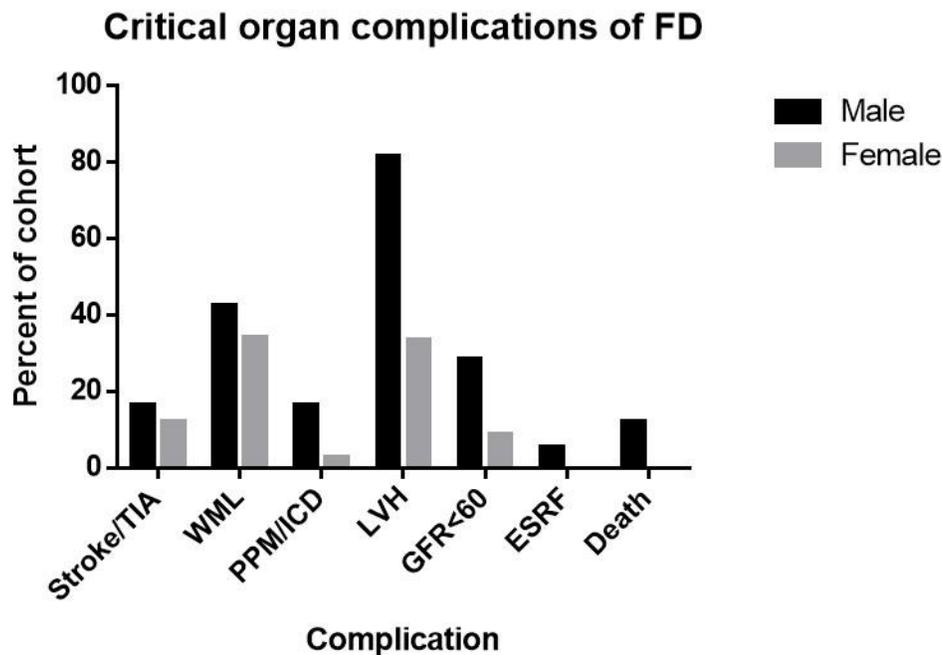

Figure 4-6: Prevalence of critical organ complications in males and females with FD

Relationship to age

The median age at stroke/TIA was 46 years (range 19-74) in males and 47 years (range 24-82) in females. As shown in **Figure 4-7** there is no difference in prevalence of stroke/TIA between males and females which gradually increases with age up to the age of 60 years, after which the rate of stroke increases. Similarly in those patients who have not suffered a stroke, there is no difference in the prevalence of white matter lesions at baseline between males and females, with a more rapid increase in both sexes after the age of 40. In contrast, renal impairment and left ventricular hypertrophy are more

136

prevalent at baseline in males and occur at an earlier age. In females, these complications were extremely rare below the age of 40 years, after which they increased rapidly with increasing age. Correlations between baseline GFR/LVMI and age at baseline show that whilst both are significantly correlated with age in males and females ($p<0.0001$ in all cases), the correlations are stronger in females than males: GFR – males: slope -1.00, r^2 = 0.29 vs. females: slope -0.88, r^2= 0.42; LVMI – males: slope 0.57, r^2= 0.21, females: slope 0.73, r^2= 0.46.
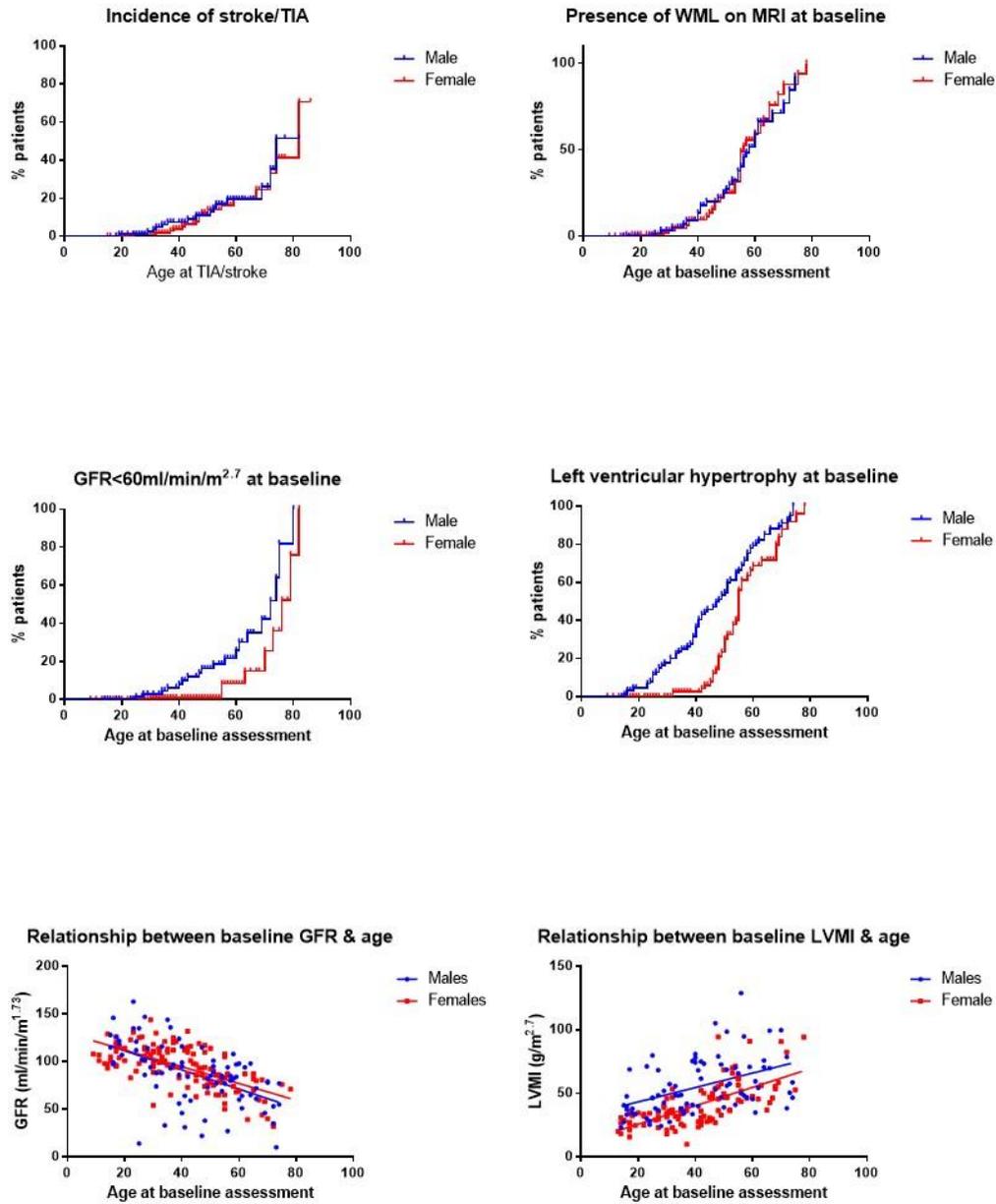
Figure 4-7: Relationship between critical organ dysfunction and age in males and females.

Relationship to other organ involvement and overall disease severity

All males who had a stroke also have left ventricular hypertrophy with 7/15 (46.7%) having a PPM/ICD or arrhythmia and the rest having conduction delays/repolarisation abnormalities on ECG. 7/15 (46.7%) of males had a GFR<60ml/min/m$^{2.7}$ and the same

number also had proteinuria. In contrast, four females (23.5%) had no evidence of cardiac or renal involvement by FD and the prevalence of LVH, conduction abnormalities and renal disease was lower in females (see **Figure 4-8**).
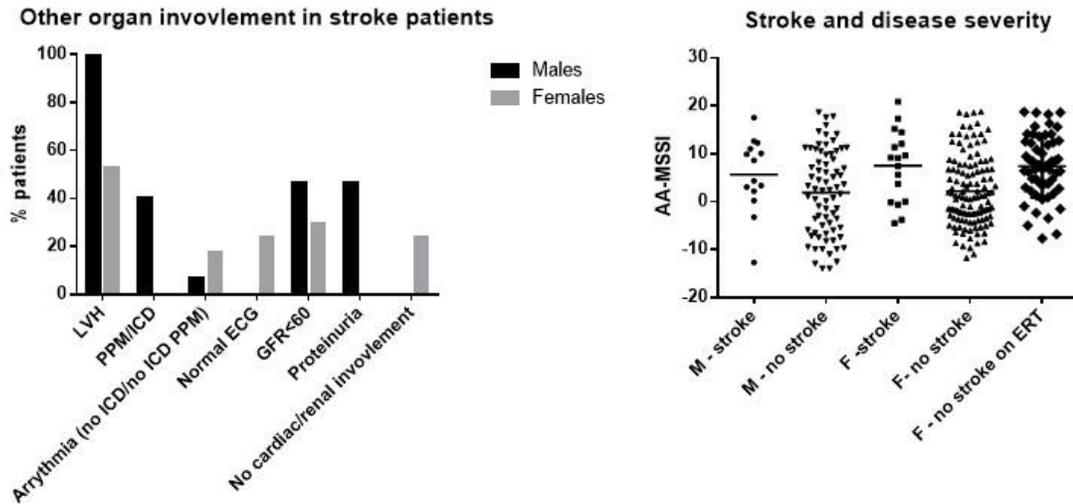

Figure 4-8: Prevalence of other organ involvement in FD patients with stroke/TIA (A). Age-adjusted disease severity in patients with and without stroke/TIA (B).

In males there was no difference in baseline age-adjusted severity score between males with a stroke and those without (6.48 vs. 2.47, p = 0.17), see **Figure 4-8**. In females, those with stroke had higher baseline age-adjusted severity scores that those without (9.12 vs. 1.01, p = 0.008), however this includes many females who are asymptomatic. When compared to females requiring ERT for other disease manifestations, there is no significant difference in disease severity between the two groups (9.12 vs. 7.54, p = 0.93).

Relationship to enzyme activity and mutation status

Plasma GLA activity data, measured at either RFH/ICH prior to commencement of ERT, was available in 73 males and 129 females. One male with the A143T mutation had plasma enzyme activity considerably higher than would be expected for an affected male, (1.5nmol/ml/hr), confirmed on repeated measurements, for reasons unknown; this male is

asymptomatic and has been excluded from the analyses related to enzyme activity.   The
normal range for plasma enzyme activity is 4.0-21.0 nmol/ml/hr.
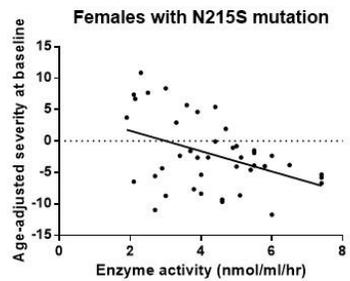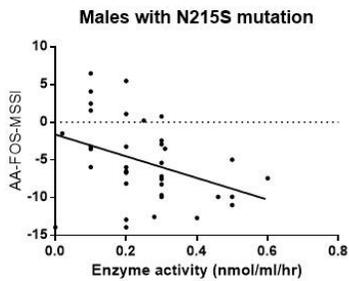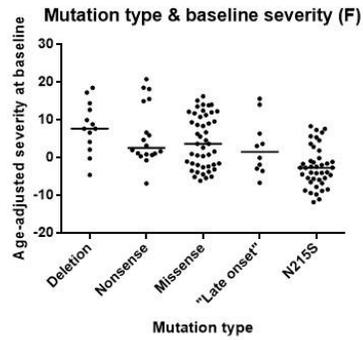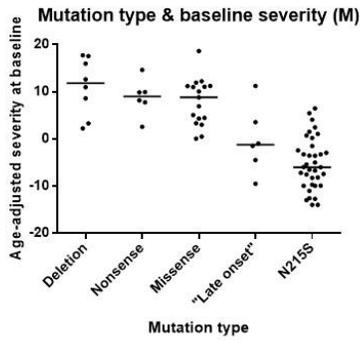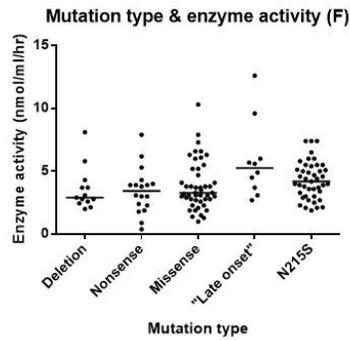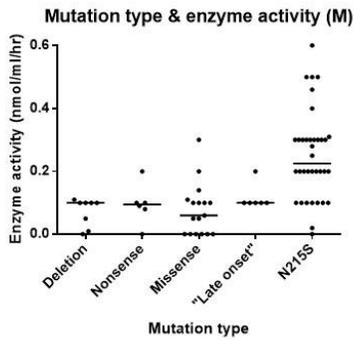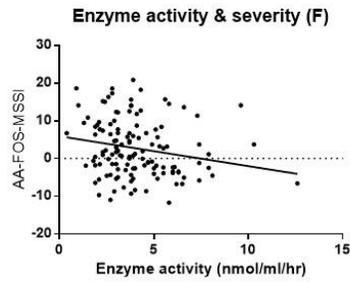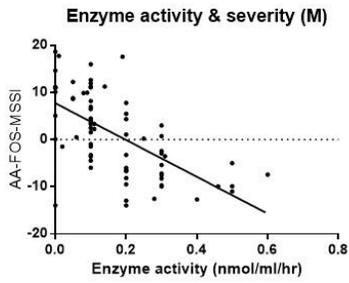
Figure 4-9: Relationship between enzyme activity, disease severity and mutation type.

In both males and females there was a correlation between baseline disease severity and plasma enzyme activity, see Figure 4-9. In males this was a highly significant correlation ($r^2 = 0.38$, $p<0.0001$) but in females although the correlation was significant it was weak ($r^2 = 0.04$, $p=0.02$). The median plasma activity for males was 0.1 nmol/ml/hr (range 0-0.6), with patients with the N215S mutation having higher plasma enzyme activity than other patients, see Table 4-12. This was concordant with a lower baseline disease severity in these patients. Even though the 36 males with the N215S mutation have an identical mutation, there was a wide range of enzyme activity seen in these patients, with the enzyme activity correlated with disease severity ($r2 = 0.13$, $p = 0.02$). The median plasma enzyme activity for females was 4.07 nmol/ml/hr (range 0.4-12.6). Females with the N215S mutation or "late onset" mutations had lower baseline severity scores than those with deletions or non-sense mutations. Amongst those with the N215S mutation, plasma enzyme activity was correlated with disease severity ($r2 = 0.18$, $p=0.005$).

Table 4-12: Relationship between enzyme activity, severity & mutation type

		Deletion	Nonsense	Missense	"Late onset"	N215S
No (%) patients	Males	8 (10.9)	6 (8.2)	17 (23.3)	6 (8.2)	36 (49.3)
	Females	13 (10.1)	18 (14.0)	46 (35.7)	10 (7.8)	42 (32.6)
Plasma enzyme (nmol/ml/hr) median & rnage	Males	0.1 (0-0.11)	0.1 (0-.2)	0.06 (0-0.3)	0.1 (0.1-0.2)	0.2 (0-0.6)
	Females	2.9 (2-8.1)	3.45 (0.4-7.9)	3.3 (1-10.3)	5.3 (2.7-12.6)	4.2 (1.9-7.4)
Baseline age-adjusted severity	Males	11.8 (2.3-17.8)	9.0 (2.6-14.7)	8.8 (0.1-18.7)	-1.2 (-9.5-11.3)	-6.0 (-14.0-6.5)
	Females	7.7 (-4.5-18.5)	2.7 (-6.8-20.9)	3.7 (-6.1-16.3)	1.59 (-6.6-15.7)	-2.6 (-11.7-8.4)

Whilst enzyme activity appears to be an important factor in overall disease severity in males, there was no difference in the median plasma activity for males with stroke/TIA compared to those without (0.1 vs. 0.1, $p = 0.24$). Similarly, there was no difference in the median enzyme activity in females with stroke/TIA compared to those without (4.30 vs. 4.07, $p = 0.69$). Amongst males, strokes were commonest in those with deletions, nonsense or missense mutations and were uncommon in those with the N215S mutation, occurring only in old age in these patients, see **Figure 4-10**. In males there was a

significant correlation between enzyme activity and age at stroke (r^2= 0.476, p= 0.013). In females, strokes occurred in patients with all mutation types with no clear relationship between age at stroke and mutation type or plasma enzyme activity (r^2= 0.098, p=0.24).
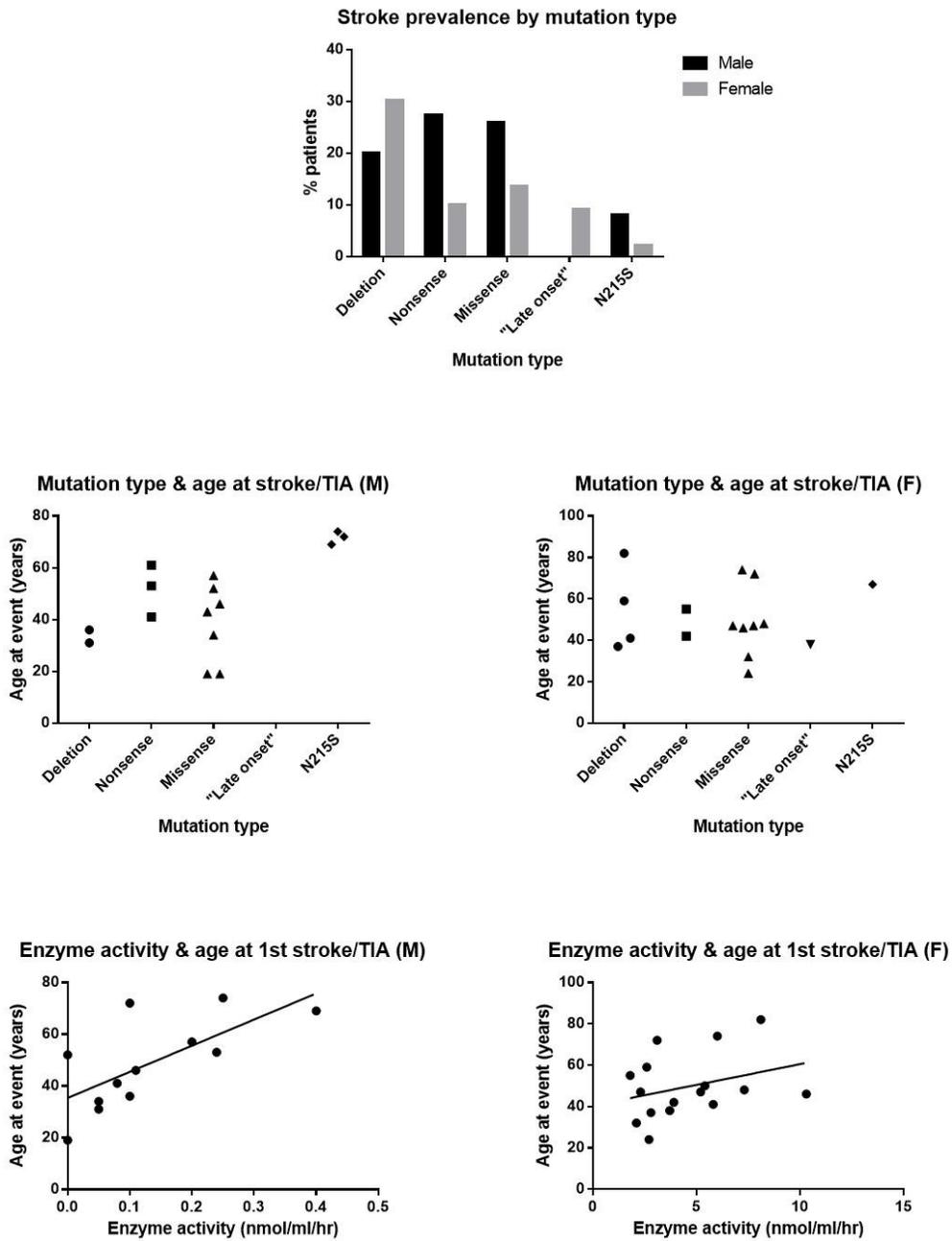
Figure 4-10: Relationship between stroke, mutation type and enzyme activity.

Vascular risk factors

Conventional risk factors for stroke include: smoking, diabetes and hypertension. Obesity and a raised lipoprotein (a) are also considered to be risk factors for vascular disease by

144

some. Amongst males there was no increase in cardiovascular risk factors amongst males with stroke compared to those without (see **Figure 4-11**). All males with the N215S mutation had vascular risk factors. Hypertension, diabetes, obesity and a raised Lp(a) were all at least twice as common amongst females who had suffered a stroke/TIA compared to those who had not. Two females had no vascular risk factors for stroke and no other evidence of other organ involvement by FD; they have the mutations A143T and Q221P.

Prognostic scores (FIPI)

The FIPI neurological score was calculated based on data from baseline evaluation and outcome censored either at the time of stroke/TIA or at latest follow up. There was no significant difference in events in patients with different prognostic scores (high, medium or low) in males (p = 0.48) or females (p = 0.45), see **Figure 4-11**.
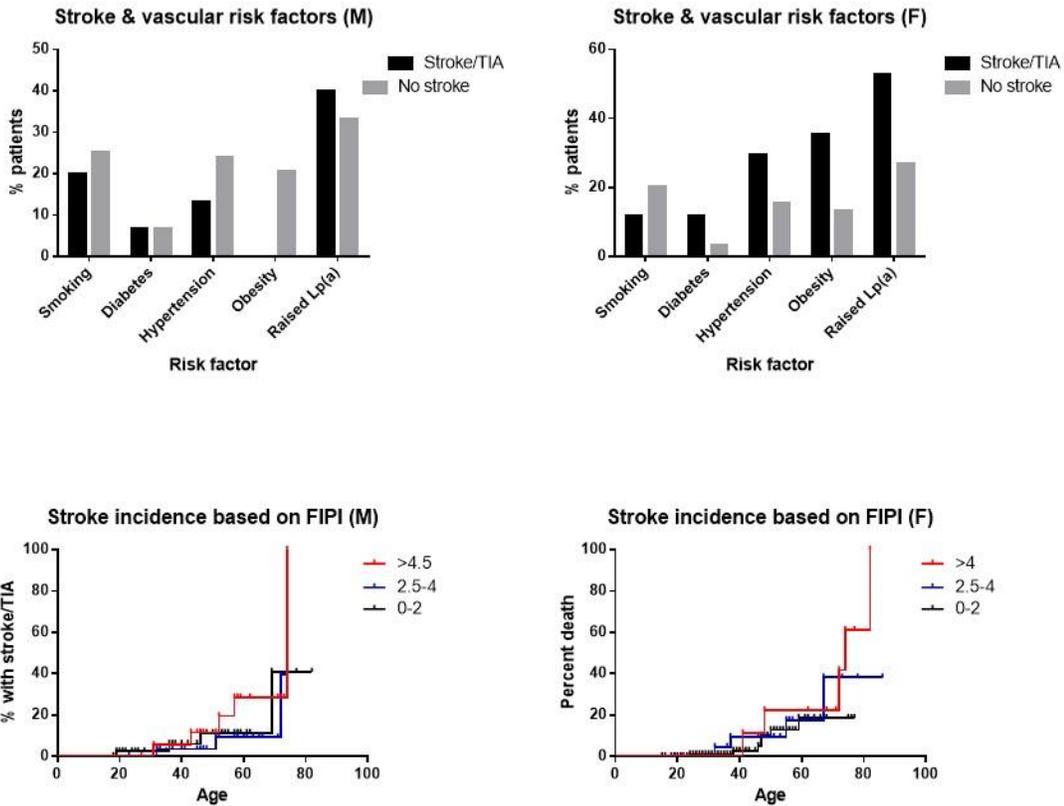
Figure 4-11: Vascular risk factors and the FIPI prognostic score

4.4.8 Plasma pro-thrombotic tendency

Nineteen male and 22 female consecutive FD patients had plasma assays performed for protein C, protein S, anti-thrombin and FVIII activities and mutational analysis for the FV Leiden and PTG20210A prothrombin gene mutations. The median age of patients was 54 years (range 19-84). All males and 18 females were receiving ERT. Seven (17.9%) patients had suffered a stroke/TIA and 61% had cerebral white matter lesions on MRI. One patient had a transient lupus anticoagulant, one patient was heterozygous for the FVL mutation and one patient heterozygous for the prothrombin gene mutation PTG20210A. One patient had a borderline reduced protein C activity (63 IU/dL); one female patient had a mildly reduced protein S activity (54 IU/dL) related to use of the oral contraceptive pill. None of these patients had had a cerebrovascular event. Five patients (12.2%) had

protein S levels above the normal range. The rest of the patients had levels of PC and PS within the normal range and all patients had normal levels of anti-thrombin.

FVIII activity was increased in 16 patients (39%, 9 male, 7 female). FVIII activity increased with age in both males in females, **Figure 4-12**; in females this correlation was significant (slope 1.25, $r^2 = 0.263$, p = 0.015); in males it was not (slope 0.91, $r^2 = 0.05$, p = 0.35), due to four males with higher VWF levels than expected for their age. Age adjusted severity scores were correlated with VWF levels, with a more significant correlation in females (slope 3.79, $r^2 = 0.471$, p = 0.0004) than in males (slope = 3.591, $r^2 = 0.316$, p = 0.012). The four males with higher VWF levels than expected for age also were outliers for severity, with higher age-adjusted severity scores. Exclusion of these four men removed the correlation between severity and FVIII levels in males. There was no difference in VWF levels between those with or without cerebrovascular disease. FVIII tended to increase as renal function declined, with a significant correlation between GFR and FVIII in females (slope -0.999, $r^2 = 0.334$, p = 0.005) but not in males (slope -0.786, $r^2 = 0.137$, p = 0.131). FVIII levels were strongly correlated with VWF levels in both sexes (males $r^2 = 0.776$, females $r^2 = 0.712$, P <0.0001).
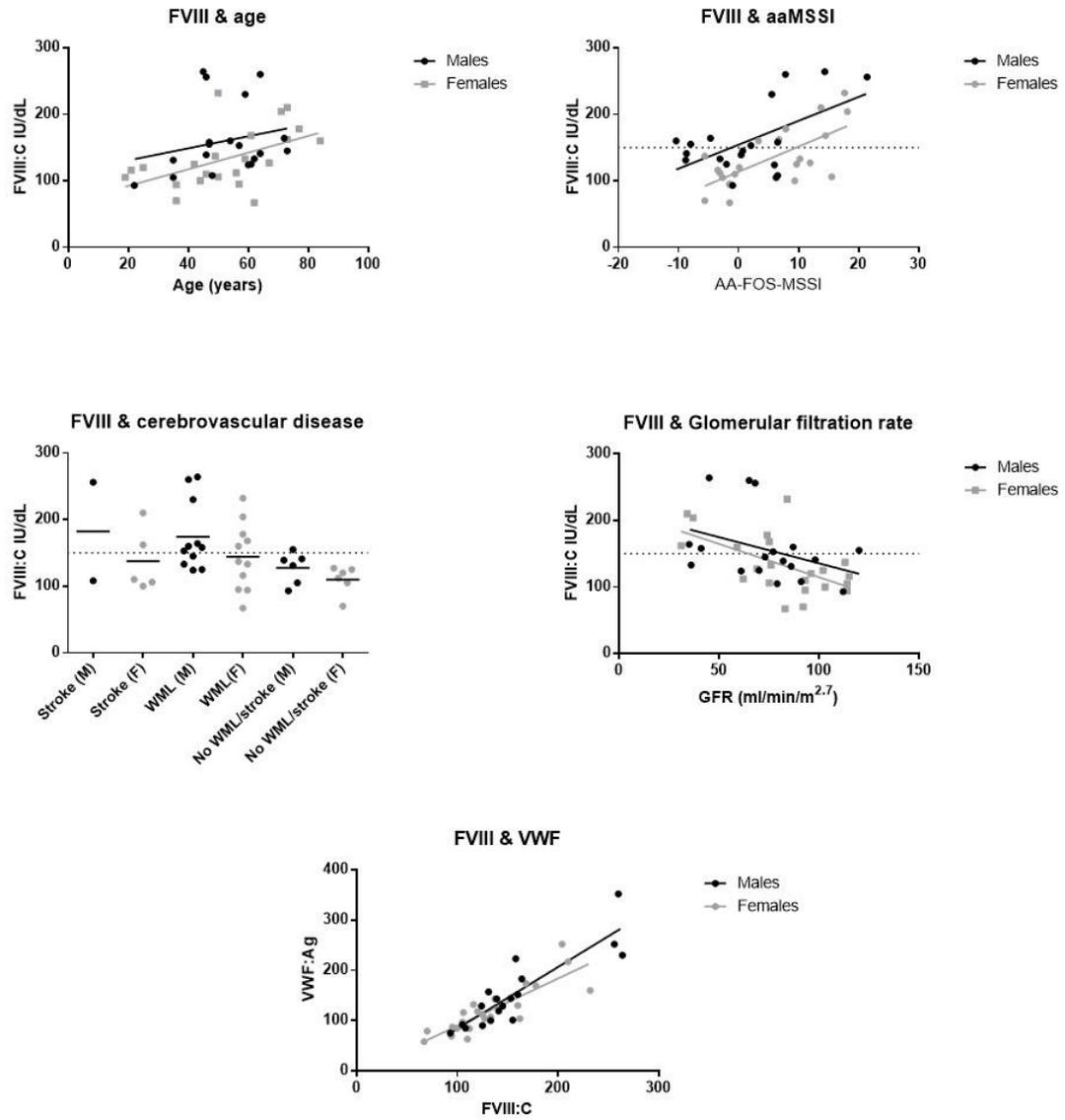
**Figure 4-12: Relationship between FVIII and disease manifestations.**

4.5   Discussion

4.5.1   Vascular manifestations are important in the presentation of FD

The data from the RFH demonstrates that vascular manifestations are important presenting features of FD in both males and females.   Cardiac manifestations, particularly arrhythmias, are common presenting symptoms in both males and females although more severe in males who also have a higher rate of pacemaker and ICD insertion.   Renal presentations with proteinuria occur in both genders, but progression to end stage renal failure did not occur in any female.   In contrast to cardiac and renal disease, stroke was a commoner presentation in females than males, being the initial symptom of FD in five females but no males.

Early diagnosis offers the opportunity for initiation of ERT with the aim of preventing develop or progression of critical organ disease, although evidence of its efficacy in this respect is still lacking[217;218]. In addition to ERT, there is the opportunity to optimise supportive therapies, for example ACE inhibitors, blood pressure control and ICD insertion for primary prophylaxis of ventricular tachycardia[211]. Data from the cohort suggests that all males are at risk of these complications and therefore improving the diagnostic pathway in these patients would be beneficial.

Many patients experienced long delays between symptom onset and the diagnosis of FD and this was true both of index cases and those diagnosed on family screening.   The number of patients diagnosed with FD on family screening who had already been investigated for manifestations of FD supports the notion that symptomatic FD remains un-diagnosed.   Those whose initial symptom was acroparasthesia experienced the longest delays, with a median delay of >10 years.   A number of factors may contribute to this: deducing the underlying cause of neuronopathic pain can be difficult, particularly when there may be few clues from physical examination or routine laboratory investigations[219]. Whilst the diagnosis was often made in males by dermatologists following the appearance of angiokeratoma, the rash often appears first in the bathing trunk region and may be missed unless directly asked about/ looked for by the consulted physician.

149

Delays were shorter in those patients who developed major organ involvement although a number of patients had been erroneously diagnosed with hypertrophic obstructive cardiomyopathy prior to arrival at the correct diagnosis. Awareness of the potential differential of FD amongst cardiologists, nephrologists and stroke physicians is likely to have been raised in recent years by a number of screening studies for FD conducted amongst these populations[220]. To date, no screening studies for FD in patients with neuronopathic pain have been published and it may be that awareness of the diagnosis amongst the diverse range of physicians to whom these patients present is lacking.

4.5.2   Vascular complications are important clinical events during the follow up of patients of FD

Eleven of the 91 males in the cohort (12.1%) have died and in all apart one case this was due to either cardiac or cerebrovascular complications, with a median age at death of 53 years. Therefore, improving our understanding of how these complications arise and how they may best be prevented is vital to improving the prognosis of FD in men. Despite the initiation of ERT, vascular complications continue to occur during follow up, particularly in those presenting with "classical" features of acroparasthesia and/or angiokeratoma.

4.5.3   Stroke in FD – just another organ complication or different aetiology?

Cerebrovascular disease stands out from other organ complications in having an almost identical prevalence in both male and female patients with FD. In males, stroke arises in the setting of other disease manifestations. It is more common in those with deletions or nonsense mutations and the age at first stroke is correlated with enzyme activity. Therefore, in males, stroke can be considered a direct complication of the disease process.

The scenario in female patients is different. Stroke can arise in the absence of disease manifestations in other organs. Whilst the development of cardiac or renal disease appears to be strongly correlated with age, this is not the case for stroke which occurs most commonly in females in the fifth decade of life. Whilst stroke is commoner in females with deletions, it occurs in all mutation types and there is no relationship between plasma enzyme activity and age at stroke. Vascular risk factors seem to be more important in the

development of stroke in females than males. There are two females with a *GLA* mutation and stroke but no other disease manifestations or risk factors for stroke. The fact that this scenario is not seen in male patients raises questions about the mechanisms which link a *GLA* mutation with the occurrence of stroke in these patients and whether it is truly causative or there are other, as yet unknown, factors to which the stroke might be attributable.

4.5.4   Fabry disease in men – a spectrum or distinct subtypes?

The incidence of FD has increased substantially in the past decade, mostly due to an increase in diagnoses of patients with the N215S mutation. These patients have a higher residual enzyme activity and a significantly lower overall disease severity score with a predominance of cardiac involvement. The "classical" phenotype of acroparasthesia and angiokeratoma in adolescence is rare in these men. Although there are exceptions to this with two males with the N215S mutation presenting with proteinuria and end stage renal failure in their 20s/30s the N215S mutation potentially represents a distinct subtype of FD with different disease manifestations and prognosis to other patients. This is potentially important for understanding prognosis in these patients – many of these patients are diagnosed at an age exceeding the expected life expectancy of males in FD reported in historical cohorts[37] and are likely to have an improved prognosis. This is also important for interpretation of clinical studies, particularly where comparisons to historical natural history cohorts are drawn[221], as the natural history of these patients is likely to be significantly better than that of patients with more "classical" disease manifestations.

Position 215 on the GLA enzyme represents an N-linked glycosylation site and glycosylation is important for the correct trafficking of proteins intracellularly. Understanding why a mutation at this site results in cardiac disease in later life may shed new insights in the pathogenesis of FD in different organ systems. The range of enzyme activity seen in men with this mutation suggests that there are other factors which modify enzyme activity, either at the gene expression level or through a translational/post-translational mechanism. The correlation between enzyme activity and disease severity would suggest that these factors are important modifiers of disease[222].

151

4.5.5 Fabry disease in women – disease causing or disease predisposing?

The *GLA* gene is located on the X chromosome and therefore historically females were considered carriers rather than affected heterozygotes. Our cohort data is consistent with that of studies performed in the last 15 years which demonstrate that many females develop clinical manifestations of FD[38;223]. On the other hand, many females remain asymptomatic throughout life. Particularly in light of the large number of females diagnosed on family screening, understanding which females may go on to develop clinical manifestations of FD and why is of key importance both for counselling these women regarding genetic testing and understanding how best to manage them.

Age appears to be an important factor in the development particularly of cardiac and renal disease with the incidence of these disease manifestations increasing rapidly after the age of 40 years (left ventricular hypertrophy) and 60 years (renal impairment). The stronger correlations between age and LVMI and GFR in females than males suggests that age-related factors have more of an impact on disease in females than males. In the general population cardiovascular disease is unusual in pre-menopausal women[224] with a significant increase being seen after the menopause, although this has been attributed in part to an increase in other cardiovascular risk factors[225]. Whether menopausal changes play a role in FD has not yet been investigated. FVIII levels, which have also been associated with risk of vascular disease, increase with age in both males and females but are more closely correlated with age in females than males and are correlated with disease severity in females but not males. This would support the hypothesis that age related changes in the vasculature in females impact on disease manifestations.

Unlike men, conventional cardiovascular risk factors and obesity were much commoner in women with cerebrovascular disease than those without suggesting that in females additional risk factors may impact on the development of vascular disease. Obesity has been demonstrated to be an additive risk factor in combination with other vascular risk factors[226]. The importance of other risk factors is also supported by studies of polymorphisms in genes related to inflammation and coagulation where associations between polymorphisms and cerebrovascular disease were stronger in females than

males[92;93]. We did not find an increase in the presence of the prothrombotic mutations or abnormalities of the anticoagulant system in our cohort. In both males and females a raised lipoprotein (a) was commoner in those with cerebrovascular disease than those without. Serum levels of Lp(a) are primarily genetically determined[227] and increased Lp(a) is a risk factor for both cardiac[228] and cerebrovascular disease[229]. Potential mechanisms for this increased risk include up-regulation of the adhesion molecules ICAM-1 on leukocytes[230] and activation of coagulation[231]. Lipoproteins are the carrier proteins for Gb3 in the circulation[232] and therefore increased lp(a) may also exacerbate disease mechanisms mediated by increased Gb3. Investigation of lp(a) polymorphisms may reveal a new genetic modifier of FD.

Taken together, these factors suggest that in females a *GLA* mutation, rather than being disease causing, may act to pre-dispose females to the development of cardiac, cerebrovascular or renal disease with additional factors required for complications to occur.

4.6 Conclusions

This study confirms that vascular disease is important not only in the clinical presentation but also the long term morbidity and mortality of patients with FD. In male patients these complications become more prevalent with increasing age, with disease severity correlating with enzyme activity and mutation type. The presence of an N215S mutation with a higher residual enzyme seems to limit the development of disease manifestations predominantly to the heart with cerebrovascular disease occurring only in the presence of additional risk factors. Females have less severe disease with many remaining asymptomatic. The onset of cardiac and renal disease occurs at a later age and to a lesser extent in females than males and it may be that in females additional events are required at the cellular/organ level for disease manifestations to develop. In contrast, the pathophysiology of cerebrovascular disease would appear to be different, occurring at a similar age and prevalence in males and females.

The next two chapters explore potential disease processes at the cellular level, focussing on processes related to cellular adhesion and coagulation occurring at the blood-endothelial interface to try to shed new insights onto the pathogenesis of these disease manifestations in patients with a *GLA* mutation.

Chapter 5   Cellular adhesion in Fabry and Gaucher

5.1   Introduction

The endothelium provides the critical interface between the blood and its constituents and tissues. During steady state conditions there are resident populations of leucocytes, including lymphocytes (both B and T cells), dendritic cells and macrophages, in the tissues; these leucocytes have important roles in immune surveillance. During activation of inflammation communications between the endothelium and circulating leukocytes enable the adhesion of leukocytes to the vessel wall and their egress from the circulation into the surrounding tissues.   This process has been implicated in a wide variety of diseases including vascular diseases (e.g. stroke)[233;234], haematological malignancies[235] and disorders which affect the microcirculation (e.g. sickle cell disease)[236;237].

Abnormalities of leukocyte adhesion have been implicated in the vascular complications associated with FD[126] including cardiovascular and cerebrovascular complications. The commonest abnormal findings to date are increased ICAM-1 and VCAM-1 (see 1.8.1), molecules central to firm leukocyte adhesion to the endothelium, in either the serum or in endothelial models. A single study of monocytes in male FD patients showed increased expression of CD11b[152]. Previous studies have concentrated on adhesion molecule expression and there is no data on whether these abnormalities result in increased adhesion activity or how they relate to clinical disease manifestations. Studies of leukocyte adhesion in GD are limited; although endothelial adhesion is required for egress of monocytes from the circulation to become tissue macrophages, it is not known whether this process is abnormal in GD. Additionally, the mechanisms underlying bone infarction, which bears some similarities to infarcts seen in sickle cell disease, and the increased risk of haematological malignancies in GD are poorly understood.

Cellular adhesion is a closely regulated process and cytokines are the primary regulators. Increased levels of pro-inflammatory cytokines have been found in both FD and GD (see **Table 1-6**) which may up-regulate cellular adhesion in these patients. Additionally the glycosphingolipids LacC and S1P can act to increase adhesion molecule expression[10;142].

155

Gb3 has been shown to up-regulate endothelial VCAM1 and ICAM1 mRNA[151] but the effect of other glycosphingolipids on endothelial-leukocyte adhesion is not known.

5.2   Purpose of this chapter

This chapter examines the expression and functional activity of adhesion molecules involved in leucocyte-endothelial adhesion, concentrating on firm adhesion using static adhesion experiments, as this is the part of adhesion where most abnormalities have been suggested in FD. Utilising the ability of monocytes, but not lymphocytes, to adhere to tissue culture plastic[238], the adhesion capacity of monocytes is assessed. Monocytes are the key abnormal cells in GD and the leucocytes in which abnormalities of adhesion molecule expression have been found in FD. Expression of leucocyte adhesion molecules in subsets of mononuclear cells is examined by flow cytometry.

5.2.1   Development of endothelial cell models of FD and GD

In order to examine the role of decreased GLA and GBA activity on the expression and functional activity of adhesion molecules on the endothelial surface, an endothelial cell model was developed. Endothelial cells perform a vast array of functions, which can vary according to their location, including active transport of small molecules, regulation of blood pressure, leucocyte adhesion and transmigration, angiogenesis and haemostasis. Whilst human umbilical vein cells (HUVEC) are the most studied primary endothelial cell isolate, they lose their phenotype after a small number, or even a single passage. For this work the EA.hy 926 endothelial cell line, which is a hybridoma of HUVEC and the human lung carcinoma cell line A549 was used. This cell line shows a number of endothelial cell characteristics including von Willebrand factor expression[184] and, particularly pertinent to this project, up-regulation of ICAM-1 and VCAM-1 in response to TNFα[239].

Pharmacological inhibition

Conduritol β epoxide (CBE) binds covalently to the active site of GBA, causing irreversible inhibition of enzyme activity[240] and has been widely used in the study of GD. The search for a similarly efficacious inhibitor of GLA, including using high throughput screening technologies, has been less fruitful[241]. 1-deoxygalactonorijimycin (DGJ) is an

iminosugar, a small organic compound mimicking carbohydrates but which contains a nitrogen atom instead of an oxygen atom in the ring system[242]. DGJ is biochemically selected to reversibly inhibit the active site of GLA but sufficiently distinct from carbohydrates to avoid processing by other carbohydrate-modifying systems. At low concentrations (upto 20μM) it acts as a chaperone and has been demonstrated to increase enzyme activity in FD fibroblasts[243]. An inhibitory effect on enzyme activity, presumptively due to competitive inhibition, has been demonstrated at higher concentrations of 400-500μM[244].

Chloroquine hydrochloride is a lysosomotropic agent which has also been used to inhibit GLA. It inhibits lysosomal enzymes to different degrees at different concentrations, with more inhibition of GLA than other lysosomal enzymes at low concentrations[245]. Chloroquine has a number of effects on the lysosomal system including increasing lysosomal pH, altering mannose-6-phosphate (M6P) receptor cycling and disrupting phagocytic pathways[246].

Amiodarone is another lysosomotropic drug but with no known direct effects on GLA or GBA activity and was used as a control for lysosomal dysfunction. At physiological pH, amiodarone is un-ionised and passively diffuses across the lipid bilayers of intracellular organelles. At acid pH, amiodarone becomes ionised and less able to diffuse out of the lysosome[247] causing expansion of the lysosomal system. Amiodarone impairs trafficking through the late endosomal pathway[248] and alters M6P receptor recycling[249]. It also inhibits lysosomal phospholipase A1 & A2 causing intralysosomal accumulation of phospholipids.

RNA interference using small hairpin RNA

Due to the lack of availability of an effective specific pharmacological inhibitor for GLA ribonucleic acid (RNA) interference (RNAi) was used as an additional method to reduce intracellular GLA activity. RNAi results in sequence specific gene silencing at the messenger RNA (mRNA level). Small interfering RNAs (siRNA) are short double stranded RNA oligonucleotides, 25 nucleotides in length, processed internally by the

enzyme DICER and then split into two separate strands. One of these strands binds a complementary mRNA sequence on the target gene which is then cleaved and degraded by the RNA-induced silencing complex (RISC), preventing protein translation. siRNA sequences can either be introduced into cellular cytoplasm as pre-synthesised oligonucleotide strands using lipid-mediated transfection or using a viral vector DNA construct which integrates into cellular DNA (transduction). The transcribed DNA encodes a short hairpin RNA (shRNA) which is then processed by DICER to siRNA. As siRNA is inherently unstable and usually completely degraded within 48 hours, a shRNA approach using a lentiviral vector was used for this project to enable sustained suppression of GLA protein synthesis.

5.3   Hypotheses

1. Increased expression of leukocyte adhesion molecules occurs in FD and GD and is associated with vascular manifestations in FD and the presence of bleeding and gammopathies in GD. *Rationale:* FD and GD are associated with a pro-inflammatory state which results in up-regulation of adhesion molecules

2. Lipid loading of endothelial cells, but not reduction in enzyme activity alone results in increased endothelial adhesion activity. *Rationale:* results to date suggest that Gb3 rather than GLA deficiency is responsible for endothelial dysfunction. Glycosphingolipids LacC and S1P have been found to up-regulate adhesion molecule expression and other glycosphingolipids may also perform this function.

5.4   Methods

5.4.1   Monocyte adhesion assay

This assay is investigates static adhesion processes and utilises the adhesion properties of monocytes whereby they are able to adhere to tissue culture plastic (TCP) surfaces. This process is predominantly mediated by integrins and therefore calcium dependent, although monocytes may also adhere to TCP through scavenger receptors in a calcium-independent manner. Both monocytes and platelets adhere to TCP and therefore an iodixanol flotation

barrier was used to isolate monocytes resulting in a monocyte-enriched, platelet deplete mononuclear cell fraction.

Adhesion of monocytes to TCP is time and concentration dependent, being linear over a range of $0.5\text{-}2\text{x}10^5$ monocytes per well and is also serum dependent (see **Figure 5-1**), where adhesion molecules within serum adhere to TCP and then monocytes adhere to these. 10% serum was used for the assays; to assess solely the leucocyte component cells were suspended in pooled normal human serum (PHS) whereas to investigate the role of serum constituents, cells were suspended in heat inactivated autologous serum (AHS). To account for differences in staining between assays, the results are expressed as the ratio of absorbance of the test well to the PHS serum blank well (see 2.7.1).

The relationship between disease manifestations of GD/FD and adhesion ratio was evaluated by linear regression analysis. The clinical data was obtained as described in the preceding chapters.
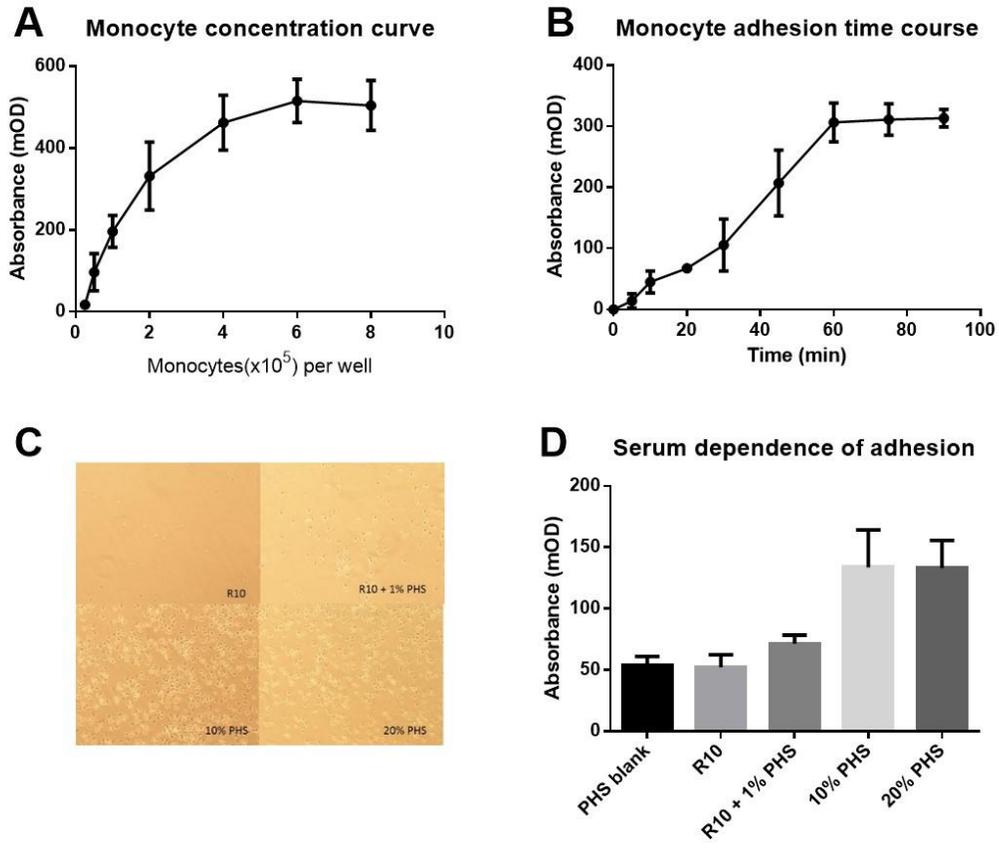
Figure 5-1: Development of monocyte adhesion assay. A: absorbance of solubilised Giemsa stain is dependent on cell concentration. B: time dependence of adhesion. C &D: dependence of adhesion on the presence of human serum.

5.4.2   Leukocyte adhesion marker expression

Surface expression of adhesion molecules on mononuclear cells was assessed using flow cytometry. The adhesion molecules investigated and the rationale for their selection is shown in **Table 5-1**.

Table 5-1: Adhesion molecules assessed

Antigen	Leucocytes expressed on			Reason selected
	Mono	CD14-	Lymph	
CD11a	Y	Y	Y	Ligand for ICAMs 1-4; ICAM1 raised in some FD studies, other ICAMs not assessed
CD11b	Y	X	X	Ligand for ICAM-1. Up-regulated in monocytes on a previous study in FD
CD11c	Y	Y	X	Shared β subunit with CD11a and CD11b but not ligand for ICAM-1
CD50	Y	Y	Y	Ligand for CD11a (LFA-1)
CD54	Y	Y	X	ICAM-1. Increased serum levels in FD
CD102	Y	Y	Y	Ligand for LFA-1 (CD11a)
CD49d	Y	Y	Y	Ligand for VCAM-1 which is increased in some FD studies
CD62L	Y	Y	Y	Predominant selectin on leukocytes – for rolling prior to firm adhesion
CD36	Y	X	X	Binds lipids; abnormal lipid profiles in FD/GD may cause upregulation

Gating strategy and analysis

Initial gating was performed on forward (FSC) and side scatter (SSC) characteristics. This differentiates lymphocytes from cells which fall within the monocyte gate. Within the monocyte gate, CD14+ monocytes were identified by gating on CD14+ strongly positive cells. Within the FSC-SSC monocyte gate there is also a population of CD14 weak-negative cells; whether these cells represent a sub-population of monocytes (e.g. $CD14^{low}CD16^{high}$) or dendritic cells is not possible to establish from the cellular markers used. They have a different pattern of expression of adhesion molecules from the CD14+ monocytes and are therefore analysed as a separate cell population. The median fluorescent intensity (MFI) of adhesion molecules on each cell population was measured using FlowJo analysis software (see 2.6). CD62L is expressed on all mononuclear leukocytes but is more strongly expressed on a subpopulation of lymphocytes. Therefore

the size of this subpopulation and the MFI of cells in this subpopulation was also calculated.

5.4.3   Development of cellular models to look at endothelial adhesion

The EA.hy 926 cell line was used to develop and endothelial model of FD and GD. Confocal imaging was performed to establish if uptake of DiI labelled acetylated low density lipoprotein (DiI-acLDL) occurred and whether von Willebrand factor was synthesised, both characteristics of endothelial cells. Confluent monolayers of EAhy.926 were stimulated with a range of doses of TNFα for 0-24 hours to assess for up-regulation of adhesion molecule expression. This was assessed by two methods: 1) adhesion of DiI labelled murine monocytic cells (RAW 264.7) to the endothelial monolayer and 2) ICAM and VCAM expression by Western blot.

For pharmacological inhibition, stock solutions of the four pharmacological inhibitors were made as detailed in the Methods chapter. They were added to EAhy.926 cells at the concentrations indicated. MTT assays were performed to measure the effect on cell viability. To measure the effect of the drugs on enzyme activity, the cells were lysed and then activity assays performed. The protein concentration of the lysate was measured and enzyme activity expressed as nmol/mg/hr.

The UCL pGIPZ shRNA library was searched for sequences that target the *GLA* gene. Three sequences were selected:

FD1 (V2LHS_82973): antisense: ATAAAGAGGCCACTCACAG

FD2 (V2LHS_82794): antisense: AAATCCCTAGCTTCAGTCC

FD5 (V3LHS_391616): antisense: AGACATGAATAAAGGAGCA

Analysis of the sequences using the bioinformatics tool, Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov) confirmed that each sequence targeted only the *GLA* gene with 100% alignment and that potential off-target effects for each sequence were different (see **Table 5-2**).

162

Table 5-2: Results of BLAST search for targets of the shRNA sequences

Gene	Query cover	E value	Function
FD1			
Alpha galactosidase A (*GLA*)	100%	0.02	
Mannose receptor C, type 1 (*MRC1*)	84%	0.11	Mediates endocytosis is glycoproteins by macrophages
Zinc finger protein 552 (*ZNF552*)	78%	0.43	Unknown
Family with sequence similarity 114 member A1 (*FAM114A1*)	73%	1.7	Unknown
Leucine rich repeat containing 15 (*LRRC15*)	73%	1.7	Unknown
Polycystic kidney disease 1 (*PKD1*)	73%	1.7	Regulates calcium permeable cation channels and intracellular calcium homeostasis.
FD2			
Alpha galactosidase A (*GLA*)	100%	0.002	
Nitrogen fixation homolog (*NFS1*)	73%	1.7	Supplies inorganic sulphur to iron-sulphur cluster (required by many cellular enzymes)
Patain-like phospholipase domain containing 4 (*PNPLA4*)	68%	6.7	May be involved in triglyceride homeostasis
Cyclic nucleotide gated channel beta 3 (*CNGB3*)	68%	6.7	Encodes beta subunit of a cyclic nucleotide-gated ion channel. Involved in function of cone photoreceptors
Sulfatase modifying factor 2 (*SMF2*)	68%	607	Involved in post-translational generation of C-α-formylglycine at catalytic site of sulphatases
FD5			
Alpha galactosidase A (*GLA*)	100%	0.002	
tRNA methyltransferase 1 homolog (*TRMT1L*)	78%	0.43	May be involved in neurological functions, including motor co-ordination & exploratory behaviour
Ankyrin repeat domain 1 (*ANKRD1*)	73%	1.7	Localised to nucleus of endothelial cells and induced by IL-1 and TNFα. Likely to function as a transcription factor
Dishevelled associated activator of morphogenesis 1 (*DAMM1*)	100%	6.7	Regulates cell growth through stabilisation of microtubules

Sequencing confirmed the presence of the correct transcript (see **Figure 5-2**). Restriction digest confirmed that the correct packing vectors were isolated (see **Figure 5-3**). EAhy.926 cells were infected with the lentiviral constructs and puromycin selection carried out, using a dose of 1μg/ml (derived from MTT assays – see **Figure 5-4**) to yield

a culture of GFP positive cells. Enzyme activity, using fluorimetry, and protein level, using Western blot, were assayed to confirm knockdown of *GLA* expression.
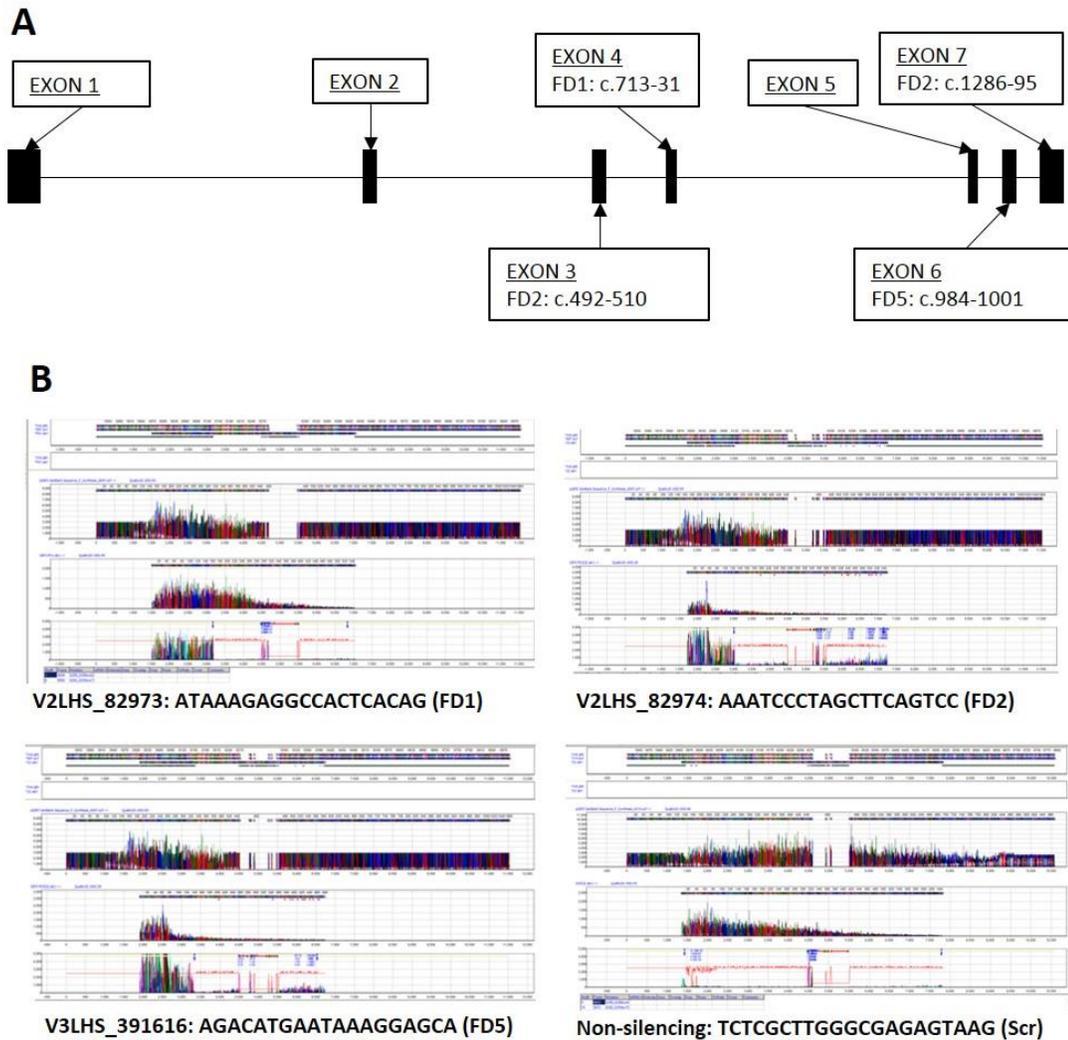

Figure 5-2: shRNA sequences. A: target sites. FD1 targets a sequence on exon 4 which forms part of the active site. In addition to a target sequence on exon 3, FD2 also targets a short sequence on exon 7. B: confirmation of shRNA sequences from isolated plasmid DNA showing insertion of shRNA sequence in the GIPZ plasmid.

Figure 5-3: Confirmation of envelope and packaging vectors by restriction digest. A: vector map for pMDG highlighting restriction sites for BamHI and EcoR1. B: vector map for p8.91 highlighting restriction sites for EcoR1 and BglIII. C: Restriction digest fragment sizes (1% agarose gel, 1V, 75 minutes).

Figure 5-4: GFP expression & puromycin toxicity. A: HEK293T cells producing lentivirus (x20); B: GFP expression in EAhy.926 cells 48 hours after infection (x20); C&D: GFP expression in EA.hy 926 cells after puromycin selection (x20,x40). E puromycin toxicity to EA.hy 926 cells (MTT assay), n=2, each experiment performed in triplicate.

5.4.4 Functional endothelial cell adhesion assay

To measure the functional adhesion capacity of monocytic cells to the endothelial cell model, the murine monocyte-macrophage cell law RAW 264.7 was used. The use of a cell line enabled the availability of a high number of cells of multiple experiments and a homogeneous cell population for use across assay. As detailed in the methods chapter (see 2.7.2) , RAW cells were labelled with DiI, a fluorescent dye, and the fluorescence of RAW cells adherent after 1 hour incubation measured. The results were expressed as the ratio of the fluorescence of test samples to the control samples.

Adhesion of RAW, as determined by fluorescence, was dependent on a) the number of RAW cells added b) the density of the EAhy monolayer and c) activation of endothelial cells with TNFα (see **Figure 5-5**). A concentration of 200 000 RAW per well was selected for the assays and a seeding density of 40 000 per well for EAhy. TNFα was used at 2nM concentration for 8 hours, which allows up-regulation of both ICAM and VCAM and increased adhesion, whilst aiming to minimise the effects of prolonged TNFα on other cellular functions. No decrease in viability or induction of apoptosis was seen at this concentration and duration. To enable delivery of lipids to cells, the lipids were dissolved in methanol.

5.4.5 Western blot for VCAM/ ICAM expression

Western blots were performed to measure the expression of ICAM-1 and VCAM-1 by endothelial cells, selected as being the most important adhesion molecules in static adhesion which is the aspect of adhesion assessed in the functional adhesion assays of this chapter.
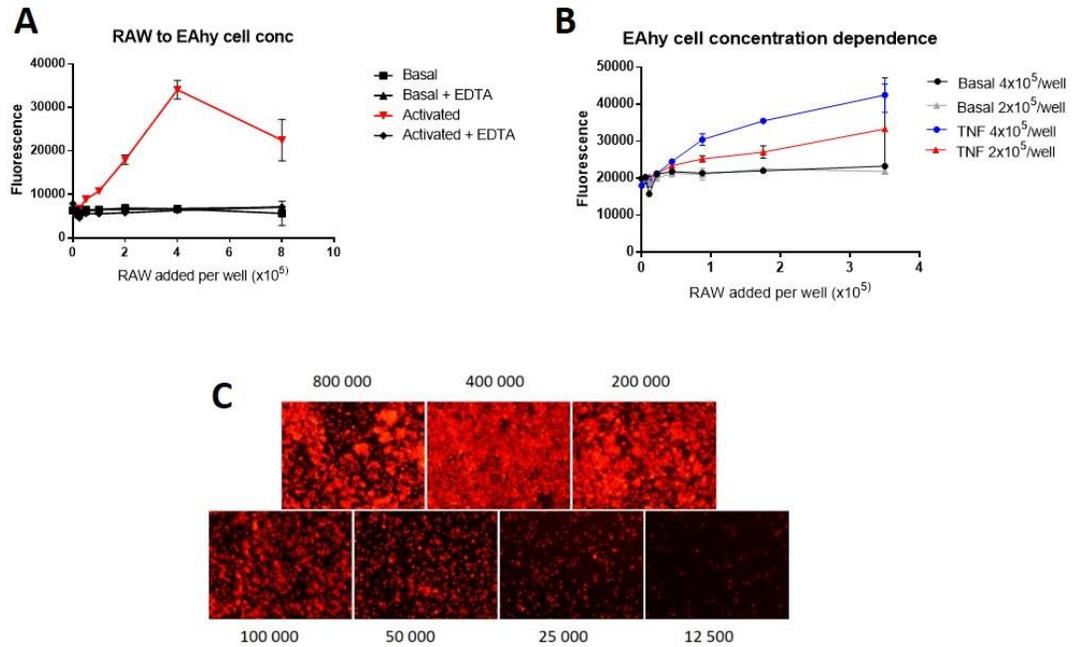
Figure 5-5: Development of RAW to EAhy functional adhesion assay. Activated EAhy cells stimulated with 2nM TNFα for 8 hours. RAW labelled with DiI fluorescent stain. A: fluorescent readout of RAW adhesion to activated EAhy is related to concentration up to 400 000 cells/well. Beyond that spatial inhibition occurs, the cells clump and dislodge during washing of the plate. There is no adhesion to basal EAhy cells or in the presence of EDTA. B: adhesion is also dependent on EAhy cell density, being increased when there is a confluent monolayer. C: fluorescent microscopy images of adhesion of RAW to EAhy with addition of different numbers of RAW cells per well.

5.5 Results

A summary of the demographics of patients recruited to the monocyte adhesion studies (flow cytometry and functional adhesion) is shown in **Table 5-3**. The number of female GD patients recruited was small due to recruitment difficulties.

Table 5-3: Patients/controls recruited to monocyte adhesion studies

	Fabry		Gaucher		Control	
	M	F	M	F	M	F
No	10	19	15	5	9	12
Age	46.1 (20-73)	45.5 (19-77)	47.8 (18-78)	36.8 (22-48)	34.2 (27-54)	44.9 (26-68)
ERT	10 (100%)	15 (79%)	15 (100%)	4 (80%)	N/A	N/A
Mean adhesion ratio (PHS)	1.91 (1.09-2.96)	2.35 (1.10-5.77)	1.69 (1.04-2.76)	1.64 (1.18-2.29)	1.62 (0.87-2.84)	1.43 (0.75-2.09)
Mean adhesion ratio (AHS)	2.06 (1.13-3.09)	2.32 (1.13-5.16)	1.78 (1.14-3.04)	1.65 (1.19-2.20)	1.56 (0.71-3.86)	1.40 (0.75-2.09)

5.5.1 Monocyte adhesion to tissue culture plastic

Adhesion in pooled human serum in Fabry patients was significantly greater than in controls (mean adhesion ratio 2.23 vs. 1.52, $p = 0.0076$) and Gaucher patients (mean adhesion ratio 2.23 vs. 1.68, $p = 0.037$) (see **Figure 5-6**). There was no difference in adhesion between Gaucher patients and controls (mean adhesion ratio 1.68 vs. 1.52, $p = 0.30$). If the patients were separated into males and females, the adhesion ratio in Fabry men was slightly higher than in Gaucher or control, but the difference was not significant ($p = 0.2$), see **Table 5-3**; from means and standard deviations of the data a sample size >40 patients would be required for the observed differences to be significant. In females, adhesion was significantly greater in Fabry patients than in controls (mean adhesion 2.35 vs. 1.44, $p = 0.023$). There was no difference between Gaucher patients and controls (mean 1.64 vs. 1.44, $p = 0.23$). The sample size of female patients with Gaucher was small due to only a few patients consenting to blood sampling.

There was no visible adhesion on phase contrast microscope or with Geimsa staining in the presence of EDTA. There was no difference in the ratio of adhesion in pooled serum to autologous serum between the patient groups, suggesting that the differences observed were due to an increased adhesion capacity of the monocytes rather than serum factors.

The relationship between age and adhesion was analysed in all patient groups (see **Figure 5-7**). A significant correlation between increasing age and adhesion was found in females with FD but not in females with GD or female controls. There was no correlation between age and adhesion in males.
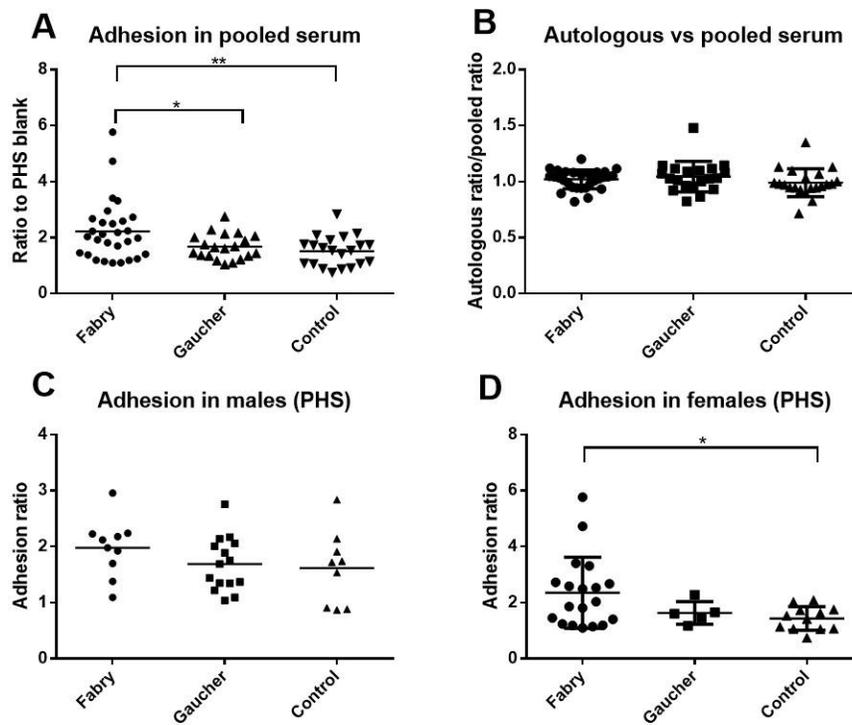
**Figure 5-6: Adhesion of monocytes to tissue culture plastic.** A: Overall adhesion in PHS. B: ratio of adhesion in PHS to adhesion in autologous serum. C: adhesion ratio in males. D: adhesion ratio in females. Lines indicate mean adhesion.
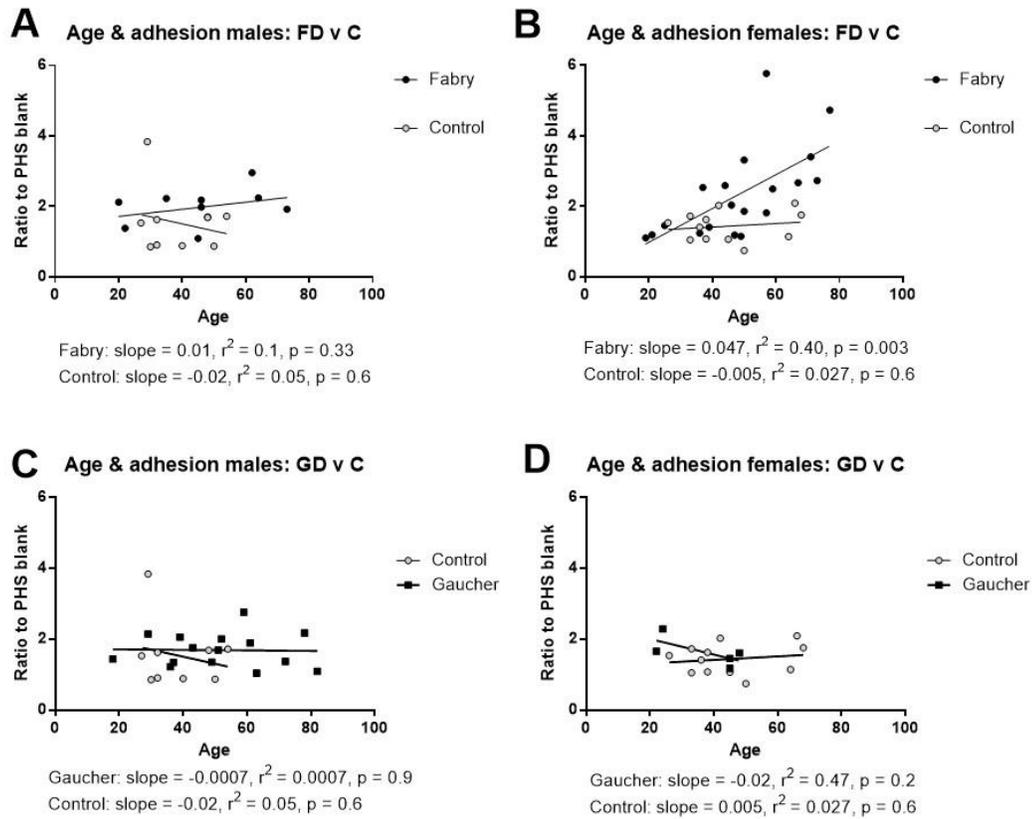
Figure 5-7: Relationship between age and adhesion. A: Males Fabry vs. control; B: Females – Fabry vs. control; C: Males – Gaucher vs. control; D: Females – Fabry vs. control.

5.5.2 Adhesion and disease features in Fabry disease

To assess the relationship between monocyte adhesion and clinical manifestations of FD linear regression analyses were performed (see **Table 5-4**). As well as disease manifestations, relationships between lipoprotein (a) and adhesion were examined as a raised Lp(a) was common amongst FD patients with cerebrovascular disease and has been demonstrated to increase expression of adhesion molecules[230]. All male patients and most female patients were receiving ERT at the time of the study; the effect of exogenous enzyme on leucocyte adhesion is unknown. Therefore both baseline and current disease parameters were analysed, with the baseline parameters serving as a surrogate for untreated disease severity in each of the assessed manifestations. Within the female FD patients, the patient with the highest adhesion ratio was an outlier across all parameters except neurovascular disease and exclusion of this patient from analyses had a big impact on the significance of the results obtained. It is uncertain why the adhesion ratio was so high is this patient and the ratio in this patient was >1 greater than the next highest result and almost three times the mean ratio. It was therefore considered that it might be appropriate to exclude this patient from the analyses and the results with this exclusion are also shown in **Table 5-4**. Exclusion of this one outlier resulted in increased strength and significance of correlation between adhesion and disease parameters in females.

There was a significant relationship between age and severity in females but not in males based both on current severity score but also on baseline severity (see **Figure 5-8**). As age is a key modifier of severity, age-adjusted severity scores were also assessed; the significant relationship was also found for age adjusted scores in females, but only after exclusion of the outlier and with a smaller r^2 and p value.

Table 5-4: Relationship between adhesion ratio and clinical FD parameters

Parameter	Time point	Male/female	Slope	R^2	P value
FOS-MSSI	Baseline	Male	0.002	0.001	0.923
		Female	0.060	0.307	*0.017*
		F (excl outl.)	0.058	0.495	*0.002*
	Current	Male	-0.006	0.014	0.759
		Female	0.054	0.261	*0.030*
		F (excl. outl)	0.060	0.562	*0.0005*

Age-adjusted MSSI	Baseline	Male	-0.056	0.330	0.083
		Female	0.064	0.150	0.112
		F (excl outl.)	0.073	0.339	*0.014*
	Current	Male	-0.015	0.143	0.316
		Female	0.051	0.103	0.194
		F (excl. outl)	0.073	0.358	*0.011*
GFR	Baseline	Male	-0.051	0.356	0.090
		Female	-0.059	0.220	*0.050*
		F (excl outl.)	-0.050	0.375	*0.009*
	Current	Male	-0.006	0.061	0.522
		Female	-0.021	0.171	0.089
		F (excl. outl)	-0.024	0.358	*0.011*
LVMI	Baseline	Male	0.005	0.088	0.439
		Female	0.070	0.320	*0.018*
		F (excl outl.)	0.061	0.419	*0.007*
Lipoprotein (a)	Current	Male	0.507	0.156	0.259
		Female	1.464	0.306	*0.017*
		F (excl outl.)	1.079	0.269	*0.033*

In both males and females there was a negative relationship between adhesion and glomerular filtration rate, with adhesion increasing with a decline in renal function. The relationship, however, was only significant in females (see **Figure 5-9**). The correlation between GFR and adhesion was stronger for baseline GFR results for both males and females. Left ventricular mass index is also correlated with adhesion in females but not males.

Cerebrovascular manifestations, stroke and the presence of white matter lesions, were also examined. There was no difference in the adhesion ratios between patients with or without stroke or with or without white matter lesions either at baseline or currently (see **Figure 5-9**). Serum Lp(a) was correlated with adhesion, in part due to the outlier, as significance was borderline if they were excluded (p = 0.033). Three out of four patients with lipoprotein levels >1.0 were members of the same family.
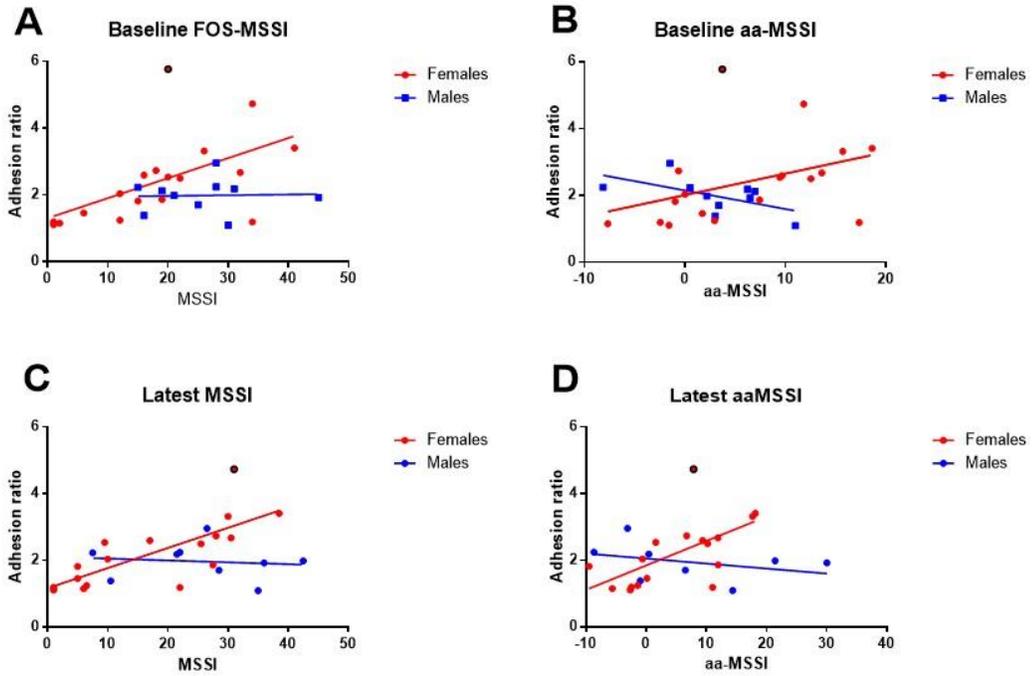
**Figure 5-8: Relationship between disease severity and adhesion.** A: baseline FOS-MSSI. B: baseline age-adjusted MSSI. C: current FOS-MSSI. D: Latest age-adjusted MSSI. Female outlier identified by black outline.
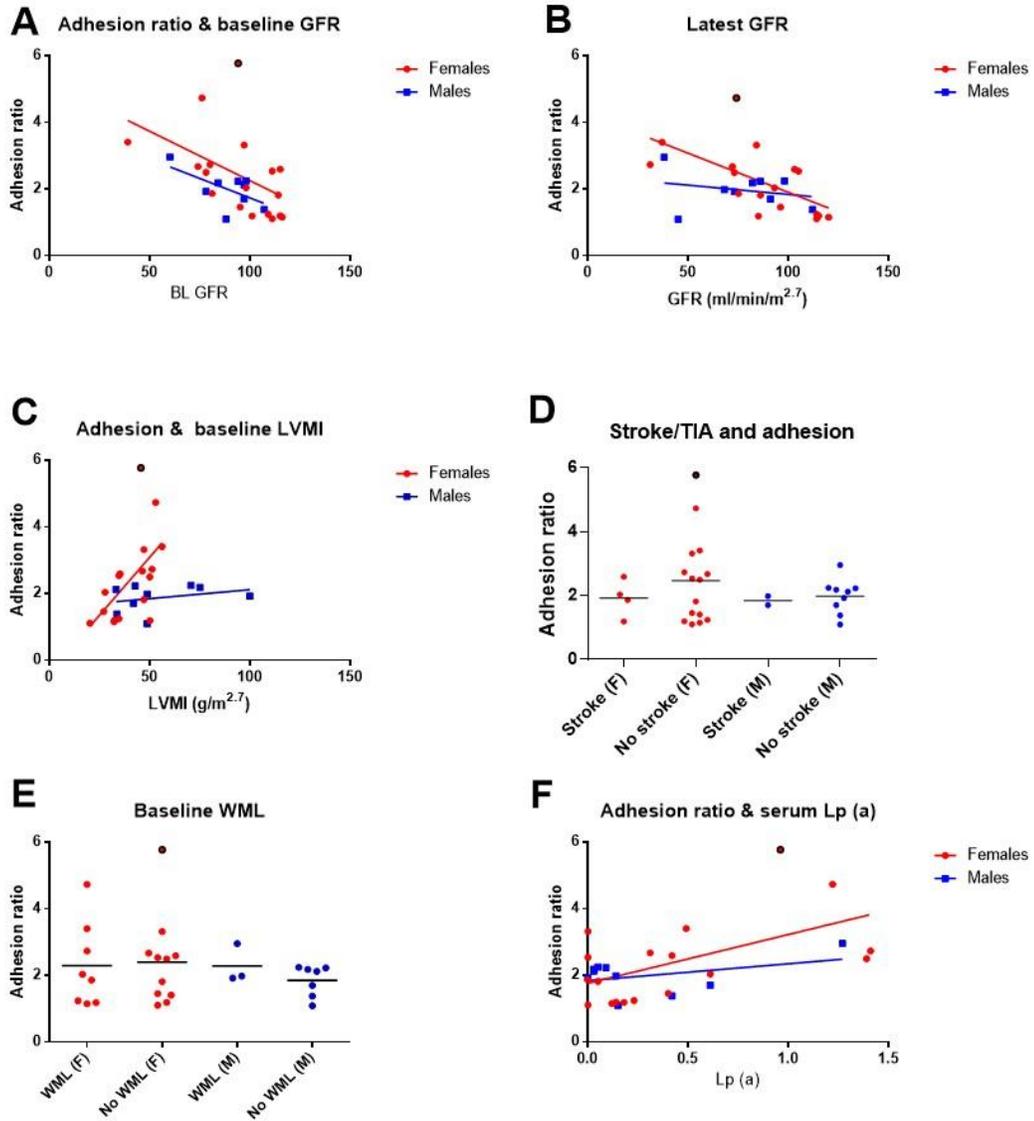
Figure 5-9: Relationship between age and organ function in FD. A: baseline GFR; B: current GFR; C: baseline LVMI; D: stroke/TIA; E: baseline WML; F: serum lipoprotein (a). Female outlier identified by black outline.
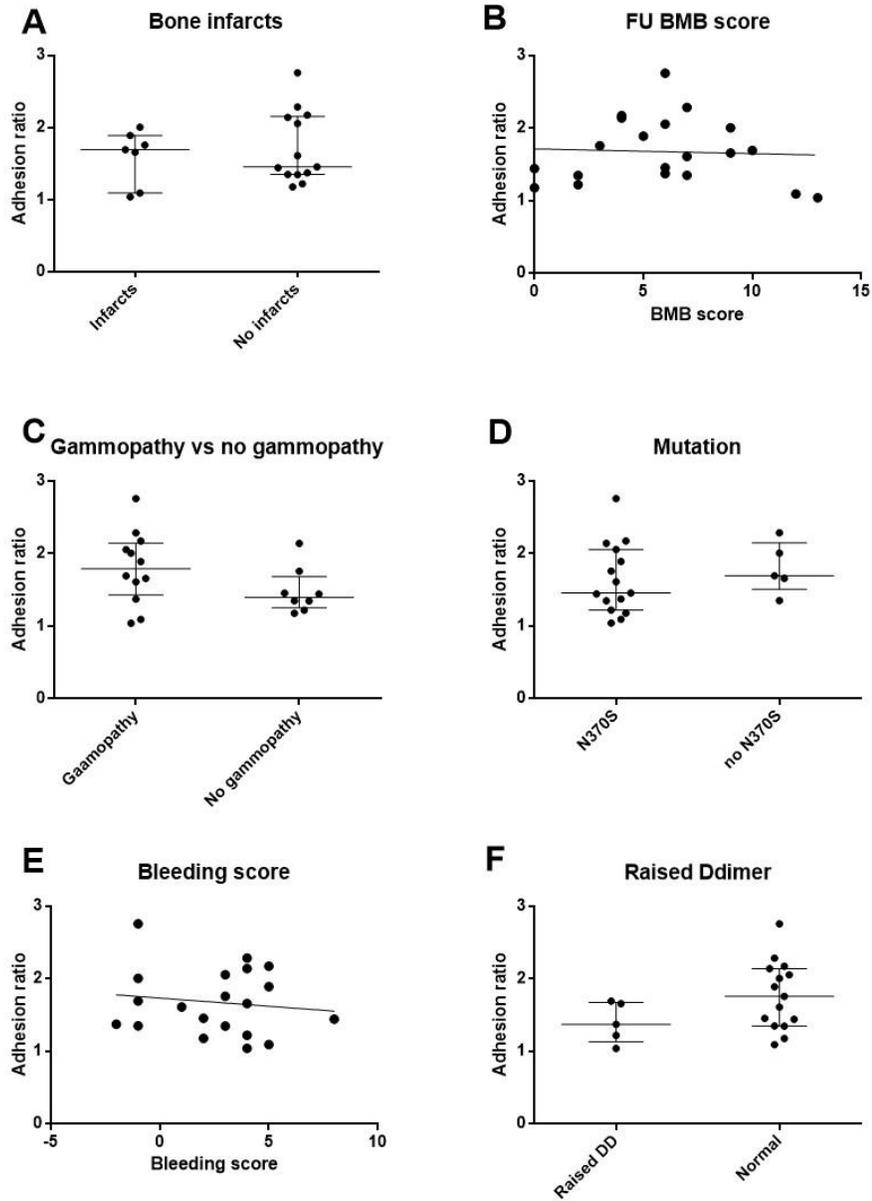
Figure 5-10: Relationship between adhesion and GD complications. A: presence of bone infarcts on MRI; B: bone marrow burden scores; C: presence of a gammopathy; D: presence of at least on N370S mutation; E: bleeding score; F: raised D-dimer. A, C, D,F: line = median; error bars = interquartile range.

176

5.5.3 Adhesion and disease features in Gaucher disease

There was no difference in adhesion between patients with or without bone infarcts on latest MRI (median 1.693 vs. 1.455, p= 0.67) and no correlation between adhesion and total bone marrow burden scores on MRI (slope = 0.004, r^2 = 0.002, p = 0.84). Adhesion was increased in those with a gammopathy but this difference was not significant (median 1.79 vs. 1.40, p = 0.15, see **Figure 5-10**). There was no correlation between bleeding score and adhesion (slope = -0.022, r^2 = 0.017, p = 0.58) nor was adhesion higher in those with a persistently raised D-dimer (median 1.37 vs. 1.76, p = 0.13). The presence of at least one N370S mutation did not affect the adhesion ratio (median 1.455 vs. 1.693, p = 0.395).

5.5.4 Expression of adhesion markers

The MFI and standard deviations for adhesion molecules on the mononuclear cell subsets are shown in **Table 5-5** and the significant findings are summarised in **Table 5-6**.

Table 5-5: MFI of adhesion molecules on leukocytes (mean ±SD)

CD	CD14+ monocytes			CD14- monocytes			Lymphocytes		
	GD	FD	C	GD	FD	C	GD	FD	C
CD11a	428 ±128	441 ±117	352 ±68	576 ±162	567 ±131	381 ±129	246 ±66	226 ±54	139 ±48
CD11b	1638 ±579	2019 ±542	1697 ±784	X	x	x	x	x	X
CD11c	159 ±70	206 ±94	175 ±71	252 ±92	298 ±102	226 ±70	x	x	X
CD49D	404 ±127	413 ±110	424 ±100	1033 ±181	916 ±158	1009 ±110	283 ±77	224 ±46	202 ±49
CD54	160 ±48	185 ±41	148 ±33	196 ±50	237 ±46	197 ±42	x	x	X
CD102	710 ±155	594 ±138	768 ±209	928 ±134	865 ±102	913 ±160	354 ±107	309 ±58	304 ±56
CD50	672 ±133	654 ±132	560 ±153	450 ±76	421 ±86	364 ±76	289 ±57	257 ±45	217 ±59
CD36	2213 ±824	2111 ±524	2127 ±702	341 ±419	205 ±95	318 ±296	x	x	X
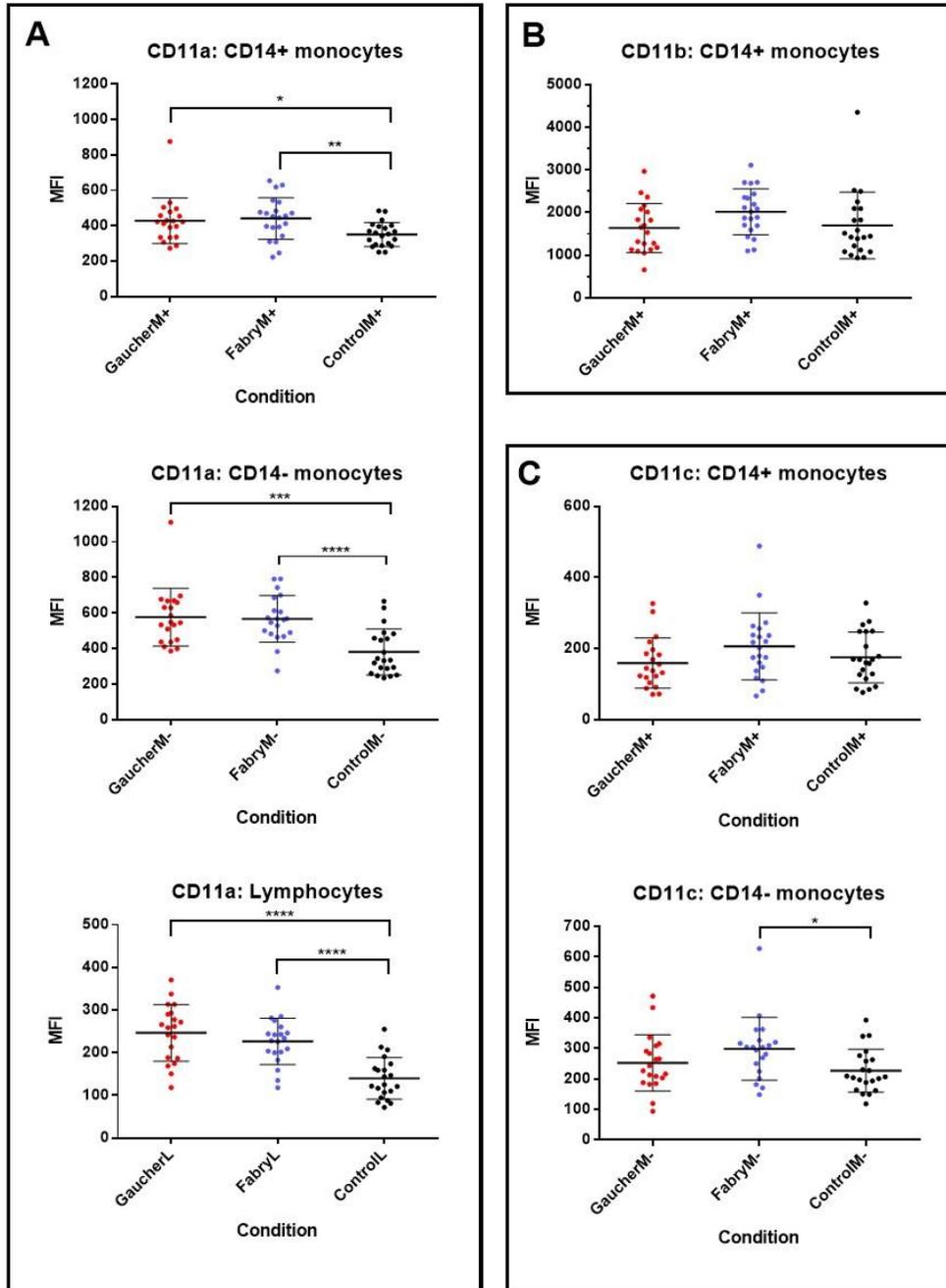
Figure 5-11: Median fluorescent intensity of the alpha subunits of the alpha-beta1 integrins. A: CD11a; B: CD11b; C: CD11c. Lines indicate the mean; error bars, standard error of the mean.
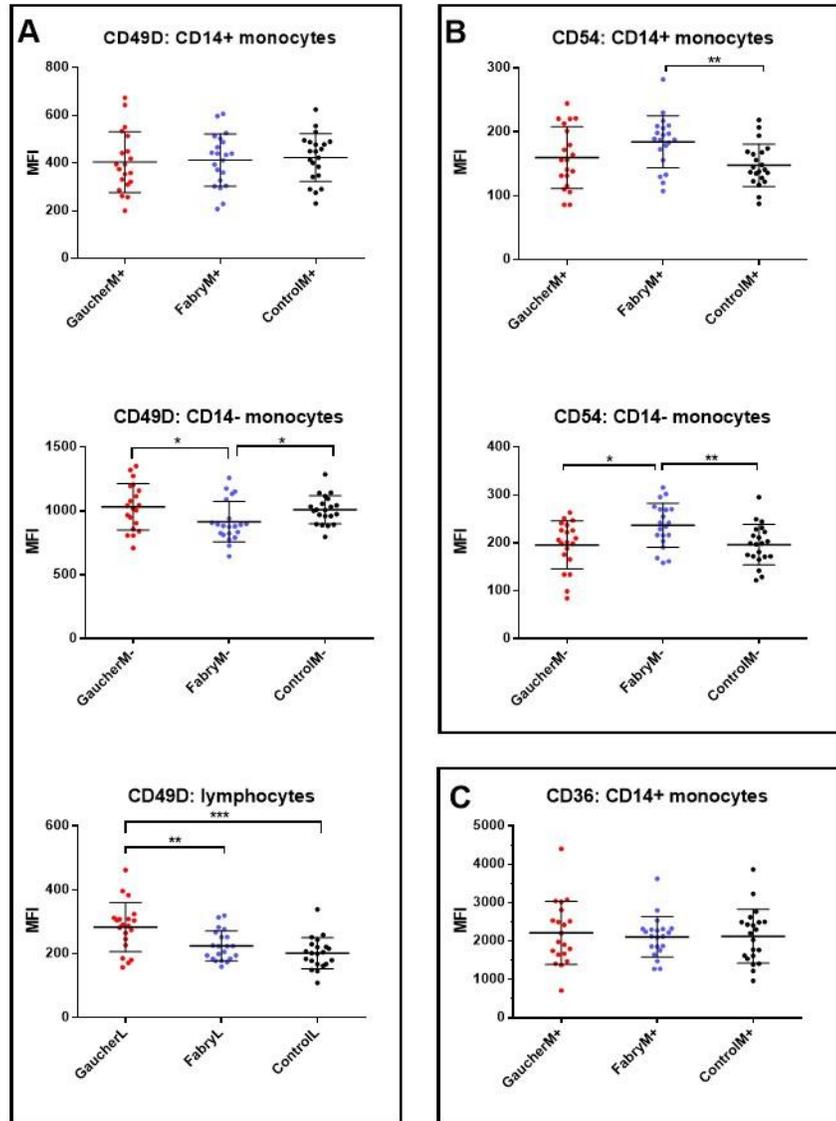
Figure 5-12: A: Expression of CD49d, alpha subunit of VLA-4; B: expression of CD54 (ICAM-1); C: expression of scavenger receptor B (CD36).
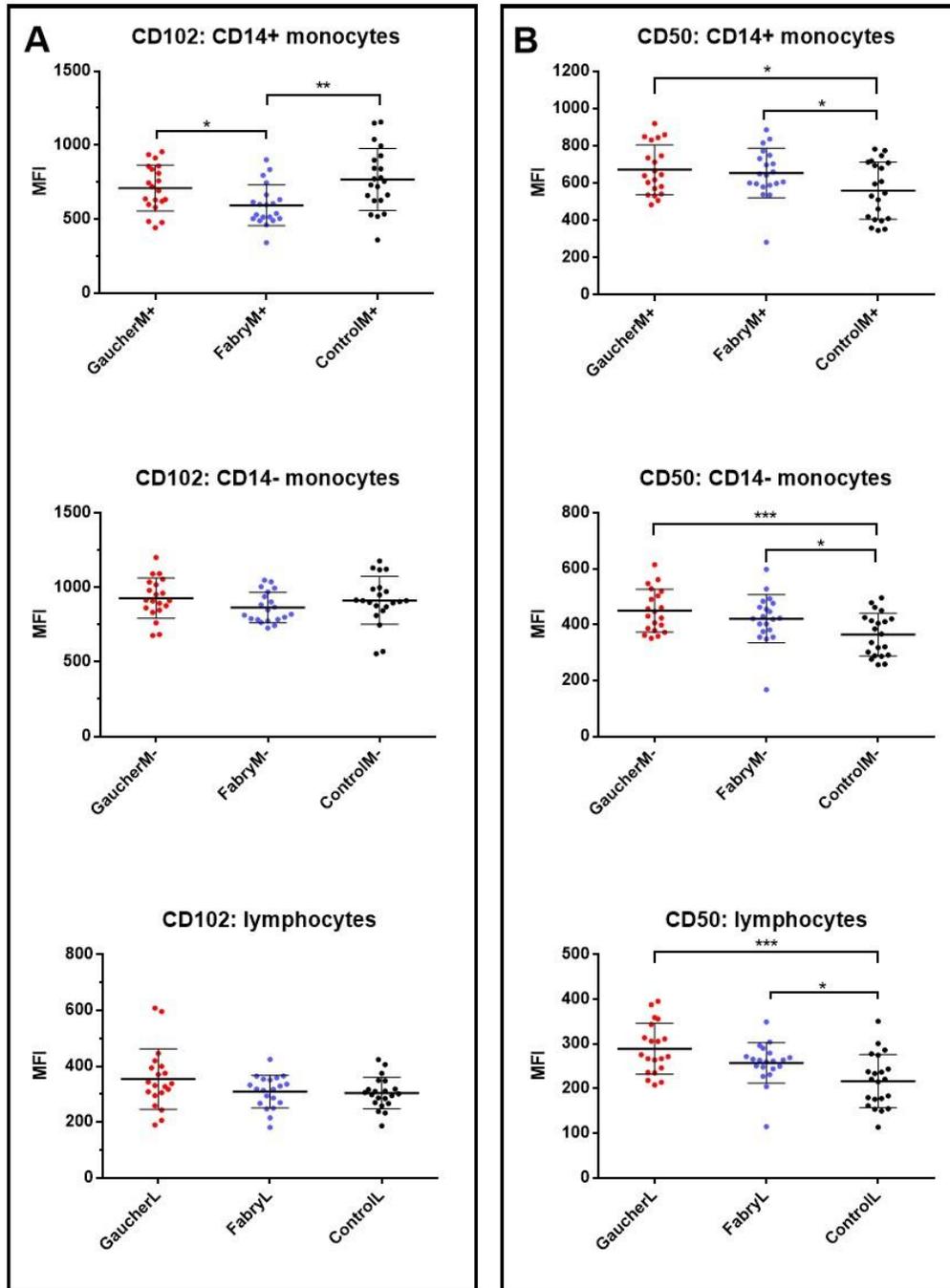
Figure 5-13: Expression of CD102 (ICAM-2) and CD50 (ICAM-3) on mononuclear cell subsets.
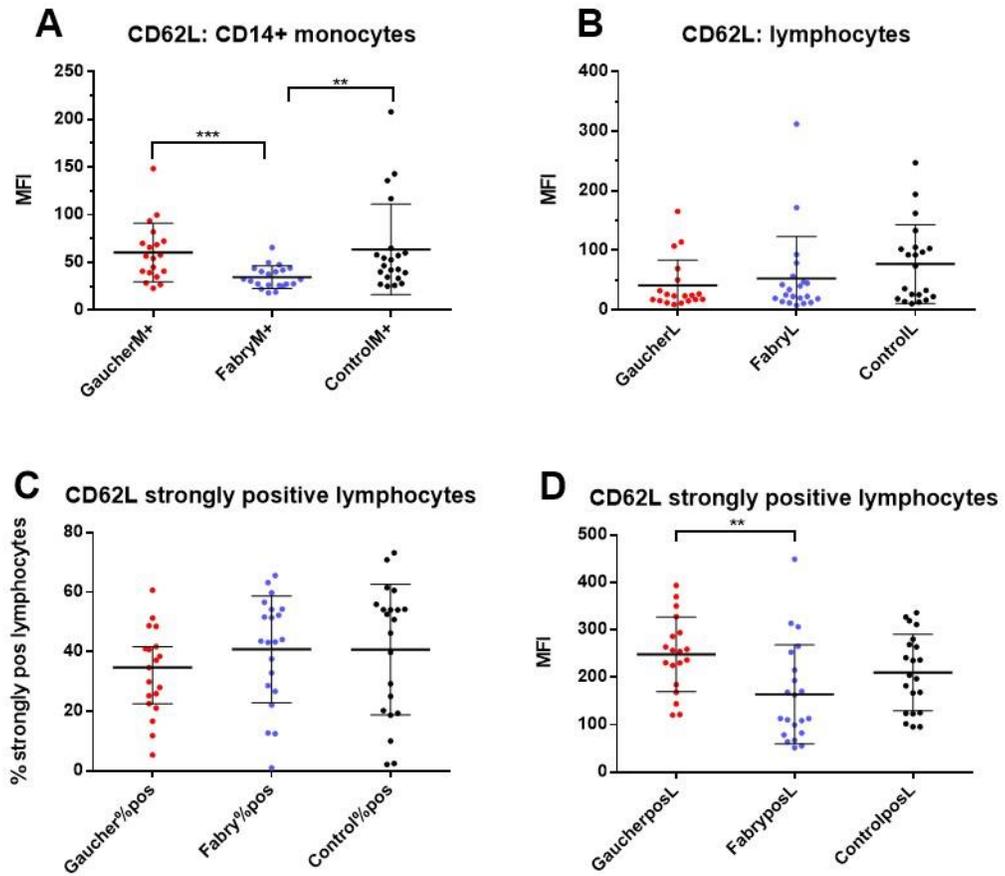
Figure 5-14: CD62L expression. A: expression on CD14+ monocytes. B: expression on overall lymphocyte population. C: percentage of lymphocytes with high CD62L expression. D: MFI of CD62L on lymphocytes with high expression.

Integrins

Expression of CD11a (the alpha subunit of LFA-1) was increased in FD and GD compared to controls across all leukocyte subsets, with the most significant differences seen in CD14- monocytes and lymphocytes ($p<0.0001$) rather than monocytes (p 0.004 for FD v C and $p = 0.019$ for GD vs. C), see **Figure 5-11**. There was no difference in the expression of CD11b. There was an increase in expression of CD11c on CD14- monocytes in FD compared to controls ($p = 0.013$). CD49d is significantly increased on lymphocytes in GD patients compared to both controls ($p = 0.0002$) and FD ($p = 0.0049$). There is a slight reduction in expression of borderline significance in FD CD14- monocytes ($p = 0.03$).

Immunoglobulin superfamily

CD54 (ICAM1) expression is increased on FD CD14+ and CD14- monocytes relative to controls ($p = 0.003$ and 0.006), see **Figure 5-12**. CD102 (ICAM2) expression was reduced in FD compared to GD or controls in CD14+ monocytes ($p = 0.018$ and 0.0034 respectively), but not in CD14- monocytes, which overall had a higher MFI, or lymphocytes, see **Figure 5-13**. CD50 (ICAM3) expression was significantly increased in GD compared to controls on lymphocytes and CD14- monocytes ($p = 0.0003$ and $p = 0.0009$ respectively), and also CD14+ monocytes ($p = 0.017$). There was a slight increase in FD compared to controls across all subsets, most marked in lymphocytes ($p = 0.019$).

L selectin

CD62L is expressed at low levels on CD14+ monocytes and was decreased in FD compared to both GD ($p = 0.0009$) and controls ($p= 0.009$), see **Figure 5-14**. Expression on CD14- monocytes was low and there were no differences between the conditions. The percentage of strongly positive CD62L lymphocytes was slightly reduced in Gaucher disease, although the MFI of CD62L on these lymphocytes was increased in GD compared to FD ($p = 0.0069$).

Scavenger receptor

There was no difference in expression of CD36 on CD14+ or CD14- monocytes.

182

Table 5-6: Summary of changes in adhesion molecule expression (relative to controls)

Adhesion molecule	GD			FD		
	M+	M-	L	M+	M-	L
CD11a (LFA-1)	↑	↑	↑	↑	↑	↑
CD11b (CR3)	-			-		
CD11c (CR4)	-	-		-	↑	
CD49D (VLA-4)	-	-	↑	-	↓	-
CD54 (ICAM1)	-	-		↑	↑	
CD102 (ICAM2)	-	-	-	↓	-	-
CD50 (ICAM3)	↑	↑	↑	↑	↑	↑
CD36 (SRB)	-			-		
CD62L	-	-	↑	↓	-	-

Linear regression analysis of monocyte MFI for CD11a, CD54 and CD50 (the adhesion molecules where significant differences in expression on monocytes were found) did not show any correlation between MFI of the molecules and the adhesion ratio.

Adhesion molecule expression and clinical disease manifestations
Increased expression of CD50, 54 or 49d was not associated with GD complications of bone disease or gammopathies. In FD CD11a and CD54 and CD50 MFI was not correlated with age-adjusted disease severity, renal function or left ventricular mass.

5.5.5 Endothelial cell model
Confocal microscopy confirmed uptake for DiI labelled acetylated LDL and synthesis of von Willebrand factor (see **Figure 5-15**); however, VWF did not form discrete Weibel Palade bodies as occurs in HUVEC cells. EAhy.926 showed inducible expression of ICAM-1 and VCAM-1 in response to stimulation with TNFα with a dose and time dependent increase in adhesion of RAW cells to EAhy.926 (see **Figure 5-16**).
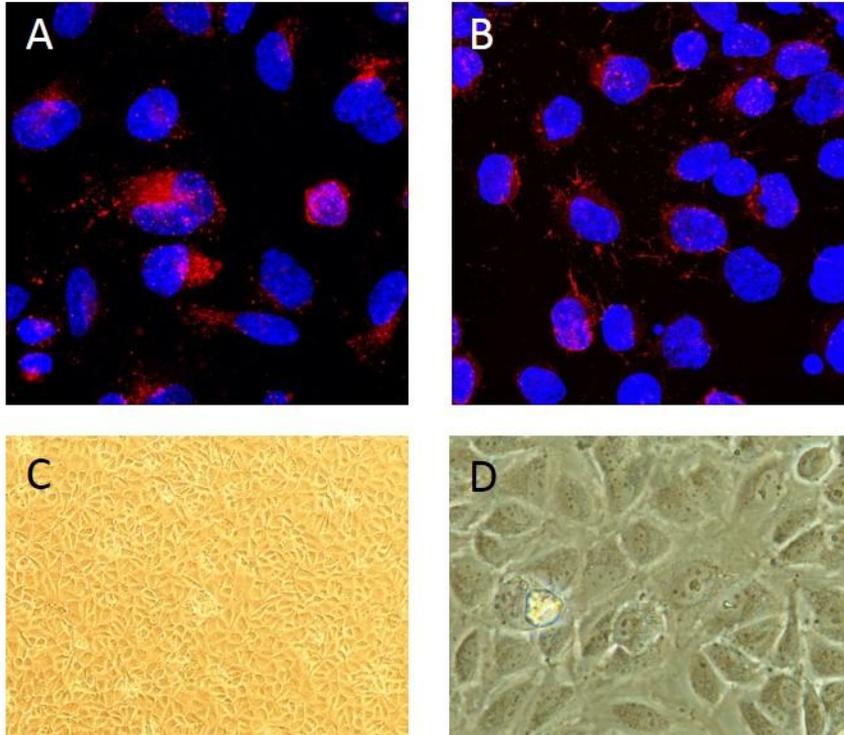
Figure 5-15: Endothelial characteristics of EAhy cells. A: Uptake of DiI-labelled acetylated LDL (x40); B: von Willebrand factor production (x40), but without forming classical Weibel Palade bodies; C & D: cobblestone morphology (x10 and x40).
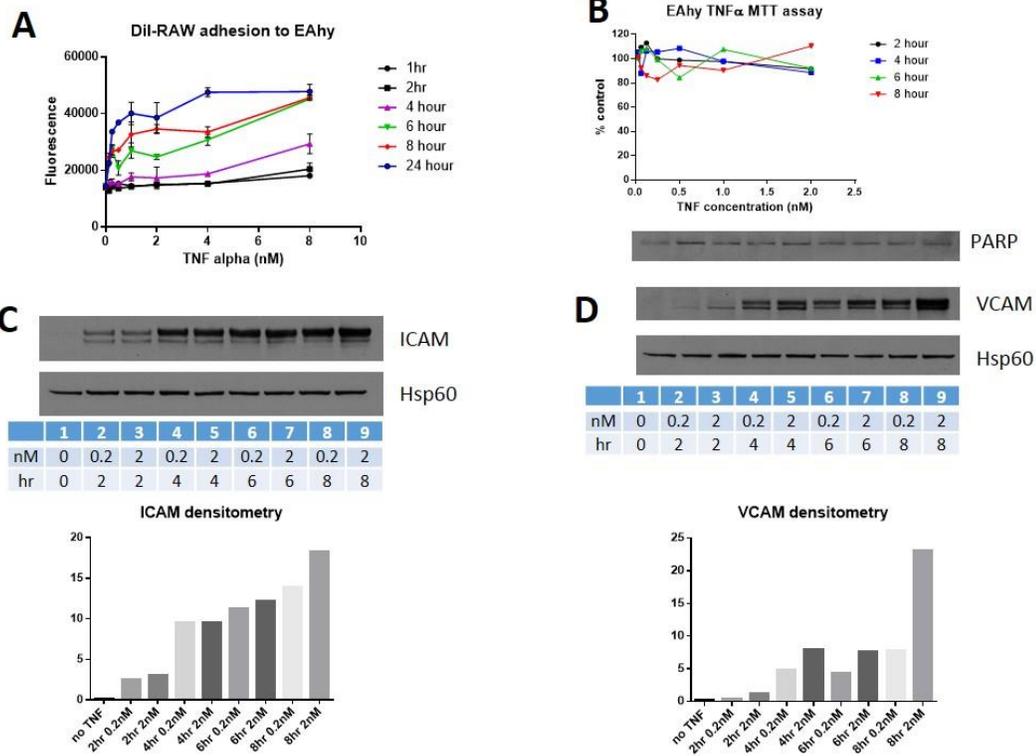
Figure 5-16: Effect of TNFα on expression of adhesion molecules of EAhy.926 monolayers. A: adhesion of DiI-labelled RAW to EA.hy926 monolayers activated with different doses of TNFα for different time periods. B: toxicity of TNFα measured by MTT assay and by PARP cleavage on Western blot. C: increase in ICAM-1 protein levels on activation of EA.hy with TNFα. D: increase in VCAM-1 protein levels on activation of EA.hy with TNFα.
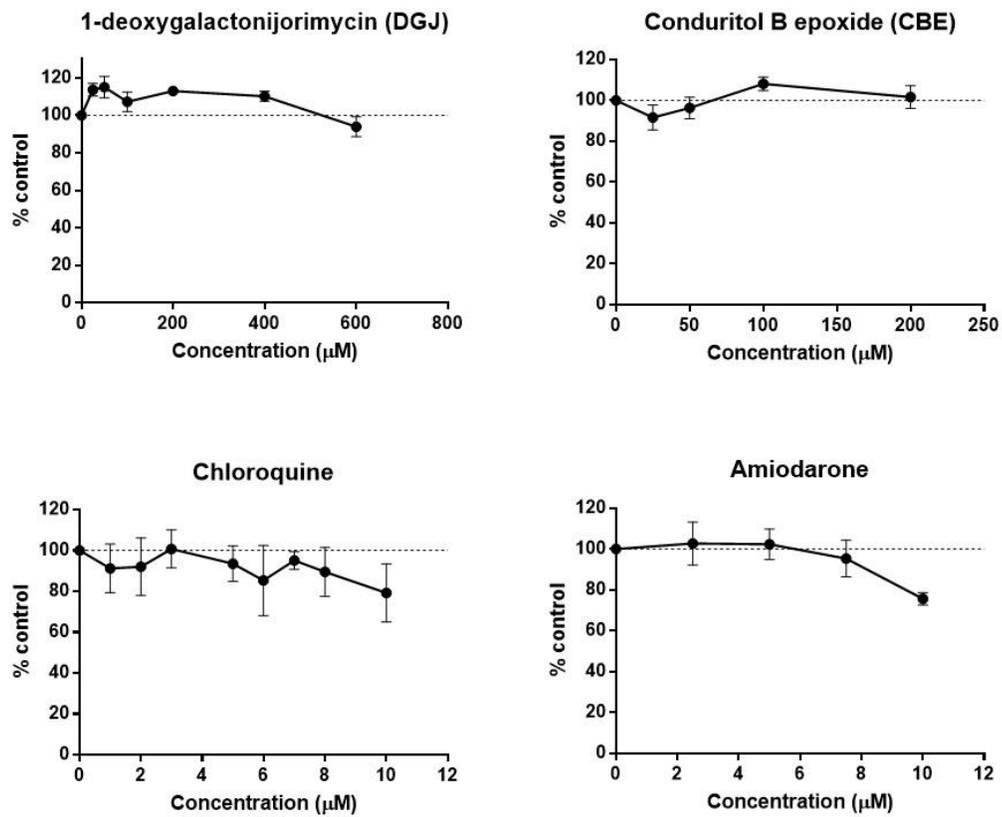
Figure 5-17: Toxicity of inhibitors on EA.hy 926 cells assessed by MTT dye reduction assay.
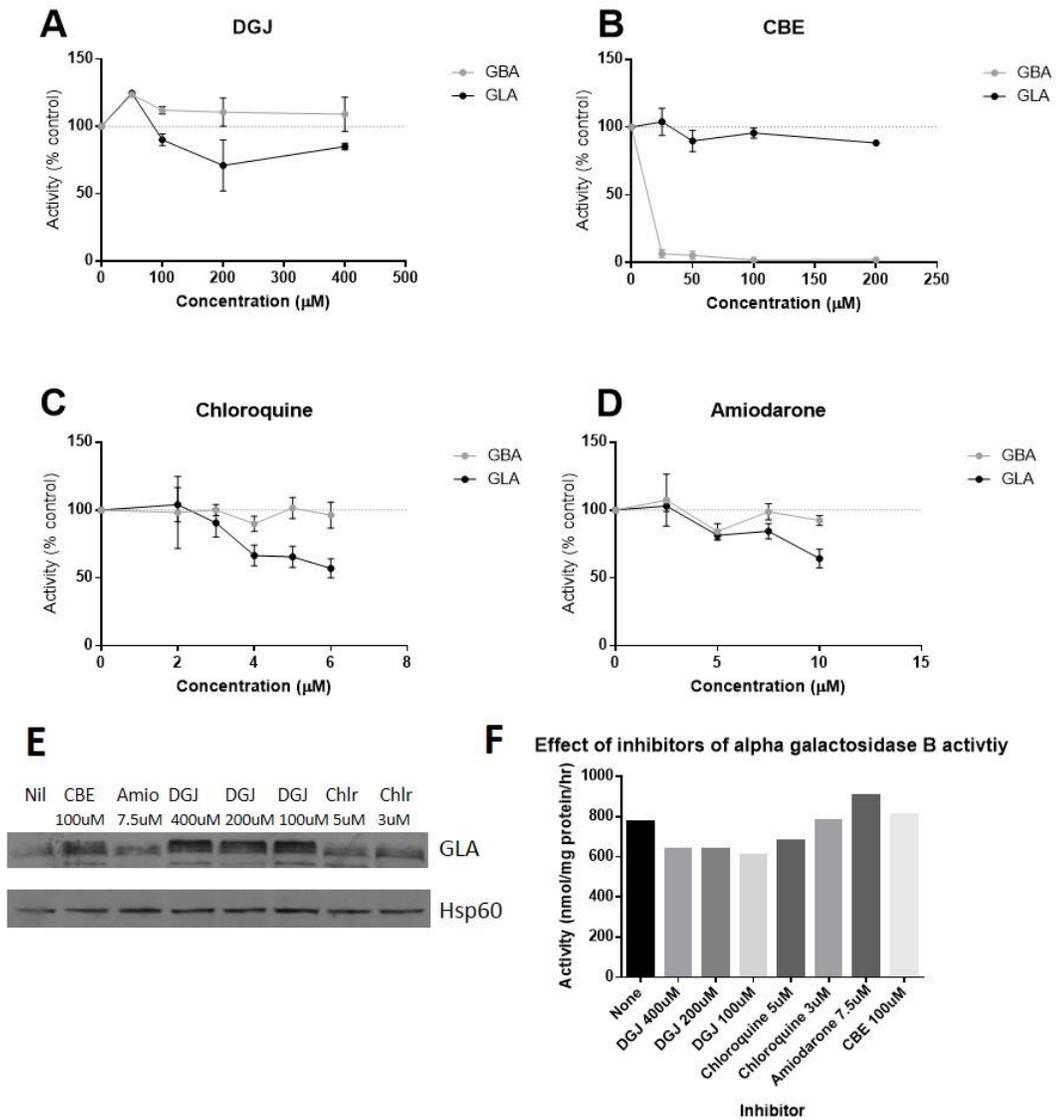
Figure 5-18: Effect of pharmacological inhibitors on GBA and GLA activity after incubation of the cells with inhibitors for 7 days. A range of concentrations were utilised to establish if there was a concentration dependent effect. A: DGJ; B: CBE; C: chloroquine; D: amiodarone. E: effect of inhibitors on GLA protein level after incubation for 7 days. F: effect of inhibitors on alpha galactosidase B enzyme activity.

Development of pharmacological model

MTT assay, showed a slight reduction in metabolic activity at higher (10µM) concentrations of chloroquine and amiodarone, implying reduced cell viability, but no reductions with DGJ or CBE (see **Figure 5-17**). GBA activity was minimal after addition of CBE, with almost undetectable enzyme activity at 100 and 200µM. The other inhibitors had little effect on GBA activity.

CBE had no effect on GLA activity. Both amiodarone and chloroquine resulted a concentration dependent decrease in GLA activity, maximal with 6µM chloroquine with 57% control activity and with 10µM amiodarone at 64% control activity. Incubation with DGJ showed a slight increase in activity with 50µM DGJ and mild reduction in activity at higher concentrations, maximal with 200µM DGJ at 71% control activity (see **Figure 5-18**). Western blot for GLA protein levels showed an increase in GLA in cells treated with DGJ compared to other inhibitors. Measurement of α galactosidase B activity showed a mild reduction in cells treated with DGJ and chloroquine but not amiodarone or CBE.

For endothelial assays the doses of pharmacological inhibitors used were: chloroquine 5µM, amiodarone 7.5µM, CBE 100µM and DGJ 200µM

shRNA mediated knockdown of alpha galactosidase A

Measurement of GLA enzyme activity showed a reduction in enzyme activity to 87.9% of control activity with the FD1 shRNA sequence, 26.4% with FD2 and 30.8% with FD5. Western blot confirmed marked reduction in GLA protein levels with FD2 and FD5 but not FD1. FD1 was therefore not used for further experiments. Interestingly treatment of FD2 and FD5 cells with DGJ 200µM resulted in partial restoration of enzyme activity and protein levels (see **Figure 5-19**). The shRNA sequences had no effect on alpha galactosidase B activity, which shares >50% sequence homology with the *GLA* gene[250].
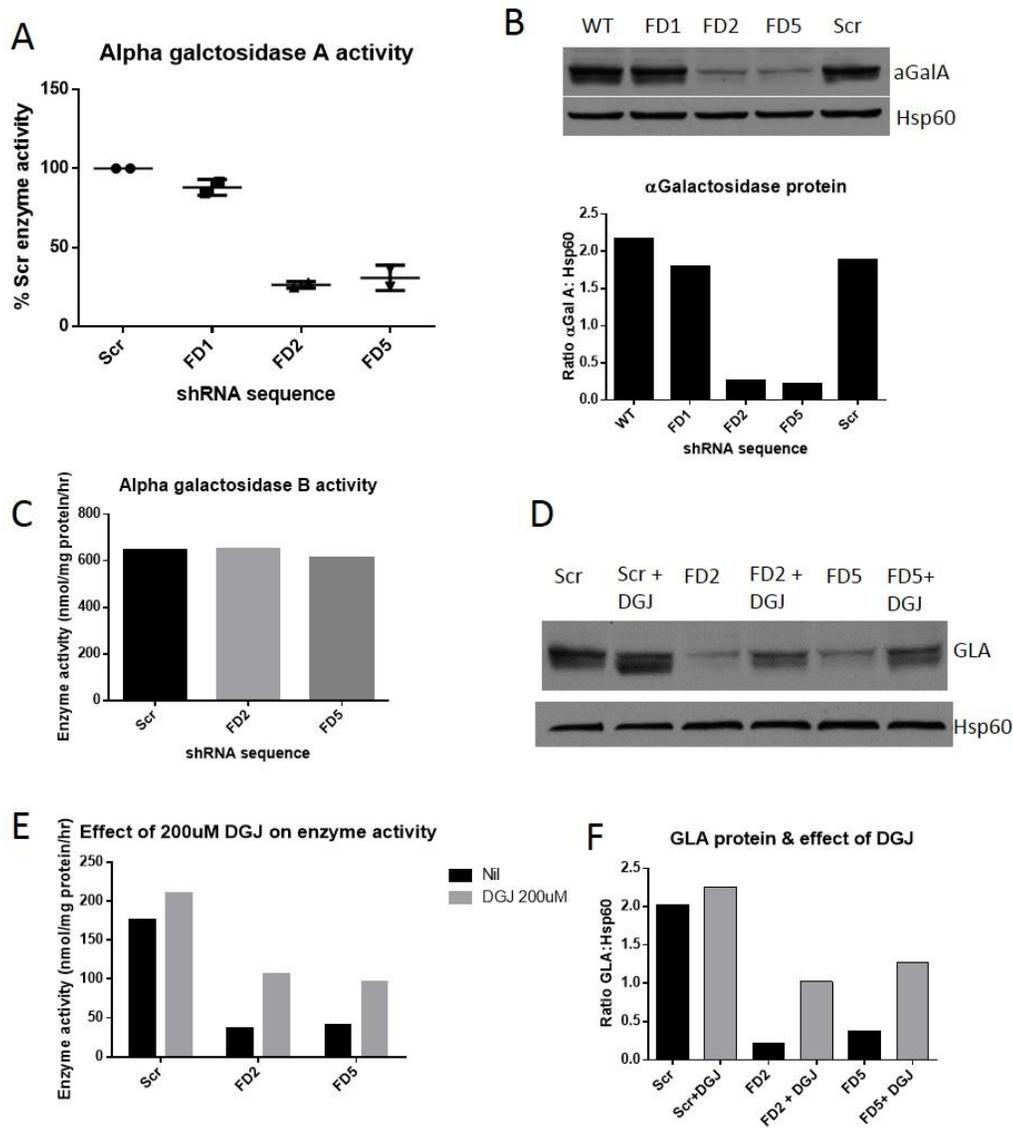
Figure 5-19: Effect of shRNA knockdown.

A: GLA activity expressed as percentage of the scrambled sequence enzyme activity; B: GLA protein levels in cell lysates; C: effect of shRNA knockdown of GLA on alpha galactosidase B activity; D: effect of treatment of cells with 200μM DGJ for 7 days on GLA protein levels (D,F) and enzyme activity (E).

5.5.6   Effect of altered glycosphingolipid metabolism on endothelial adhesion

Reduction in GLA activity as the result of shRNA mediated gene silencing did not result in increased adhesion of RAW cells to the endothelial cell monolayer (see **Figure 5-20**). Cells treated with pharmacological inhibitors showed no difference in adhesion to untreated cells, except a slight reduction in adhesion to cells treated with CBE after 4hr of TNFα stimulation but not basally or after 8 hours.

Western blots were performed to measure the protein levels of VCAM-1 and ICAM-1 in the shRNA cells to see if reduced GLA altered expression of these molecules. EAhy cells do not express VCAM-1 basally; the increased expression of VCAM-1 in response to TNFα stimulation did not differ between cells with normal GLA activity (Scr) and those with reduced activity (FD2 & FD5), see **Figure 5-20**. Basal expression of ICAM-1 was the same in all three cell types and there was no difference in responses to TNFα stimulation. Minimal adhesion occurred in all cell types in the presence of the calcium chelator EDTA, confirming that adhesion is calcium dependent which is in keeping with integrins and members of the immunoglobulin superfamily being the principle mediators.

5.5.7   Effect of glycosphingolipid addition on endothelial adhesion

Wild-type EAhy cells were incubated with different glycosphingolipids at 10μM concentration and the effect of glycosphingolipid loading on adhesion evaluated. There was no difference in functional adhesion activity following TNFα stimulation. There was a minimal increase in basal adhesion with lactosylceramide, sphingosine-1-phosphate and lysoGb3. Western blot for ICAM and VCAM did not reveal any differences in expression of these adhesion molecules with brief (8hr) or prolonged (72hr) incubation with glycosphingolipids or following TNFα activation of lipid loaded cells (see **Figure 5-21**).

Adhesion of lipid-loaded RAW to endothelial monolayers

Glycosphingolipid excess has been suggested as the mechanism for increased leukocyte adhesion. RAW cells were incubated with glycosphingolipids at 10μM concentration or methanol vehicle control for 72 hours and their adhesion to EAhy cells, both normal EAhy and those treated with CBE/shRNA, assayed. Amongst the lipids associated with GD

190

(S1P, GSP and LacC), there was an increase in adhesion RAW cells incubated with GSP compared to methanol vehicle to both normal EAhy and those treated with CBE following stimulation with TNFα. LacC resulted in an increase in adhesion only to normal EAhy treated with TNFα. Incubating RAW with lipids associated with FD (Gb3, S1P and lysoGb3) did not alter their adhesion properties compared to methanol vehicle, see **Figure 5-22**.
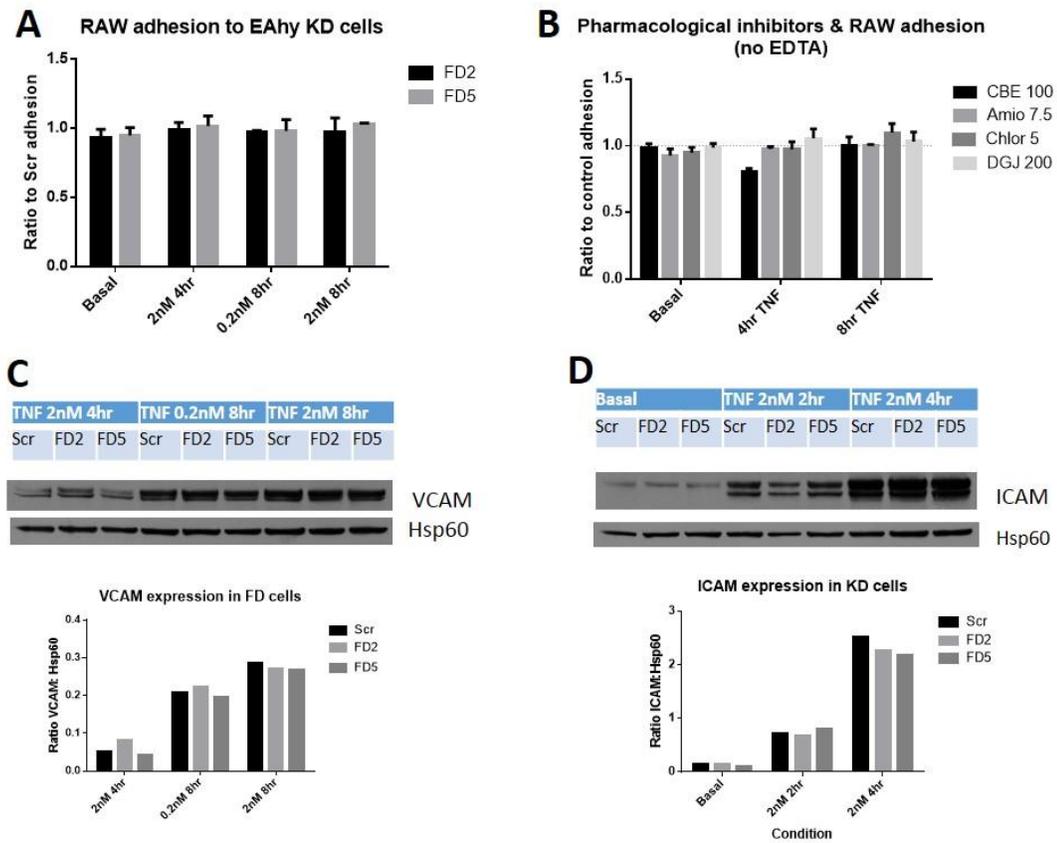
Figure 5-20: Effect of enzyme inhibition on endothelial adhesion. A: no increase in adhesion of RAW cells to monolayer in FD2 and FD5 compared to Scr (control). B: no differences in adhesion of RAW to cells treated with pharmacological inhibitors for 7 days. No difference in increase in expression of VCAM-1 (C) or ICAM (D) in response to TNFα stimulation in FD2 or FD5 compared to Scr.
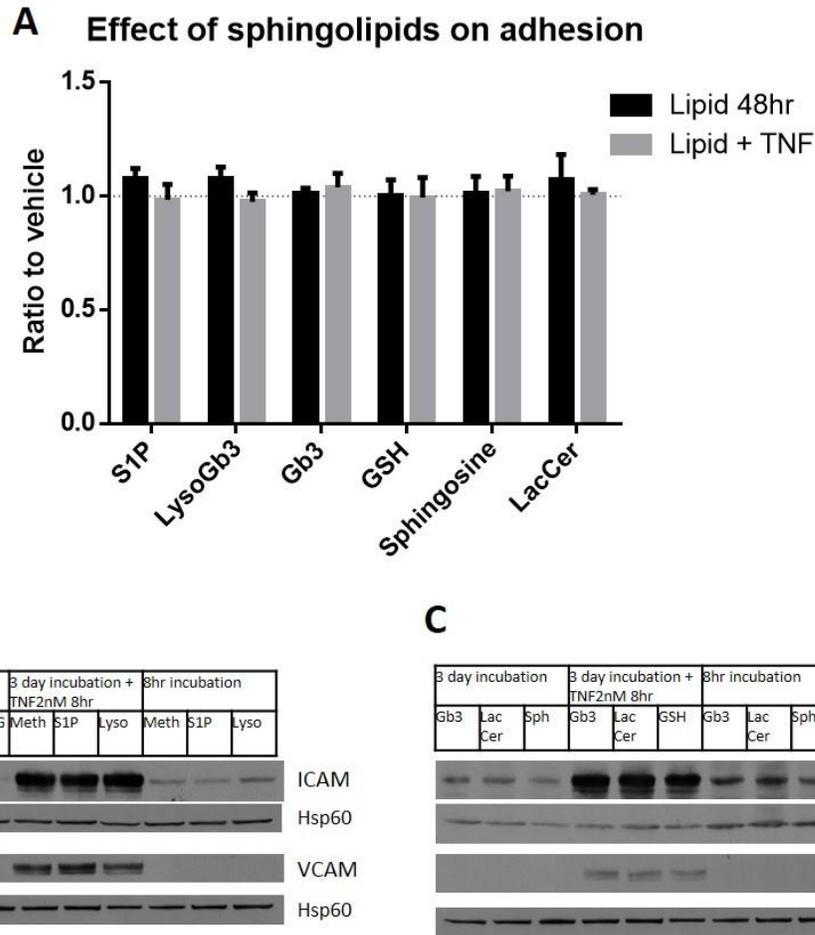
Figure 5-21: Effect of glycosphingolipids on adhesion

Effect of incubating EAhy cells with glycosphingolipids (10μM) for 72 hours on adhesion basally and following TNFα activation (2nM, 8 hours). A: adhesion ratio to vehicle (methanol) treated EAhy basally and following TNFα activation. B: Western blot for ICAM-1 and VCAM-1 expression following 72 hour lipid incubation +/- TNFα activation or 8hr lipid incubation.
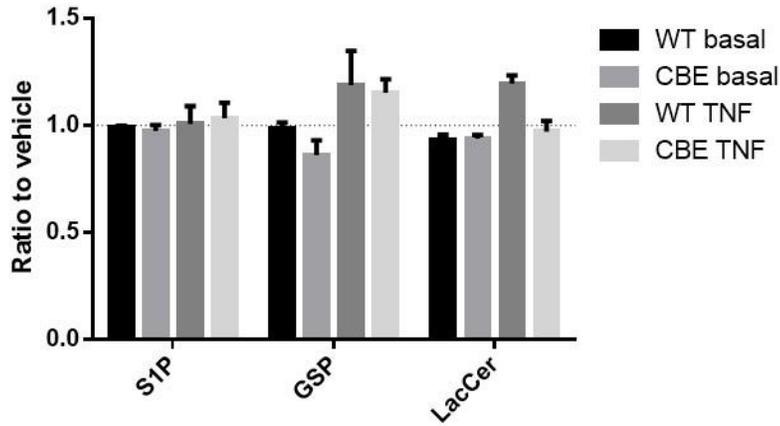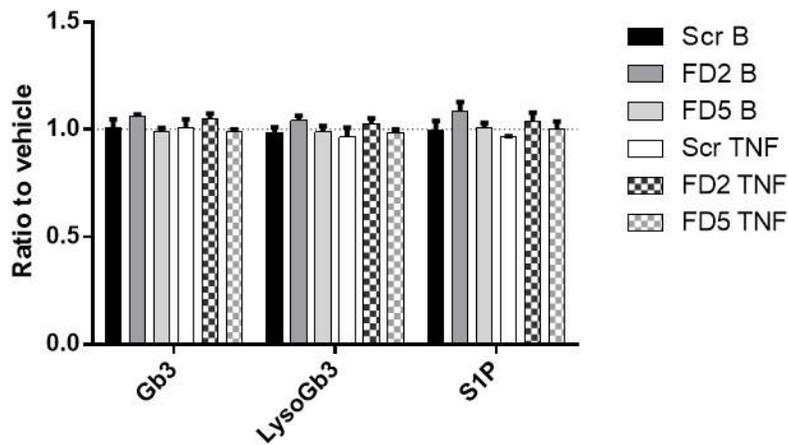
Figure 5-22: Effect of lipid-loading RAW on their adhesion to EAhy cells with reduced enzyme activity.

5.6   Discussion

5.6.1   Leukocyte adhesion in FD

Female patients with FD showed an age-dependent increase in monocyte adhesion not seen in males or in controls. Adhesion in females, but not males, correlated with disease severity, left ventricular mass and Lp(a) concentration. One significant outlier influenced the results – the reason for increased adhesion in this patient is uncertain. Exclusion of this patient resulted in significant correlations also with GFR. Unlike cardiac and renal disease, there was no relationship between adhesion and cerebrovascular disease. As discussed in Chapter 4, ageing appears to be a much more important factor in the development of disease complications in females than males and these results suggest that an age-related increase in monocyte adhesion may contribute to some of these disease manifestations. The processes that would underlie an age-dependent increase in adhesion, not seen in controls or male FD patients are uncertain. In many males the onset of disease manifestations is considerably earlier than in females and therefore it might be that as similar age-dependent effect might be seen in males in childhood-adolescence.

Dysfunction in lysosomal trafficking pathways has been reported with ageing, affecting both autophagy[251] and endocytosis[252]. ICAM1 and potentially other adhesion receptors are recycled through the endocytic pathway[253]. One hypothesis would be that females with FD are more susceptible to age-related changes in the cellular trafficking pathways that are involved in the recycling of these adhesion molecules, leading to increased concentration of activated molecules on the cell surface

Increase serum levels of adhesion molecules are associated with renal failure[254] and therefore the negative relationship between GFR and monocyte adhesion seen in both males and females was expected, although it was only significant in females.

The increase in ICAM1 expression on monocytes in FD suggests that the increased serum ICAM1 found in previous studies[152] originates, at least in part, from leukocytes. CD11a, the α-subunit of LFA-1, which is a ligand for both ICAM1 and ICAM3, which may

augment the interaction between LFA-1 and ICAM-1 was also increased, although to a lesser degree. As well as playing a role in leukocyte adhesion, interactions between LFA-1 and ICAM1 have other important roles including mediating B[255] and T cell activation[256]. LFA-1 on mononuclear cells has also been shown to accelerate TGF-β induced renal epithelial-mesenchymal transition in renal cells[257], a pathological process implicated in renal disease in FD[62].

5.6.2   Leukocyte adhesion in GD

No differences were seen in monocyte adhesion in GD and only a slight increase in two adhesion molecules (CD11a and CD50) was seen on CD14+ monocytes in GD and CD49d (the α-subunit of VLA-4) and CD62L on lymphocytes. Whilst GD has traditionally been considered a macrophage-centric disorder, recent studies of the bone marrow microenvironment[258] as well as animal studies[60] suggest that abnormalities also occur in other cell lineages. The role of ICAM3 is less well defined than that of ICAM1 and it may be more important in signalling between leukocytes in inflammation than in cellular adhesion. It is important in the activation of both lymphocytes[259] and polymorphonuclear cells (e.g. granulocytes)[260] but has also been implicated in macrophage phagocytosis of leukocytes[261]. VLA-4 is important in the homing of lymphocytes to lymph nodes and bone marrow. It is also implicated in the pathogenesis of myeloma[262] particularly of bone disease by promoting osteoclast activity[263]. The pathogenesis of bone disease in GD and the mechanisms underlying the increased risk of myeloma are poorly understood. The altered pattern of lymphocyte adhesion molecule expression seen in this study may contribute to these disease processes, although no differences were seen in expression of these molecules in GD patients with and without gammopathies or bone disease in this study.

5.6.3   Endothelial adhesion

Reductions in GBA or GLA activity did not alter adhesion capacity or the expression of ICAM1 or VCAM1; the increased ICAM1 seen on leukocytes was not seen in the endothelium. Studies on endothelial cells in FD have suggested Gb3 loading of the endothelium, rather than GLA deficiency up-regulates ICAM1 and VCAM1[64;151]. We did

not replicate this finding which could be for a number of reasons. These studies found increased mRNA levels, but in the study where protein levels were also measured by Western blot[64] no difference was found. Mode of delivery of lipids may also make a difference. *In vivo* glycoproteins predominately circulate complexed to lipoproteins[232] and some studies have delivered lipids by complexing them to albumin, rather than dissolving them in methanol. Lactosylceramide has been found to increase ICAM1 expression on endothelial cells[10], but we did not replicate this finding. Glucosylsphingosine increased RAW adhesion to activated endothelial cells and warrants further investigation.

5.6.4 Experimental limitations

Endothelial cell model

Endothelial cells cover a vast surface area, lining all the blood vessels and lymphatics of the body, developing different characteristics depending on the vascular bed and the stimuli they received. In addition to this heterogeneity, laboratory study of endothelial cells is challenging due to their tendency to de-differentiate and change phenotype in culture, even at a low passage numbers. The use of a HUVEC-carcinoma hybridoma provided a more stable phenotype[184], but the cells do lack some characteristics of HUVEC. Whilst they do express VWF, they do not form characteristic Weibel-Palade bodies; they do up-regulate ICAM1 and VCAM1 in response to TNFα, the key adhesion phenotype being investigated in this study. Additionally HUVEC are macrovascular, being sourced from the umbilical vein, whereas many of the disease manifestations of GD and FD occur in the microcirculation. Sources of microvascular cells are limited, most commonly sourced from the dermis, and have similar phenotypic instability to other endothelial cells.

A further limitation of the disease model was the effects of enzyme inhibition. Whilst CBE almost completely inhibited GBA, it was not possible to achieve a similar level of inhibition for GLA either pharmacologically or using shRNA. Additionally, these methods do not re-capitulate the disease state fully in vitro as there is no mutant enzyme present. The GBA and GLA activity in endothelial cells of patients with GD/FD is not

known and therefore it is uncertain whether sufficient inhibition was achieved. Measurement of intracellular glycosphingolipids (e.g. Gb3) to see if accumulation occurs may be helpful. The most promising strategy to overcome all these limitations would be the culture of primary endothelial cells from GD/FD patients and the relatively new techniques of culture of blood outgrowth endothelial cells and induced pluripotent stem cells may facilitate this.

Leucocyte-endothelial adhesion

Cellular adhesion is a complicated process with multiple modifiers including circulating cytokines and other molecules, blood cells (erythrocytes and platelets as well as leukocytes) and vascular tone. The functional assays in this chapter look at mononuclear cells and endothelial cells in isolation, under conditions that may bear little resemblance to the *in vitro* process. Only the functional adhesion of monocytes was examined although flow cytometry suggested more marked abnormalities within the lymphocytes. Static adhesion assays are commonly used in laboratory settings but the circulation is never static and flow rate is an important modifier of adhesion.

Unavoidably, all GD patients and almost all FD patients had been commenced on ERT prior to entry into the study. Samples were not collected whilst enzyme was being infused and the extent to which ERT might have affected the assay results is uncertain. In GD, where treatment has been shown to be particularly efficacious for the visceral and haematological manifestations of the disease, this may have masked potential correlations with bleeding manifestations. The effect of ERT on bone disease is less well established nor is it known if ERT reduces the risk of myeloma. Recombinant GBA is targeted to cells via the mannose receptor which is expressed on cells of the monocyte-macrophage lineage but not lymphocytes which may explain in part why abnormalities in adhesion molecule expression in GD were most marked in lymphocytes. The efficacy of ERT in FD remains to be established; patients continue to develop new disease complications (e.g. stroke) despite receiving ERT.

5.7 Conclusion

Investigation of leukocyte adhesion revealed an age-dependent increase in monocyte adhesion in females with FD, with correlations with renal and cardiac but not cerebrovascular disease, but the main abnormalities found were in the expression of adhesion molecules on lymphocytes in both GD and FD. In both disorders, increased levels of the α-subunit of LFA-1 and ICAM3 were found with additional increases in ICAM1 in FD and the α-subunit of VLA-4 in GD. These abnormalities may be implicated in organ involvement seen in these disorders, particularly bone disease in GD and renal abnormalities in FD. Further investigation of lymphocytes within GD and FD, both to examine the mechanisms for the increased antigen expression (e.g. cytokine or lipid abnormalities) and to understand the functional impact of these abnormalities may shed new lights on to these disease processes. No abnormality in endothelial function was found in these experiments, but there were marked limitations of the endothelial cell model and further exploration using either microvascular cells or primary endothelial cells from FD patients may be more fruitful.

Chapter 6   Cellular support of coagulation in Fabry and Gaucher

6.1   Introduction

Coagulation encompasses the pathways involved in blood clot formation and dissolution and their regulation. Under normal physiological circumstances, these pathways are tightly regulated so that clot formation is rapid but localised to sites of vessel injury and that, once the vessel wall defect is repaired, clot dissolution occurs and normal vessel patency returns. Abnormalities in any of these haemostatic pathways (procoagulant, anticoagulant or fibrinolytic) can result either in unintended bleeding or inappropriate thrombus formation.

As demonstrated in Chapter 3, bleeding is a common symptom in the presentation of GD and is predominately mucocutaneous in nature. Whilst bleeding maybe contributed to by thrombocytopenia, bleeding manifestations are often out of proportion to the reduction in platelet count. Decreased platelet aggregation in response to low dose agonists has been reported in a number of studies[172-174] but the mechanisms underlying this remain unknown. Coagulation factors have also been shown to be decreased (see **Table 1-11**), with variable decreases in coagulation factors found in different studies, and with abnormalities in anticoagulant pathways and fibrinolysis also being reported. These findings point to a more global dysfunction across the coagulation pathways and it has been hypothesised that chronic activation of coagulation is responsible for this[119].

Conversely, some of the end organ complications of FD have been attributed to inappropriate microthrombus formation. Whilst this is most obvious within the cerebrovascular system, microvascular disease akin to that seen in metabolic disorders such as diabetes and also sickle cell disease, might contribute to development of proteinuria and cardiac conduction defects. These disease manifestations may be present at diagnosis but may also develop following institution of enzyme replacement therapy, particularly as patients age. Investigations of a haemostatic defect within FD to date have predominately focused on the protein C anticoagulant pathway, but increased soluble tissue factor has been found in some studies (see **Table 1-10**).

6.1.1 Purpose of this chapter

This chapter explores the role of cellular components of the blood (monocytes and platelets) and the endothelium in supporting generation of thrombin – the final effector enzyme of the coagulation cascade. As well as converting fibrinogen to fibrin, thrombin activates platelets and also activates the protein C anticoagulant pathway and stimulates release of pro-angiogenic cytokines[264].

Monocyte tissue factor expression and procoagulant activity

Tissue factor (TF) is an important activator of thrombin formation. Intravascular tissue factor is mostly expressed on myeloid cells, particularly monocytes[265]. Much of the tissue factor expressed on the cell surface is thought to be encrypted – in an inactive form[266], with activation (e.g. rise in cytosolic calcium) required for full activation[267]. In this chapter the ability of monocytes to initiate coagulation is investigated by measuring expression of tissue factor on the cell surface and by a procoagulant activity assay to assess the ability of monocytes to initiate plasma coagulation.

The platelet membrane in haemostasis

Following vessel wall injury, macromolecules such as collagen become exposed on the sub-endothelial membrane allowing circulating platelets to adhere. Following adhesion, platelets become activated and clump together (or aggregate) at the site of injury to form the primary platelet plug. Platelet activation also results in exposure of anionic phospholipids, predominately phosphatidylserine, in the outer membrane of the platelet, on which the enzyme complexes of the coagulation cascade form. Thrombin produced by the coagulation cascade acts as an additional platelet activator. In quiescent platelets, phosopholipids are asymmetrically distributed over both leaflets of the plasma membrane, with PS almost exclusively located in the cytoplasmic leaflet. On platelet activation, when intracellular calcium increases, a scramblase, which facilitates movement of phospholipids between the membrane leaflets is activated. Concomitant inhibition of an aminophospholipid translocase prevents movement of surface PS back to the inner leaflet, resulting in accumulation of PS in the outer leaflet. This process is called the platelet

procoagulant response, although the exact pathways leading to its activation are not fully understood.

This fluidity of the phospholipid membrane depends on its lipid composition, including cholesterol content (cholesterol reduces movement), fatty acid composition and saturation of phospholipid. Whether altered glycosphingolipid composition alters this membrane fluidity and activation of the platelet procoagulant response not known. This chapter examines the ability of platelet to support thrombin generation in the presence of patient plasma, using platelet rich plasma and the platelet membrane procoagulant response using a prothrombinase assay in which phospholipid procoagulant activity is the rate-limiting factor for thrombin generation.

The endothelial cell membrane in coagulation

As well as its role in cellular adhesion, the endothelial cell membrane plays an important role in haemostasis, having both anticoagulant and procoagulant properties. Quiescent endothelial cells provide an anticoagulant surface with additional procoagulant properties acquired on activation of the endothelium (e.g. by TNFα). Activation of the anticoagulant protein C pathway takes place on the endothelial cell membrane, where the endothelial protein C receptor (EPCR) is the key receptor regulating the activity of activated protein C. The APC pathway has been implicated in disease processes beyond thrombosis including inflammation[268] and ischaemic stroke[269]. Physiological activation of protein C by thrombin occurs on the surface of endothelial cells, involving two EC membrane receptors: EPCR and thrombomodulin[270]. Thrombin binds to thrombomodulin which promotes activation of PC by thrombin. Localisation of protein C to the EC surface by the EPCR enhances this reaction[271]. APC inactivates FVa and FVIIIa effectively terminating thrombin generation. Abnormalities of the APC pathway have been reported in both GD[119] and FD[152]. This chapter investigates whether endothelial dysfunction as a result of enzyme deficiency and/or lipid loading results in abnormal thrombin generation on the endothelial surface.

6.2 Hypotheses

1. Monocytes from FD and GD have increased tissue factor expression and are procoagulant. *Rationale*: tissue factor expression is induced by pro-inflammatory cytokines. FD is associated with a procoagulant state and laboratory studies in GD suggest that chronic activation of coagulation occurs.

2. The platelet membrane in FD and GD has altered procoagulant capacity. *Rationale*: glycosphingolipid accumulation within lipid rafts in the platelet membrane may also membrane dynamics required for assembly of coagulation enzyme complexes (e.g. the prothrombinase complex).

3. The endothelium in FD is procoagulant and supports increased thrombin generation. *Rationale*: microvascular lesions occur in FD and have been hypothesised to be due to impaired regulation of the procoagulant pathway.

6.3 Methods and assay development

6.3.1 Monocyte tissue factor procoagulant activity

Monocytes express tissue factor on the cell surface, which is present at low levels in un-stimulated cells. This tissue factor can be used to initiate coagulation of citrated plasma following re-calcification. A coagulation assay, sensitive to very low levels of tissue factor was developed whereby tissue factor is the rate-limiting factor and the source of the tissue factor is added cells; the methodology is detailed in 2.11.1. This assay assesses the ability of cells to support thrombin generation via TF/FVIIa initiated coagulation. The assay uses a KC4 coagulometer with a mechanical end point. The reaction occurs in a cuvette containing a metal ball-bearing. When the reaction mixture is fluid, a magnet holds the ball-bearing on its base point whilst the cuvette rotates. As a fibrin clot forms, the fibrin stops the ball-bearing moving and it is moved away from the magnet as the cuvette rotates; this loss of contact stops the clock and gives the coagulation time.

Assay development and validity

A standard curve was constructed using serial dilutions of a thromboplastin (Recombiplastin, Diapharma) to initiate coagulation. Serial dilutions, down to a dilution of 1 in 64000 of Recombiplastin were made, using Tris-HCl-1%BSA buffer as the diluents, demonstrating the sensitivity of the assay to small amounts of tissue factor (see **Figure 6-1**). A standard curve was constructed with a 1 in 1000 dilution of Recombiplastin being allocated an arbitrary value of 100 units of tissue factor.

Increasing concentrations of monocytes resulted in a decrease in the coagulation time, consistent with an increased amount of a procoagulant factor being present on the cells. Serial dilutions of RAW 274.1 did not initiate coagulation; whilst murine monocytes express tissue factor, murine tissue factor is unable to activate human FVII. This demonstrates that it is a specific factor present on human monocytes which activates coagulation rather than just the presence of a large number of cells. From the monocyte dilutions, a concentration of 1×10^5 per cuvette of monocytes was chosen for the assay, being on the slope of the curve.

Surface expression of tissue factor was measured using flow cytometry, with the same gating strategy as described in 5.4.2. This confirmed that tissue factor was only expressed on CD14+ monocytes and the mean fluorescent intensity of the bound tissue factor antibody was measured.
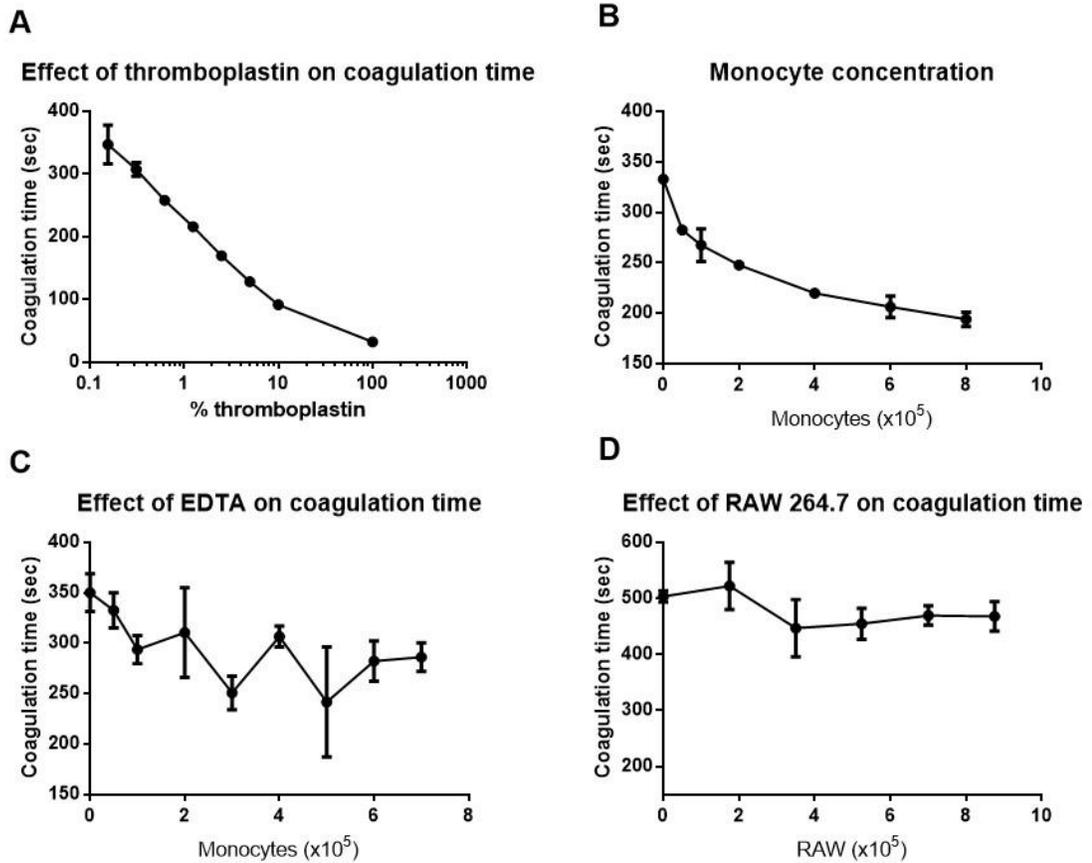
Figure 6-1: Development of tissue factor procoagulant activity. A: Effect of concentration of recombiplastin on coagulation time; 100% represents a 1:100 dilution of recombiplastin; B: Effect of monocyte concentration on coagulation time. C:Effect of use of EDTA for blood collection on coagulation time. D: Effect of RAW 264.7 cells on coagulation time.
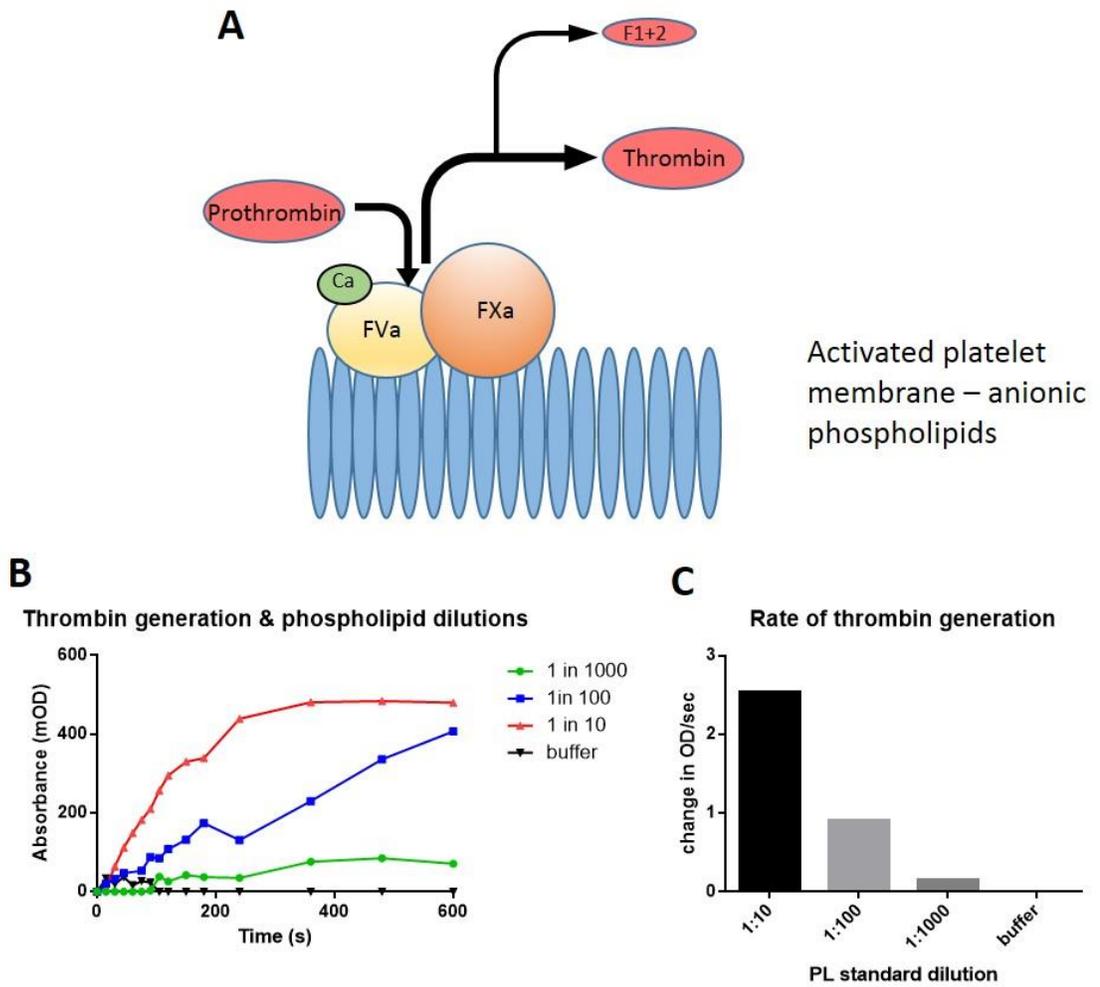
Figure 6-2: The prothrombinase assay. A: the prothrombinase complex which forms on the surface of activated platelets, converting prothrombin to thrombin. B & C: when FXa, FVa, calcium and prothrombin are added in excess, the rate of thrombin generation is dependent on the amount of phospholipid reagent added.

6.3.2 Platelet assays

Patients who were known to be taking anti-platelet agents (e.g. aspirin, ibuprofen) were excluded from these assays. Citrated whole blood was collected and centrifuged to yield platelet rich plasma (PRP). As well as measuring the platelet count for the assays, the platelet size parameters were recorded: mean platelet volume (MPV), platelet distribution width (PDW) and platelet large cell ratio, see **Figure 6-5**.

6.3.3 Platelet rich plasma thrombin generation

Thrombin generation assays, using a calibrated automated thrombogram were performed using standard methodology as described in 2.11.2. Thrombin generation is considered a global haemostatic assay which is affected by both the procoagulant and anticoagulant potential of a sample by measuring real time thrombin generation[272]. The area under the curve gives the total amount of thrombin generated (endogenous thrombin potential) and the peak thrombin, the maximum thrombin at any time, are recorded. Two time parameters are also measured: the lag time – the time period before measurable thrombin is generated and the time to reach peak thrombin generation.

Utilising PRP, the platelets provide the phospholipid surface required for thrombin generation whilst the plasma contains the necessary coagulation factors with the reaction being initiated by the addition of 1pM tissue factor and calcium ions. PRP thrombin generation is reduced in patients with platelet function defects and has been found to be increased in over half of patients following a stroke[273]. In this study PRP thrombin generation was performed to see if there was a qualitative difference in the ability of platelet to support thrombin generation. Therefore the platelet count was adjusted to be identical in all samples using platelet poor plasma. Platelet counts of $150x10^9$/ml and $100x10^9$/L were selected to see if thrombin generation was impaired at lower platelet counts as encountered in GD.

6.3.4 Prothrombinase activity

The prothrombinase assay is designed to assay the procoagulant activity of the platelet membrane. The prothrombinase complex consists of the serine protease FXa and its

cofactor FVa and calcium ions assembled on the phospholipid membrane of platelets and converts prothrombin to thrombin (see **Figure 6-2**). When FXa, FVa, calcium and prothrombin are added in excess, phospholipid becomes the rate-limiting factor. This was confirmed using a phospholipid reagent, Bell and Alton platelet substitute at a range of concentrations and the full method is described in 2.11.4.

To remove plasma coagulation factors, platelets from PRP were washed, using an acidified buffer to prevent platelet activation[274], and then re-suspended in Tris-HCl-0.1% BSA buffer at concentrations of 100×10^9/L and 50×10^9/L, selected to ensure that the platelet phospholipid membrane was the rate-limiting factor of the reaction (confirmed in **Figure 6-7**).

6.3.5 Endothelial thrombin generation

The EAhy endothelial model described in Chapter 5 was used to investigate thrombin generation on the endothelial surface. This cell line has previously been used to study endothelial-dependent thrombin generation using the calibrated automated thrombogram[275]. Under basal conditions EAhy cells express thrombomodulin and EPCR and activate protein C[276]; they also express low levels of tissue factor following TNFα stimulation[277]. Increased levels of tissue factor serve to increase the rate of thrombin generation (increasing the amount of thrombin generated and speeding up the time course over which this occurs) whilst increased activation of APC has the opposite effect.

Assay development and validity

Under basal conditions, thrombin generation occurs slowly with a small endogenous thrombin potential and peak thrombin. Activation of EAhy cells by TNFα increases thrombin generation, almost halving the lag time and doubling the endogenous thrombin potential (see **Figure 6-3**), supporting the presence of an inducible procoagulant response by endothelial cells. There was little difference in thrombin generation over the range of doses of TNFα assessed in control studies suggesting that this procoagulant response is induced by relatively low concentrations of TNFα.

To assess the effect of excess glycosphingolipids and reduced enzyme activity on thrombin generation, cells (either normal EAhy or cells treated with CBE 100µM/ shRNA targeting GLA) were incubated with 10µM concentrations of lipids for seven days. Lipids that are implicated in each disease were utilised: FD – S1P, Gb3, lysoGb3; GD – S1P, GSP and LC as described in 5.4.4
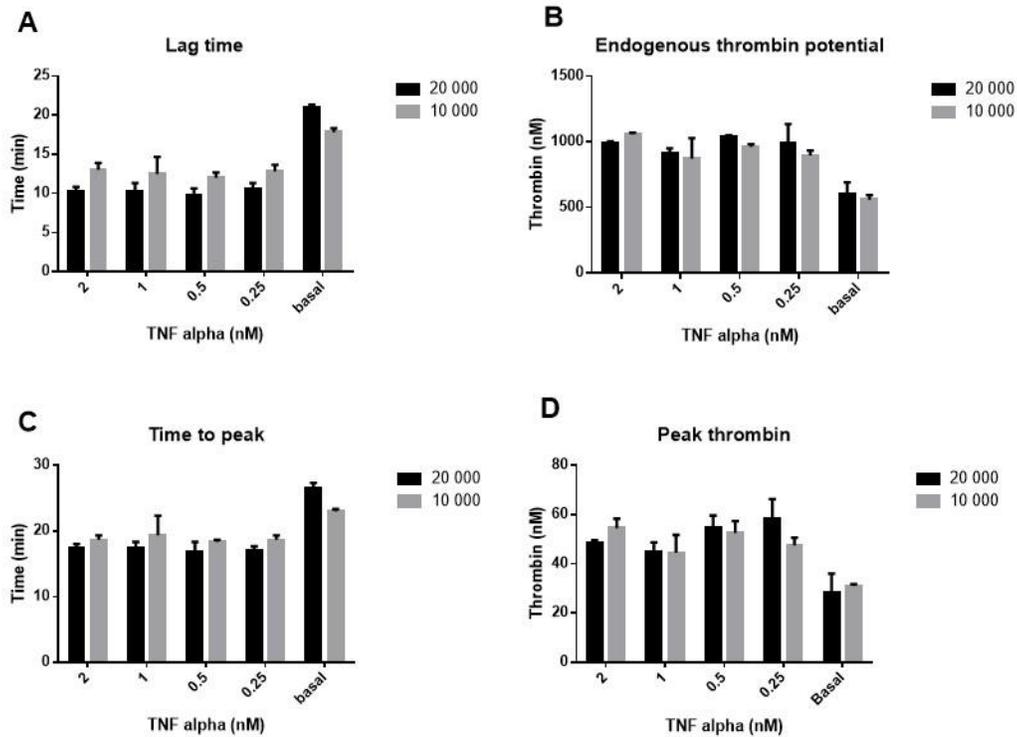
Figure 6-3: Effect of TNFα and cell concentration on thrombin generation. Cells were seeded at 20 000 or 10 000 per well and cultured for 7 days. They were activated with a range of concentrations of TNFα for 6 hours and thrombin generation measured.

6.4 Results

6.4.1 Monocytes and tissue factor

The monocyte tissue factor assays were performed on the same samples as the monocyte adhesion assays and the patient characteristics are detailed in **Table 5-3**. The mean tissue factor expression and activity of the different patient populations is shown in **Table 6-1**.

Table 6-1: Monocyte tissue factor expression and activity

	Control	Gaucher	Fabry
No. patients	21	20	29
TF MFI	68.72 ±41.14	94.18 ±61.68	74.26 ±51.59
TF activity (arbitrary units TF)	12.70 ±5.38	10.26 ±5.03	12.23 ±6.67

There was no difference in tissue factor expression, as measured by median fluorescent intensity, on monocytes between patients with GD, FD or healthy controls ($p = 0.25$). Two patients with GD and one patient with FD had much higher MFI than the others, for reasons that are unclear. The two GD patients are brothers, one of whom presented with pancytopenia, splenomegaly and a paraprotein with a past history of avascular necrosis. The other brother was diagnosed following the diagnosis in his sibling and does not have bone infarcts or a paraprotein; both are receiving ERT. The main disease manifestations in the FD patient relate to renal disease, but two other patients with lower GFRs had tissue factor MFI in keeping with those of the rest of the cohort. All three patients had no history of thrombotic events (arterial or venous), a normal coagulation screen and normal D-dimers; none of these had increased tissue factor procoagulant activity.

There was no difference in the TF procoagulant activity of monocytes between different patient populations ($p = 0.31$). There was no correlation between TF procoagulant activity and TF expression on monocytes (see **Figure 6-4**).
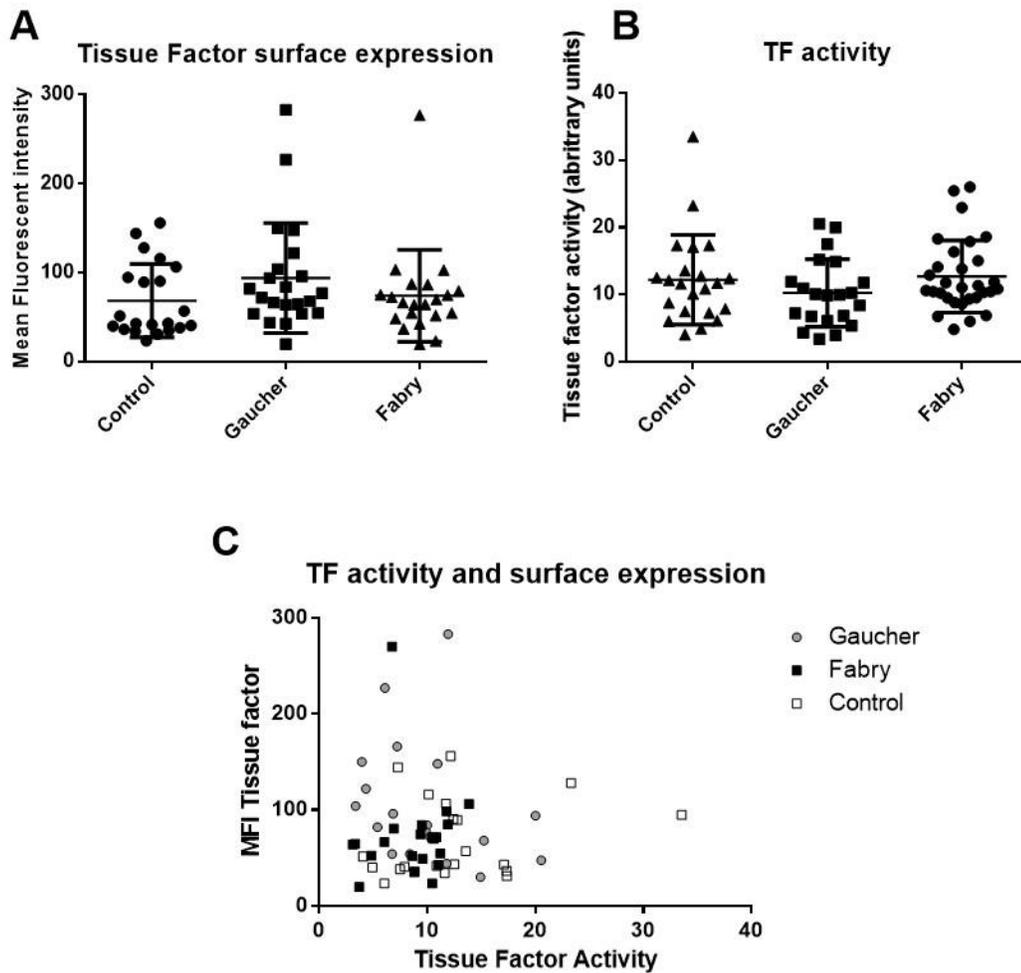
Figure 6-4: Monocytes and tissue factor. A: surface tissue factor expression. B: tissue factor coagulation activity. C: relationship between tissue factor expression and measured activity.

6.4.2 Platelets

The demographics of the patients recruited for the platelet studies are shown in **Table 6-2**; all patients were receiving ERT. Both PRP thrombin generation and the prothrombinase assay were performed on each sample. There were no differences in platelet size parameters between the patients groups (see **Figure 6-5**).

Table 6-2: Patients recruited to platelet studies and platelet parameters (mean ±SD)

	Control	Gaucher	Fabry
No.	8	7	8
Age (years)	33.0 (22-52)	46.0 (22-63)	42.0 (24-53)
M:F	4:4	3:4	5:3
MPV (fl)	9.91 (±0.48)	10.3 (±1.02)	9.67 (±0.79)
PDW (fl)	10.02 (±0.91)	11.94 (±2.12)	10.30 (±1.65)
P-LCR (%)	23.66 (±4.10)	27.83 (±8.71)	21.21 (±7.49)

Platelet rich plasma thrombin generation

The results of PRP thrombin generation are shown in **Table 6-3** . There was no difference in thrombin generation parameters between the three patient groups (see **Figure 6-6**). The lag time was longer in FD patients, which was of borderline significance, possibly due to the small sample size, which would suggest initiation of thrombin generation was slightly delayed in these patients but without alterations in the total amount of thrombin generated.

Table 6-3: PRP thrombin generation parameters (mean±SD)

Parameter	Plt (x10^9/L)	Control	Gaucher	Fabry	One-way ANOVA p value
Endogenous thrombin potential (nM)	150	1395±591	1507±319.1	1727±516	0.41
	100	1575±560	1247±267.8	1354±541.6	0.87
Peak thrombin (nM)	150	88.46±36.86	108.5±88.5	119.9±56.1	0.36
	100	94.96±33.45	84.84±14.33	117.8±77.8	0.47
Lag time (min)	150	9.23±2.24	8.78±2.71	11.62 ±2.47	0.08
	100	9.50±2.42	8.56±3.68	12.32±4.0	0.12
Time to peak (min)	150	18.93±4.16	17.88±4.86	19.12±3.73	0.84
	100	19.08±4.70	18.19±6.97	20.63±3.35	0.68

Prothrombinase activity

The results of the prothrombinase assay are shown in **Table 6-4** and **Figure 6-7**; as the results were not normally distributed the results are expressed as median with the 25-75 percentile range stated; Mann-Whitney U tests were performed to establish the significance of differences between GD/FD groups and controls. Across all parameters and platelet counts, the median prothrombinase activity was higher in FD patients than in controls or GD. There was an increase in the rate of thrombin generation of borderline significance at both a platelet count of $100 \times 10^9/L$ (p = 0.083) and $50 \times 10^9/L$ (p = 0.049). The increase in amount of thrombin generated was significant at a platelet count of $100 \times 10^9/L$ (p = 0.01) but not $50 \times 10^9/L$ (p = 0.43).

Table 6-4: Prothrombinase assay results (median and 25-75% percentiles)

Parameter	Platelet count ($x10^9$/L)	Control	Gaucher	Fabry
Rate of thrombin generation (ΔOD/sec)	100	1.24 (0.72-1.84)	1.26 (0.87-1.81)	2.27 (1.74-3.24)
	50	0.80 (0.58-0.94)	0.59 (0.45-0.82)	1.04 (0.76-1.68)
	Ratio 100:50	1.88 (1.33-2.27)	2.14 (1.95-2.35)	1.84 (1.54-2.76)
Maximum thrombin generated (OD)	100	522.5 (264.3-642.8)	508.0 (357.0-537.0)	690.0 (621.3-801.5)
	50	330.0 (236.3-426.8)	242.0 (172.0-281.0)	373.5 (336-572.0)
	Ratio 100:50	1.39 (1.10-1.80)	1.81 (1.29-2.86)	1.63 (1.10-2.05)
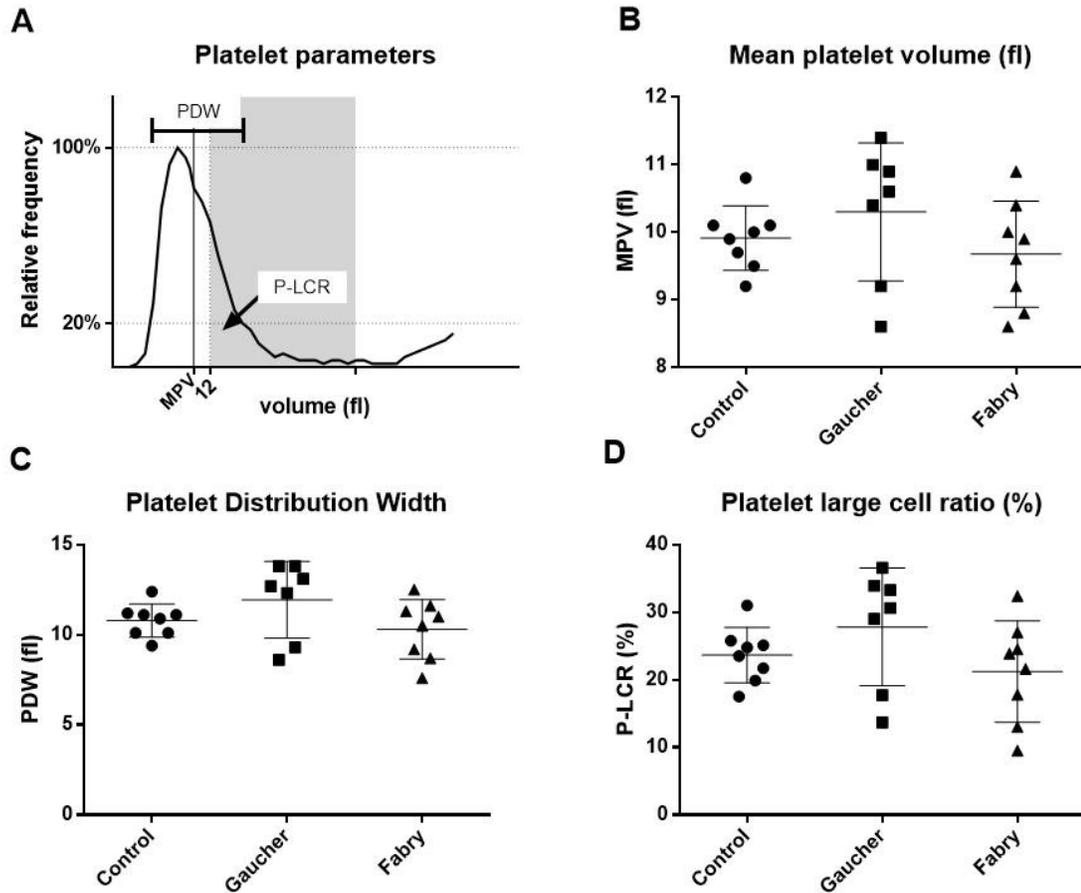
Figure 6-5: Platelet parameters of PRP. A: diagram indicating how platelet distribution width (PDW) and platelet large cell ratio (P-LCR) are derived. B: mean platelet volume; C: PDW; D: platelet LCR.
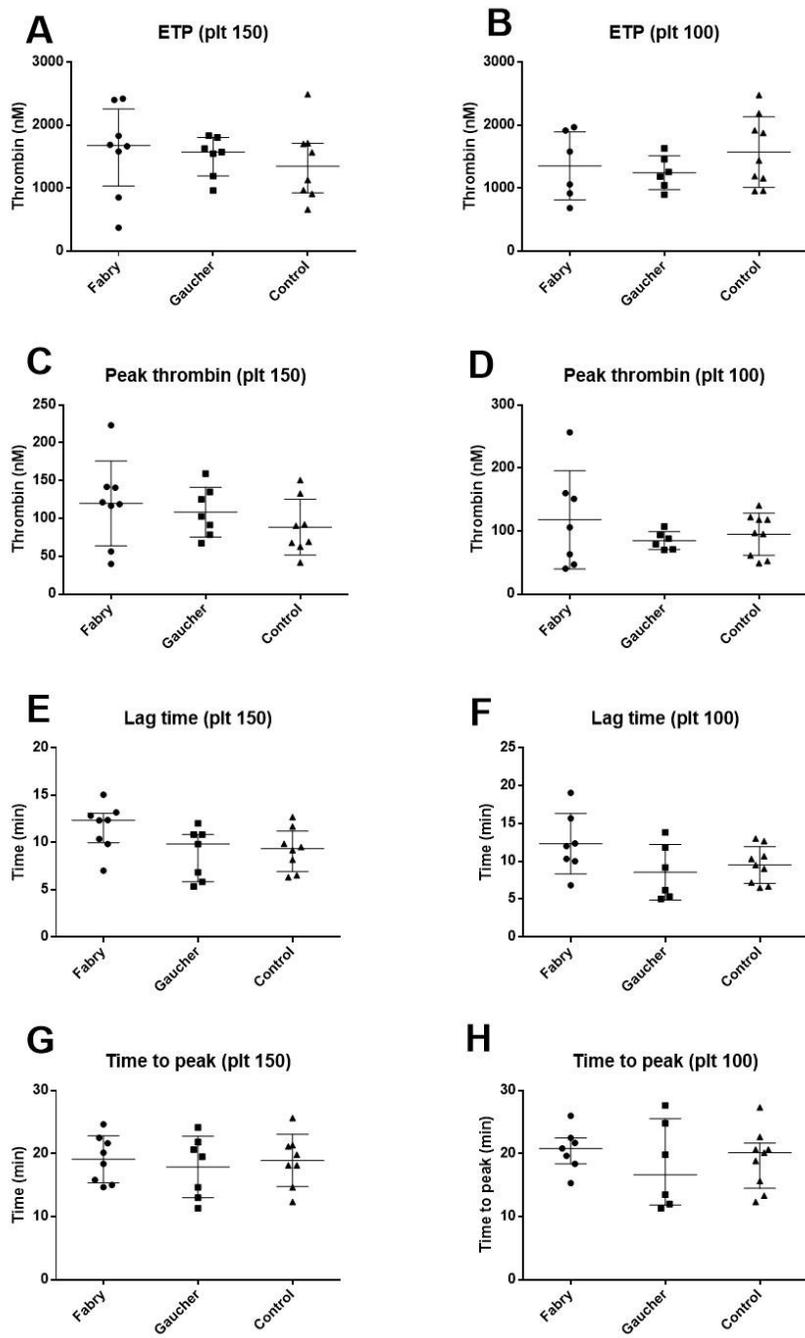
Figure 6-6: Thrombin generation of PRP. Activated with Innovin at platelet counts 150×10^9/ml and 100×10^9/ml. Endogenous thrombin potential (A,B), peak thrombin (C,D), lag time (E,F) and time to peak (G,H).
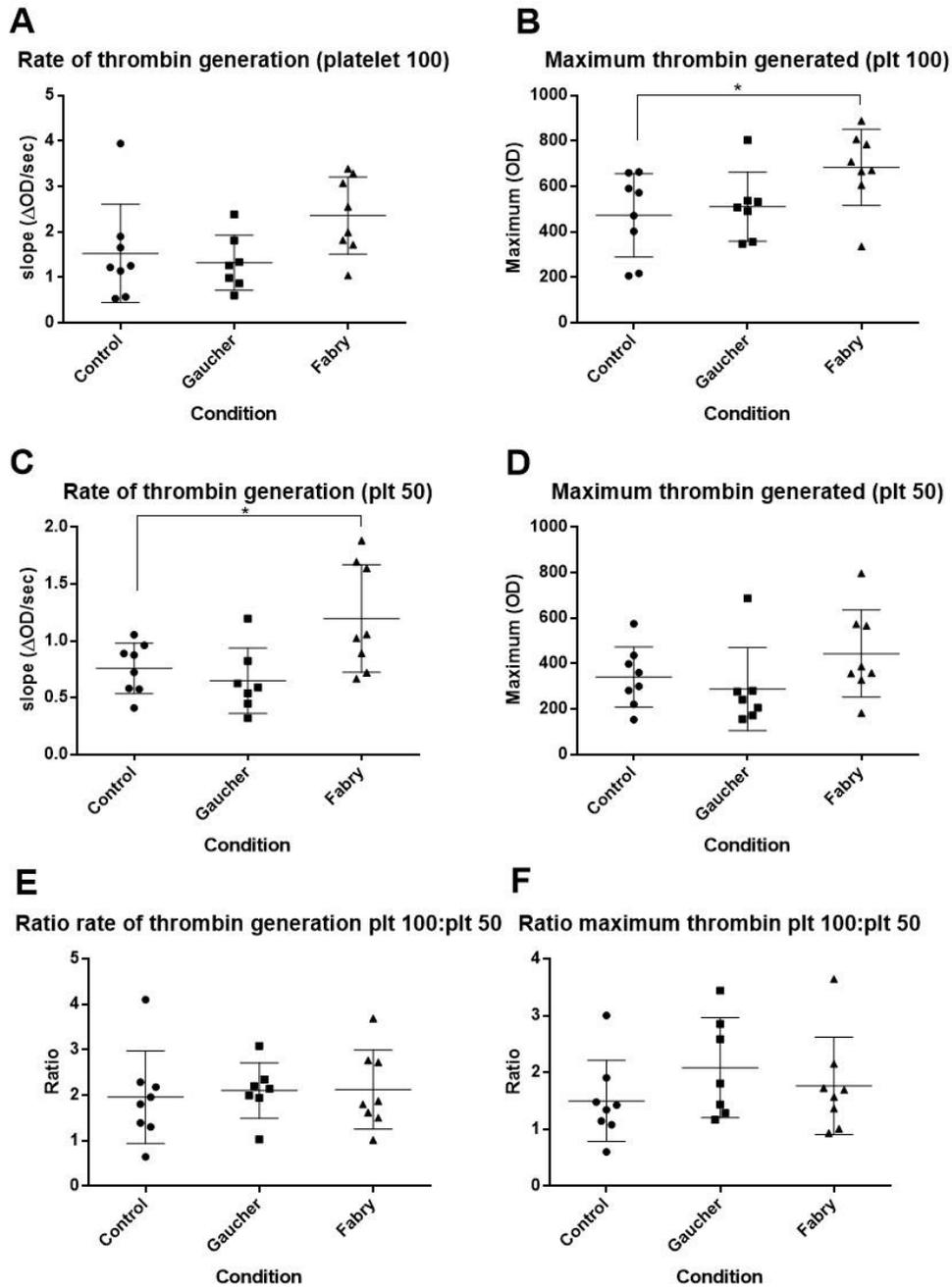
Figure 6-7: Platelet prothrombinase activity. Measured at platelet counts of 100×10^9/ml and 50×10^9/ml. Rate of thrombin generation (A,C) and maximum thrombin generation (B,D). Platelet concentration dependence of both rate (E) and maximum thrombin generated.

6.4.3 Endothelial cell thrombin generation

Gaucher endothelial model

To establish whether enzyme deficiency, glycosphingolipid excess or the presence of both conditions alters the ability of endothelial cells to support thrombin generation in GD, cells were cultured with specific glycosphingolipids (S1P, GSP and LacCer) in the presence or absence of CBE. The results are shown in **Table 6-5** and **Figure 6-8**; due to the multiple comparisons performed a Bonferroni correction was applied so that results are considered significant if $P<0.005$. Addition of glycosphingolipids to untreated EA.hy cells did not alter their ability to support thrombin generation. Lipid loading with glucosylsphingosine of cells treated with CBE increased thrombin generation, particularly after TNFα activation – increasing the ETP and peak thrombin and the speed of thrombin generation. Sphingosine-1-phosphate loading also had a similar effect on TNFα activated, CBE treated cells. Lipid loading with lactosylceramide of CBE treated cells did not alter thrombin generation, except for an increase in peak thrombin after TNFα activation, although this increase was less than that seen with GSP or LacCer.

Table 6-5: Endothelial cell thrombin generation - Gaucher cell model

Measured parameter	EAhy activation	Lipid addition	WT	CBE	P values (t test)		
			Mean ±SD	Mean ±SD	Lipid vs. vehicle (WT)	Lipid vs. vehicle (CBE)	CBE vs. WT
ETP (nM)	Basal	Vehicle	255±50	222.5±38.5	-	-	0.42
		S1P	165±31	214.5±0.5	0.06	0.74	0.06
		GSP	349±17.5	331.0±11	0.04	0.009	0.20
		LacC	210.5±53.5	176±83	0.35	0.43	0.58
	TNFα	Vehicle	477.5±9.5	522.5±34.5	-	-	0.18
		S1P	621±12.5	730±46	0.006	0.003	0.06
		GSP	730±56	699.5±16.5	0.024	0.001	0.41
		LacC	638.5±67.5	542±26	0.079	0.47	0.10
Peak thrombin	Basal	Vehicle	16.73±2.51	15.45±2.43	-	-	
		S1P	11.6±1.81	13.86±0.27	0.05	0.32	0.56
		GSP	23.62±2.38	26.46±3.48	0.026	0.01	0.10
		LacC	14.23±3.42	11.03±5.5	0.37	0.27	0.31
	TNFα	Vehicle	32.49±4.46	33.30±1.04	-	-	0.44
		S1P	44.08±4.18	51.30±1.71	0.030	<0.0001	0.77
		GSP	44.02±5.47	56.82±0.15	0.047	<0.0001	0.05
		LacC	43.42±2.77	43.52±0.13	0.022	<0.0001	0.95
Lag time	Basal	Vehicle	38.5±4.83	36.5±1.83	-	-	0.53
		S1P	44.5±3.17	35.83±0.17	0.15	0.56	0.0091
		GSP	24.67±2.83	26.33±1.67	0.01	0.002	0.43
		LacC	43.33±3.33	29.00±2.33	0.23	0.11	0.0036
	TNFα	Vehicle	9.50±0.83	8.00±0.33	-	-	0.04
		S1P	7.67±0.67	6.50±0.17	0.04	0.002	0.04
		GSP	8.17±0.5	7.67±0.33	0.08	0.29	0.22
		LacC	7.67±1.67	7.50±0.17	0.16	0.08	0.87
Time to peak	Basal	Vehicle	45.17±4.5	42.67±1.67	-	-	0.42
		S1P	52.17±2.83	43.0±0.67	0.08	0.77	0.006
		GSP	31.33±2.50	31.83±0.5	0.01	0.0004	0.75
		LacC	51.0±3.33	35.67±4.0	0.14	0.05	0.007
	TNFα	Vehicle	16.67±1	14.67±0.33	-	-	0.03
		S1P	14.33±0.5	13.0±0.33	0.02	0.003	0.02
		GSP	15.17±0.5	13.17±0.17	0.08	0.002	0.002
		LacC	14.0±2.33	13.33±0.33	0.14	0.008	0.65
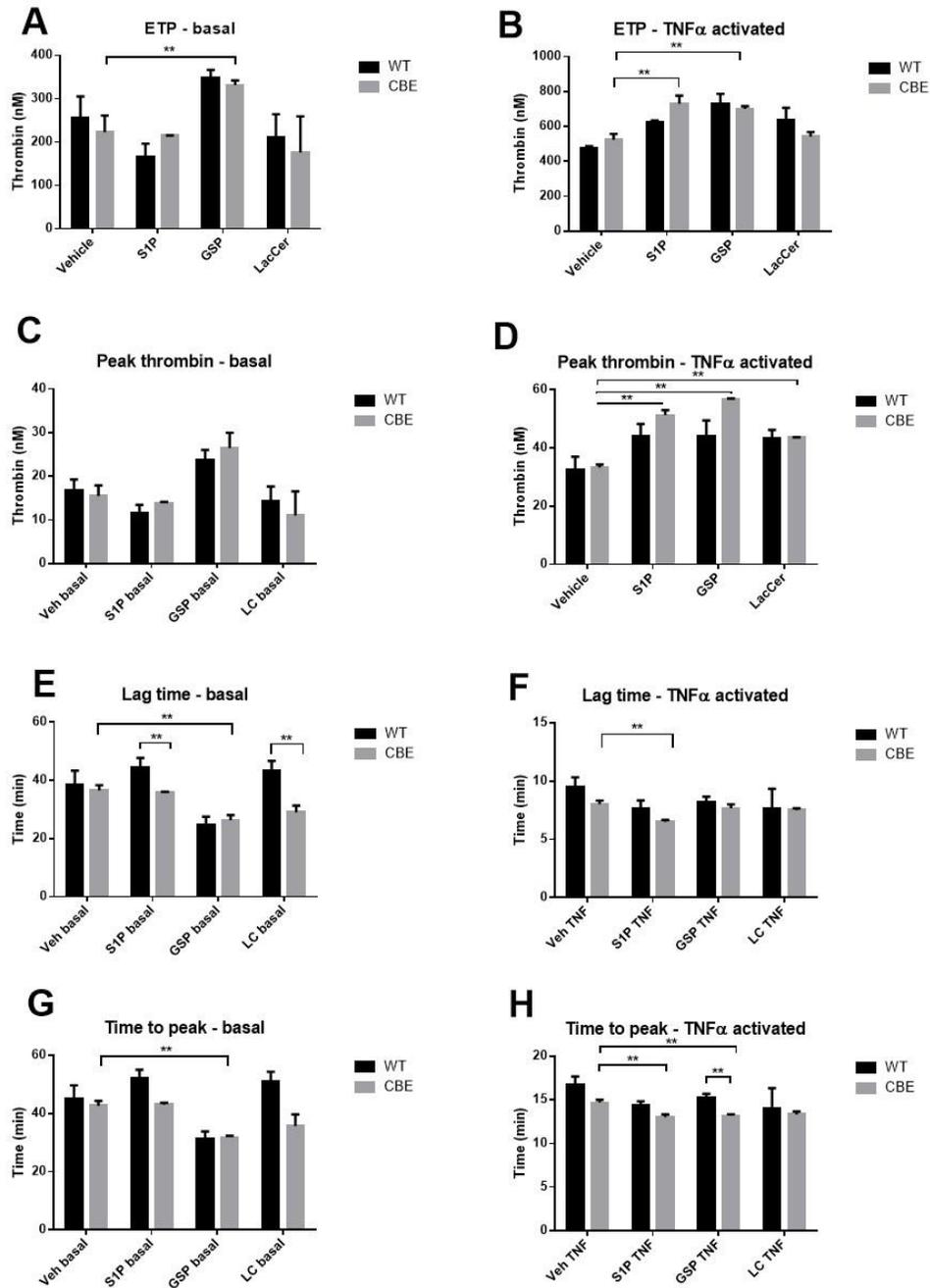
Figure 6-8: Thrombin generation – GD endothelial cell model. Effect of the addition of S1P, GSP or LC to EA.hy, either wild-type or treated with 100μM CBE for 7 days.
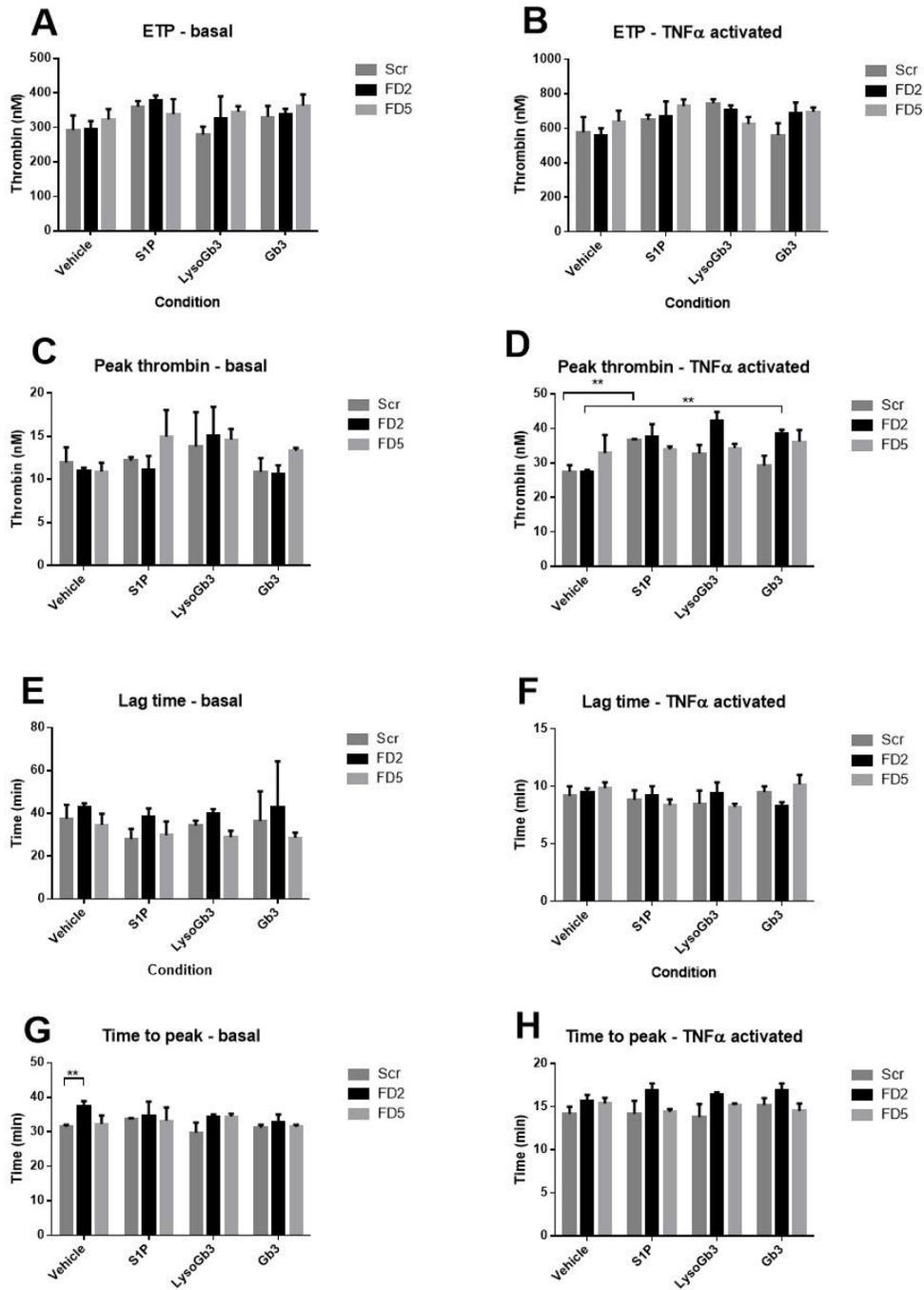
Figure 6-9: Thrombin generation – FD endothelial cell model. Effect of the addition of S1P, lysoGb3 or Gb3 to EAhy transfected with shRNA targeting the *GLA* gene (FD2 and FD5) or non-targeting control (Scr) for 7 days.

219

Fabry endothelial model

Endothelial cells containing shRNA either targeting the GLA gene or a scrambled, non-targeting control were cultured for seven days in the presence/absence of glycosphingolipids (S1P, Gb3 and lysoGb3). Neither decreased GLA activity nor glycosphingolipid loading altered the ability of endothelial cells to support thrombin generation (see **Table 6-6** and **Figure 6-9**). Peak thrombin was increased in FD2 cells loaded with Gb3 compared to those cultured with methanol vehicle only, but there were no differences in other thrombin generation parameters or in the FD5 cells.

Table 6-6: Fabry endothelial cell model thrombin generation

Parameter	EAhy activation	Lipid	Scr	FD2	FD5	P values				
			mean±SD	mean±SD	mean±SD	Lipid vs. veh Scr	Lipid vs. veh FD2	Lipid vs. veh FD5	FD2 vs. Scr	FD5 vs. Scr
ETP (nM)	Basal	Vehicle	292.5±42.1	295.0±23.5	323.5±29.75	-	-	-	0.93	0.36
		S1P	360.0±16.8	377.5±15.3	339±43.1	0.06	0.007	0.64	0.25	0.48
		LysoGb3	279.0±23.4	327.1±63.5	345.5±16.2	0.65	0.46	0.32	0.28	0.02
		Gb3	330.5±32.1	338.0±16.2	363.5±32.4	0.28	0.06	0.19	0.74	0.28
	TNFα	Vehicle	577.0±89.5	559.0±41.5	640.5±62.1	-	-	-	0.77	0.37
		S1P	649.0±30	670.5±85.5	729.0±38.5	0.26	0.11	0.10	0.70	0.05
		LysoGb3	746±22.5	707.0±27	614.5±42.0	0.03	0.007	0.73	0.13	0.02
		Gb3	561.0±69.5	688±62.5	696.0±25	0.81	0.04	0.22	0.08	0.03
Peak thrombin	Basal	Vehicle	11.97±1.75	10.96±0.39	10.91±1.01	-	-	-	0.38	0.41
		S1P	12.20±0.4	11.09±1.63	14.98±3.07	0.83	0.89	0.09	0.32	0.19
		LysoGb3	13.83±3.96	15.07±3.36	14.63±1.23	0.50	0.10	0.01	0.70	0.76
		Gb3	10.88±1.59	10.58±1.06	13.32±0.34	0.46	0.59	0.02	0.80	0.06
	TNFα	Vehicle		27.39±0.65	32.90±5.24	-	-	-	0.93	0.17
		S1P		37.64±3.61	33.99±0.83	**0.001**	0.008	0.74	0.68	0.005
		LysoGb3		42.21±2.63	34.32±1.32	0.044	0.0007	0.67	0.009	0.39
		Gb3		38.58±1.06	36.24±3.32	0.43	**<0.0001**	0.40	0.31	0.05
Lag time	Basal	Vehicle	37.5±6.5	43.17±1.5	34.67±5.24	-	-	-	0.21	0.59
		S1P	28.0±4.76	38.67±3.67	29.83±6.5	0.11	0.12	0.37	0.04	0.71
		LysoGb3	34.33±2.33	39.71±2.33	28.88±3.0	0.47	0.09	0.17	0.05	0.07
		Gb3	36.5±13.83	42.67±21.67	28.67±2.33	0.92	0.97	0.14	0.70	0.39
	TNFα	Vehicle	9.17±0.83	9.52±0.3	9.86±0.5	-	-	-	0.52	0.29
		S1P	8.83±0.83	9.19±0.83	8.37±0.5	0.64	0.55	0.02	0.62	0.46
		lysoGb3	8.5±1.13	9.36±1	8.19±0.3	0.45	0.80	0.008	0.38	0.67
		Gb3	9.5±0.5	8.33±0.3	10.17±0.83	0.59	0.08	0.61	0.026	0.30
Time to peak	Basal	Vehicle	31.39±0.65	37.39±1.5	32.23±2.51	-	-	-	**0.003**	0.60
		S1P	33.71±0.23	34.72±4.01	33.22±3.84	0.004	0.34	0.73	0.69	0.84
		LysoGb3	29.67±3	34.39±0.67	34.39±0.84	0.39	0.03	0.23	0.06	0.06
		Gb3	31.13±0.83	32.88±2.17	31.39±0.67	0.74	0.04	0.61	0.27	0.74
	TNFα	Vehicle	14.17±0.83	15.70±0.67	15.37±0.67	-	-	-	0.07	0.12
		S1P	14.17±1.50	16.87±0.84	14.39±0.33	0.99	0.13	0.09	0.05	0.82
		LysoGb3	13.80±1.5	16.37±0.3	15.20±0.17	0.73	0.19	0.69	0.04	0.18
		Gb3	15.17±0.83	16.87±0.84	14.53±0.84	0.21	0.13	0.25	0.06	0.40

6.5   Discussion

6.5.1   Monocyte procoagulant activity

No differences were found in tissue factor expression or procoagulant activity in patients with GD or FD compared to controls. Tissue factor expression has not previously been studied in GD. Whilst plasma coagulation abnormalities are common at baseline, as shown in Chapter 3, they improved markedly following enzyme replacement therapy. The patients studied were all receiving ERT and therefore it may be possible that any abnormalities in monocyte procoagulant activity that are present in untreated disease respond to ERT. Increased plasma soluble tissue factor was found in two studies of FD patients [88;170] but only in males and was associated with renal failure.

Assay limitations

There was no association found between tissue factor expression and monocyte procoagulant activity – which may be due to limitations of both the methodological approach and the procoagulant assay itself. Freshly isolated monocytes were used for the procoagulant assay, which represent steady-state circulating monocytes. Membrane tissue factor is predominately in an encrypted, inactive form. Monocyte TF activation is induced by pro-inflammatory stimuli[278] and therefore the procoagulant activity of circulating steady-state monocytes, is likely to be much less than their tissue factor expression would suggest. Further assays could potentially be performed, activating monocytes with cytokines to assess if there were differences in TF activation between patient populations – although the no difference in surface expression of TF was found.

The procoagulant activity assay may also be too insensitive to detect differences in procoagulant activity of steady-state monocytes. The assay end point relies on fibrin clot formation and although the assay was sensitive to very low concentrations of thromboplastin, monocyte support for coagulation is more complex. As well as TF expression, monocytes can also support thrombin generation by direct activation of FXa. FX binds to the Mac-1 receptor (CD11b/CD18), triggering release of cathespin G which

directly activates FX to FXa. Additionally, monocytes express tissue factor pathway inhibitor which binds and inactivates TF.

6.5.2 Platelet support for thrombin generation

There were no significant differences in platelet size parameters from which it was concluded that a similar amount of platelet phospholipid was available in sample for the functional platelet assays. There was no difference in thrombin generation in PRP between the patients group, although there was considerable variation within all groups. The prothrombinase assay suggests that the procoagulant response may be increased in patients with Fabry disease in the small group of patients assessed. Gb3 is synthesised by megakaryocytes and found in the membrane of platelets[279]. Whether platelet membrane lipid composition is altered in platelets is not known and warrants further investigation. An increase in procoagulant activity should be confirmed on a larger sample size and by additional assessment by a tenase activity – assessing the ability of platelets to support FXa generation which is also dependent on phosphatidylserine exposure.

Assay limitations

The study of platelet function presents a number of challenges. Platelet function can be affected by a wide range of drugs, especially aspirin[280]; this poses particular problems in the study of FD patients, many of whom take aspirin or other cardiovascular medications which can affect platelet function. This limits the patients who can be studied and may bias towards more mildly affected patients. Whilst attempts were made to exclude patients taking medications known to affect platelet function, it is possible that food substances or over the counter medication may have been taken which would affect platelet function. The manual sub-sampling method of the prothrombinase assay also limits the number of samples which can be tested on any one occasion.

6.5.3 Endothelial cell thrombin generation

Neither decreased GBA nor GLA activity on its own resulted in changes in thrombin generation. Lipid loading of cells treated with CBE to inhibit GBA resulted in increased thrombin generation when cells were loaded glucosylsphingosine or sphingosine-1-

phopshate, but only after endothelial activation of TNFα; no effect was seen with lactosylceramide. Previous studies of the effect of glycosphingolipids on thrombin generation are limited. A single study of platelets found that GSP inhibited platelet thrombin generation whilst S1P had no effect, but this study only incubated the platelets with the lipids for 5 minutes[281]. The fact that thrombin generation is enhanced only in the presence of both lipid loading and CBE suggests that impaired lipid degradation is required for this effect and that this creates an environment in which the procoagulant properties of activated endothelial cells are enhanced. This could be further investigated by investigating different aspects of thrombin generation in isolation (e.g using FXII deficient plasma to look at contact activation or an anti-TF antibody to look at the effect of tissue factor) to see if increased thrombin generation could be attributed to one part of haemostasis.

Assay limitations

The general limitations of the endothelial cell model have been discussed in 5.6.4. Whilst EA.hy cells have been used to model thrombin generation and to detect abnormalities in contact activation[275] and the protein C anticoagulant pathway[282], being a hybridoma of HUVEC and lung adenocarcinoma they are likely to be difference in the haemostatic properties between these cells and the plethora of endothelial cells found *in vivo*. In particular, further investigation utilising microvascular endothelial cells, likely to be the sites of inflammation within GD, may provide more physiologically relevant information.

6.6   Conclusions

No differences were found in tissue factor expression or the procoagulant activity of monocytes from patients with GD or FD, suggesting that monocyte tissue factor is unlikely to be important in vascular disease manifestations in these disorders. FD but not GD is associated with an enhanced procoagulant response on the platelet membrane in the small sample studied to date. This may be of particular importance as many of the vascular features of FD are thought to arise in the microvasculature where platelets play an important role in coagulation. The potential contribution of platelets to the disease manifestations of FD is not well understood but clinically important. Should the finding

of increased procoagulant response be confirmed in further studies then the next question would be whether this is modified by anti-platelet agents and, by implication, whether there is evidence to support their use in patients with FD as either primary or secondary prophylaxis.

Increased thrombin generation by Gaucher-like endothelial cells following their activation would be consistent with the hypotheses from other studies that coagulation abnormalities are due consumptive consequent on chronic activation of coagulation[119] and that some disease manifestations might be due to microvascular thrombosis[183].

Chapter 7   Discussion

This project has established the importance of vascular events in the pathology of both GD and FD and undertaken initial laboratory investigations into the potential mechanisms underlying these events. Detailed description of the GD and FD cohorts at the Royal Free hospital highlights the complexity and heterogeneity of these disorders. This chapter sets these findings in the broader context of research in the lysosomal storage disorders field, discussing new insights into and the pathogenesis of FD and GD and potential future investigative directions whilst also discussing the limitations of the methodological approach taken.

7.1   Vascular features in the clinical presentation of FD and GD

7.1.1   Vascular manifestations are important presenting features of FD and GD

Within this project, the major vascular manifestations explored were bleeding symptoms in GD and, in FD, critical organ events that are believed to have at least in part an underlying vascular mechanism, namely stroke, renal disease and cardiac arrhythmias.

Bleeding symptoms are common in the presentation of GD, being the primary presenting feature in 18.6% of patients (27.6% of symptomatic patients) and present in 54% of patients overall at baseline, including post-operative bleeding in 8%. Whilst platelet counts are reduced, the lack of correlation between platelet count and bleeding severity suggests other mechanisms come into play. The presence of low FXI activity in a substantial number of patients at baseline, with significant improvements following ERT supports the presence of a disease related impairment of coagulation protein function. Whilst it had been hypothesised that bleeding severity in GD would be correlated with severity of disease manifestations in other organ systems, this was not the case with the methodological approach utilised in this study. The disease severity scoring system used[34] was the one for which data was universally available for the cohort but this and the measures of bone disease (BMB score and presence/absence of infarcts) may not be

sensitive enough to detect a relationship between disease manifestations at a cellular/organ level in different organs and haemostatic defects.

Vascular critical organ complications were common at presentation in both male and female index cases of FD, with cardiac manifestations being the commonest presenting features in both males and females. Stroke as a presenting feature was commoner in females than males. The majority of FD patients were diagnosed following family screening. Amongst males diagnosed in this way, 21% already had cardiac complications and 4% had suffered a stroke before diagnosis. Amongst females, the prevalence of pre-existing cardiac complications was lower (9%) than in males but the prevalence of stroke was similar (3%). Whilst cerebrovascular disease in men occurred in the presence of other critical organ dysfunction in men, this was not the case in females. In addition to mutation type, other cardiovascular risk factors appear important in the development of cerebrovascular disease in females. There were two females with stroke with no risk factors and no other FD manifestations. Whether these patients have some other, as yet unidentified, predisposing risk factor to stroke is unknown.

7.1.2 Improvements in diagnostic pathways are needed to reduce delays between symptom onset and diagnosis

In both GD and FD, index cases experienced substantial delays between symptom onset and arrival at the correct diagnosis. In both disease, the main reason was failure to identify an underlying cause for the clinical feature (e.g. thrombocytopenia, neuropathic pain) rather than mis-diagnosis. The potential reasons for this are numerous and include:

1. The wide differential diagnosis of presenting symptoms

2. The rarity of FD and GD

3. Lack of physician awareness of FD and GD

4. Relative physical well-being of many patients at presentation

The differential diagnosis of underlying causes of many of the clinical manifestations with which patients with GD and FD present is broad; amongst these many causes the incidence of GD/FD is rare compared to more common conditions. How rare is unknown. The true incidence of GD and FD is not known. Newborn screening studies suggest that the true incidence of FD may be far higher than previously thought[29], particularly for mutations associated predominately with disease manifestations in later life. This is supported by the data from the RFH cohort where there has been a marked increase in diagnosis of older patients with predominately cardiac manifestations in recent years. Whilst diagnosis of GD is predominately fortuitous, usually being suspected following the finding of Gaucher cells on bone marrow biopsy, such opportunities for diagnosis are rare in FD where biopsies of affected organs are rarely performed. The presenting features suggest that for FD, cardiologists, stroke physicians, dermatologists and paediatricians/paediatric neurologists are key physicians amongst whom awareness of FD should be increased. The longest delays in diagnosis, on average of 22.5 years, were amongst FD patients whose first symptom was pain, beginning in childhood or adolescence. They present a particular diagnostic challenge and more research is needed on how best to improve the diagnosis of these patients amongst whom the physical and psychological consequences of delayed diagnosis can be substantial[283].

The availability of specific therapy in the form of ERT provides added impetus to reduce diagnostic delays in symptomatic patients. Irreversible complications occur in both GD (e.g. avascular necrosis) and FD (e.g. cardiac fibrosis). There is some evidence to support early initiation of therapy in reducing these complications in GD[186] but this is not yet available in FD.

7.2   Glycosphingolipids and enzyme defects: different effects in different cells?

The heterogeneity of clinical manifestations within FD and GD and the marked differences in clinical manifestations between two disorders so closely linked on a metabolic pathway raise important questions about the roles of glycosphingolipids and their degradative enzymes within different cell types and organ systems.

Lysosomes are intracellular organelles, ubiquitous in eukaryotic cells. The vast majority of studies of cellular function in FD and GD have been performed on either fibroblasts or peripheral blood leucocytes and even basic knowledge regarding GLA and GBA expression and activity in other human cell types in lacking. Whilst in type 1 GD macrophages are the key cell implicated in disease pathogenesis, the range of cell types thought to be important in the pathogenesis of FD is much broader, including podocytes, cardiomyocytes and neurons. Perhaps further investigation of a broader range cell types might help answer some of the following unanswered questions in FD and GD, which are explored in further detail below:

Why patients with FD and GD accumulate lipids in different cell types?

Why do females develop clinical manifestations in FD?

Why do different mutations cause clinical disease manifestations in different organ systems?

Do the small number of recurrent mutations in FD and GD indicate a founder effect or survival advantage?

7.2.1   Do different glycosphingolipids and their degradative enzymes function differently in different cells?

In GD, the glycosphingolipid which accumulates in macrophages is thought to be derived predominately from phagocytosis of senescent erythrocyte membranes. Globoside is the predominant glycosphingolipid in erythrocyte membranes[284] and would be degraded by GLA before degradation by GBA. It is therefore surprising that reticulo-endothelial manifestations do not occur in FD. Alpha-N-acetylgalactosaminidase (also known as alpha galactosidase B) can remove the terminal α-galactose from Gb3 as well as the terminal α-N-acetylgalactosamine from sialoglycopeptides[285]. Deficiency of this enzyme results in Schindler's disease, the clinical manifestations of which include angiokeratoma, facial coarseness and neurodegeneration; lipid storage in endothelial cells is also reported. The first two of these clinical features are shared with FD, but neurodegeneration is not a

229

feature of FD, nor does FD unlike virtually all other LSDs have an infantile onset neurodegenerative subtype. These observations raise the questions of a) whether degradation of glycosphingolipids occurs by a single identical pathway in all cell types b) whether excess of a particular glycosphingolipid has a different effect on different cell types?

7.2.2 What is potential pathogenic role of the mutant protein?

Our cohort data is in keeping with previous studies that clearly demonstrate that amongst females heterozygous for a *GLA* mutation, a substantial proportion develop clinical manifestations of FD, but equally others live to old age with no evidence of clinical disease. This raises the question as to why females develop clinical disease when their enzyme activity is normal or only slightly reduced. Recently, it has been found that *GBA* mutation carriers have an increased risk of Parkinson disease. In both these scenarios there is the question as to whether the presence of mutant protein from transcription of the abnormal *GBA/GLA* gene is disease causing or disease predisposing. Our clinical data from FD suggests that in females, age and cardiovascular risk factors may play and important role in the development of vascular manifestations. One hypothesis is that a *GLA* mutation predisposes to development of clinical manifestations (e.g. stroke, cardiac hypertrophy, and arrhythmia) but that further factors are required for development of overt disease.

7.2.3 Are different mutations more pathogenic in different organs/ cell types?

GD has classically been divided into distinct subtypes with type 2 and 3 associated with neurodegenerative disease. Homozygosity for the L444P is associated with type 3 disease whilst the presence of at least one N370S mutation is considered protective. The particular molecular significance of mutations at these sites is unknown. Data from our cohort suggests that patients with the N215S mutation could be considered a distinct sub-type of FD, predominately presenting with cardiac disease in the later decades of life and having higher residual plasma enzyme activity. Why the heart, rather than other organs, is predominately affected in these patients is not known. The asparagine at position 215 in GLA is glycosylated and it may be that, as a result of alternative splicing mechanisms or other post-transcriptional modifications, this glycosylation is more important for structure

or activity of GLA in cardiac tissue than in other organ systems. Little is known about the regulation of the gene promoter region or alternative splicing of GBA and GLA in different cell types and further exploration of this may help explain why some mutations predispose to particular disease manifestations. The variation in enzyme activity, which correlates with disease severity, amongst patients with the N215S mutations points to additional factors other than the point mutation affecting enzyme level. Additionally, the fact that a small number of patients with this mutation present with renal disease in the absence of cardiac disease, often at a young age, suggests that other variables may affect the cellular types in which the effects of the enzyme mutation clinically manifest. Understanding the effect of a particular mutation at the molecular level would also be particularly beneficial in FD where screening studies for FD have uncovered a high prevalence of *GLA* mutations of uncertain clinical significance[48].

7.2.4 Recurrent mutations: founder effect or survival advantage

Most mutations within FD are private and occur within a single or small number of families. Within our cohort, 29 families, not known to be related, have the N215S mutation. This has yet to be reported in other cohorts but raises an important question. Is this recurrence due to a founder effect, as has been proposed for recurrent mutations in GD within the Jewish population, or does this mutation confer a survival advantage? Patients with the N215S mutation within our cohort overall have less severe disease than patients with other mutations but whether there is any survival advantage to heterozygotes to account for its frequency is not known. Cell membrane glycosphingolipids have important functional roles within the innate immune system[286], with glycosphingolipid-enriched microdomains being important for phagocytosis. Whether alterations in glycosphingolipid composition of cell membranes in "milder" mutations such as N215S in *GLA* or N370S in *GBA* results in improved functioning of the innate immune system has not to date been investigated.

7.3 Abnormalities in blood-endothelial interactions

Both bleeding events and thrombotic events arise due to abnormalities at the blood-endothelial interface, caused by either abnormalities in components of the blood,

abnormalities in the vessel wall or by more complex alterations in the interactions between the two (e.g. blood flow pattern). The methodological approach taken looked at aspects of these processes in isolation and under static conditions.

7.3.1 Impact of ageing on abnormalities in FD

Previous studies in FD had found increased levels of soluble adhesion molecules in the plasma of FD patients (see **Table 1-9**) and one study had reported increased expression of the adhesion molecule CD11b on the surface of monocytes in male patients[152]. We did not replicate this finding but did find increased expression of CD11a, ICAM1 and ICAM3 on monocytes in FD. Level of expression of these molecules on monocytes was not however correlated with functional adhesion of monocytes to tissue culture plastic under static conditions, nor were they correlated with disease manifestations. This may be because increased expression does not result in increased adhesion activity under basal conditions or because of the limitations of the methodology used. Functional adhesion activity of monocytes was increased in FD with clinically significant correlations between adhesion and clinical parameters (disease severity, organ function and age) in females. These findings support the clinical data from the cohort where age had a bigger impact on organ dysfunction in females than males.

One hypothesis for further exploration is that in females with FD age is an important modifier of disease manifestations. Autophagy is the key pathway by which intracellular macromolecules and organelles are delivered to the lysosome for degradation and there is some evidence of autophagy dysfunction in FD[73]. Autophagy also becomes impaired as differentiated cells age[287;288]. Although most studied in neurodegenerative diseases, there is evidence to support similar processes in other cell types including podocytes[289]. Therefore in females the additive effect of ageing on the presence of mutant GLA may result in cellular dysfunction.

7.3.2 Abnormal lymphocyte adhesion molecule expression in GD

Although cells of the monocyte-macrophage lineage are thought to be the primary abnormal cell in GD, increases in adhesion molecule expression were most marked in

lymphocytes. This is a potentially important finding not only because it support the presence of abnormalities in cells other than those of the monocyte-macrophage lineage but also, as discussed in 5.6.2, of the potential role of these abnormalities in the pathogenesis of bone disease and myeloma. Whilst the efficacy of ERT in improving blood counts and decreasing visceral disease volume is well established, whether ERT fully ameliorates abnormalities within the bone microenvironment contributing to bone disease or increased myeloma risk is not established.

No increases in functional monocyte adhesion were seen in GD and increases in monocyte adhesion molecule expression were modest compared to those seen in lymphocytes. The finding of increased adhesion of RAW.274 murine monocytes loaded with glucosylsphingosine and lactosylceramide to EA.hy926 cells suggests that the findings in monocytes isolated from patients may be due to treatment effect. Further investigation of monocytes from untreated patients is warranted, although the small number of patients available for such a study may limit sample size.

7.3.3   Procoagulant defects in FD and GD require further investigation

Initial investigations of the platelet membrane procoagulant response in this project suggest that the rate of thrombin generation on the platelet membrane is increased in FD. Platelets in FD remain under-investigated; confirmation a procoagulant platelet defect would be highly significant both in terms of furthering understanding of the pathogenesis of FD but more importantly in the potential therapeutic benefit of anti-platelet agents.

Endothelial support for thrombin generation was increased in endothelial cells treated with CBE. Whilst at initial glance this may seem discordant with the bleeding phenotype noted in GD, deficiencies of anticoagulant proteins have been found in patients with GD (see **Table 1-11**) and implicated in the pathogenesis of bone infarcts. As activation of the protein C pathway occurs on the endothelial surface, defects in which would increase thrombin generation, this finding warrants further investigation in primary endothelial cell isolates from GD patients.

7.4   Study limitations

7.4.1   Difficulties in studying rare inherited disorders

Research into rare inherited disorders presents several difficulties, a number of which were encountered during the course of this project. Although the patient cohorts at RFH are relatively large for FD and GD, they are still small, particularly when one considers the marked heterogeneity, especially within FD. Only small numbers of new patients are diagnosed each year and therefore, inevitably, virtually all laboratory investigations were performed in patients already receiving enzyme replacement therapy. Collaborative studies between centres might increase availability of samples on newly diagnosed, untreated patients but the effects of transport time on some of the cell types studied, especially platelets are likely to be deleterious.

7.4.2   Difficulties in undertaking mechanistic studies in FD and GD

Attempting to recapitulate GD/FD within the laboratory environment is challenging. Whilst naturally occurring animal models exist for a number of LSDs, GD and FD are not amongst these. Whilst murine models provide valuable insights into disease mechanisms, particularly at an organ level, the lack of disease features in the presence of storage material in some models raises questions about the comparability of glycosphingolipid and lysosomal biology in mice and humans.

Whilst pharmacological inhibition and siRNA silencing can be used to develop disease models, they do not allow the effect to the presence of the mutant protein to be investigated. Additionally we do not currently know the normal GLA and GBA enzyme activity in different cell types and therefore it is difficult to establish the degree of reduction in enzyme activity needed to resemble that found in patients. Advances in cell culture techniques, particularly the ability to de-differentiate cells (e.g. fibroblasts) to pluripotent stem cells and then differentiate them to a wide variety of cell types, may open the door to more research on primary cell isolates. Improved techniques in the isolation of endothelial progenitor cells from peripheral blood and podocytes from urine may provide an easier route for investigation of these cell types.

234

7.4.3 Laboratory investigation of blood-endothelial interactions

Endothelial cells line all blood vessels and lymphatics and, in adult humans, number over one trillion, covering a surface area of greater than 3000 square metres[290]. In addition to leucocyte adhesion and haemostasis, the endothelium fulfils many other roles including regulation of vascular tone and transfer of molecules across from plasma into surrounding tissues. Endothelial cells adapt to their microenvironment such that the phenotype of cells varies between the microvasculature and large vessels. Reducing this diversity to the level of a petri-dish comes with obvious limitations. The cell line used in this study is of venous macrovascular origin (human umbilical vein) whereas many of the disease manifestations of FD and GD are more likely to be microvascular in origin, where obtaining cells is more challenging.

In their *in vivo* state interactions between blood components and the endothelium occur in the presence of whole blood which is under a variable rate of flow depending on the anatomical site but is never static. Whilst the methodological approach of investigating components of blood in isolation (e.g. platelets, monocytes) allows more specific study of those cells, it over-simplifies what is a highly complex *in vivo* physiological system.

7.5 Further research avenues

7.5.1 Disease heterogeneity and modifiers

This thesis has better defined the vascular manifestations of GD and FD and their importance in the diagnosis of these disorders. It has also highlighted the vast heterogeneity within both disorders, particularly FD. The wide range of GLA activity seen in male patients with the N215S mutation suggests that there are other factors either within the gene (e.g. polymorphisms) or affecting levels of gene expression which contribute overall enzyme activity. The availability of high throughput genetic sequencing technologies facilitates the simultaneous investigation of a number of genes. As well as full sequencing of the *GLA* gene, evaluation of other genes which may contribute to vascular disease, in particular the *LPA* gene, which accounts for >90% of the differences

seen in plasma lipoprotein (a) levels[227] may be a potentially fruitful investigation as increased Lp(a) appeared to be a risk factor for cerebrovascular disease in FD patients.

Greater evaluation of potential acquired disease modifiers (e.g. hypertension, obesity) is required in FD, ideally as a collaborative study with other centres. Many females within the cohort were asymptomatic and diagnosed on family screening, but some had severe disease manifestations (e.g. stroke, cardiac arrhythmia). If the policy of family screening of females continues to be pursued then this must be coupled by concerted efforts to improve prognostic predictions and identify measures which might reduce risk of developing disease complications.

7.5.2   Blood-endothelial interactions using primary cell models

The limitations of the endothelial cell model and static adhesion have already been discussed. Further investigations are required to confirm/ refute the preliminary findings from this thesis. Ideally this would involve isolation of blood outgrowth endothelial cells from patients and measurement of adhesion of primary leucocytes (both lymphocytes and monocytes) to these endothelial cells under flow conditions. Further investigation of thrombin generation could also be performed using primary cell isolates from patients with GD.

Oxidative stress has been implicated in the pathogenesis of both FD[64;89] and GD[291;292]. This has been more widely studied in FD than in GD and dysfunction of endothelial nitric oxide synthase, secondary to Gb3 accumulation, resulting in decreased nitric oxide availability has been proposed as the mechanism. Measurement of eNOS activity and the end products of increased oxidative stress (e.g. lipid peroxidation) in FD and GD endothelial cells could provide a link between adhesion, coagulation and disease manifestations. Oxidative stress results in the up-regulation of a number of cellular adhesion molecules, particularly integrins and ICAM-1[293].

7.5.3 Further exploration of platelet function and membrane composition

Establishing whether platelets are procoagulant in FD is clinically important and warrants further investigation. Platelet studies need to be performed on a larger sample size. The current rate limiting step is the need for frequent manual subsampling during the prothrombinase assay. Should the findings be replicated in a larger group of FD patients not receiving anti-platelet agents, then study of those patients taking anti-platelet agents would be important to see if anti-platelet agents reduce this effect.

7.6 Conclusions

This project has confirmed that vascular manifestations are an important part of the pathology of both GD and FD but are heterogeneous in their clinical manifestations. Non-disease related factors, including ageing and more conventional cardiac risk factors, are important contributors to this heterogeneity in FD females. The mechanisms underlying these disease processes remain poorly understood. Preliminary results from this project demonstrate abnormal expression of certain adhesion molecules in leucocyte subsets and further investigation using primary cells and more physiological flow systems is warranted. Understanding the mechanisms underlying vascular disease is of great clinical import as it may open up new therapeutic avenues. This is particularly important in FD where the efficacy of ERT in preventing critical organ complications remains to be established.

Publications

Research papers:

Thomas AS, Mehta A & Hughes DA. Diagnosing Gaucher disease: an on-going need for increased awareness amongst haematologists. *Blood Cells Mol Dis* 2013 Mar; 50(3): 212-7.

Review articles:

Thomas AS, Mehta AB & Hughes DA. Gaucher disease: haematological presentations and complications. *Br J Haematol* 2014 Mar 3 doi 10.1111/bjh 12804 [Epub ahead of print]

Thomas AS & Mehta AB. Difficulties and barriers in diagnosing Fabry disease: what can be learnt from the literature? *Expert Opin Med Diagn.* 2013 Nov; 7(6): 589-99

Thomas AS & Hughes DA. Fabry disease. *Pediatric Endocrinology Reviews*. 2014 Jun 11 (Suppl 2) *In press*.

Conference presentations & posters:

Thomas AS, Mehta AB & Hughes DA. Diagnosing Gaucher disease: an on-going need for increased awareness amongst haematologists. 12th European Working Group on Gaucher Disease Meeting, Paris 2012.

Thomas AS, Hughes DA, Mehta AB & Gomez K. Gaucher disease: an inherited disorder with an acquired bleeding diathesis. British Society Haemostasis & Thrombosis Annual Meeting, Bath, 2012.

Thomas AS, Mehta AB, Gomez K & Hughes DA. Systematic screening detects no increase in incidence of lupus anticoagulant or heritable thrombophilias amongst patients with Fabry disease. Lysosomal Disease Network WORLD Symposium, Orlando Florida, 2013.

Thomas AS, Mehta AB & Hughes DA. Thrombocytopenia and splenomegaly in pregnancy: a common presenting feature of Gaucher disease. British Society of Haematology Annual Scientific Meeting, Liverpool, 2013.

Thomas AS, Hughes DA, Mehta AB & Gomez K. FXI deficiency and Gaucher disease: a potentiated bleeding risk? XXIV Congress of the International Society for Thrombosis and Haemostasis, Amsterdam, 2013.

Appendices

Appendix 1 Ethical Approval

NHS
Health Research Authority

NRES Committee London - Camden & Islington
REC Office
Maternity, Level 7
Northwick Park Hospital
Watford Road
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Middx HA1 3UJ

Telephone: 020 8869 2915
Facsimile: 020 8869 5222

02 March 2012

Dr Derralynn Hughes
Senior Lecturer in Haematology
Royal Free & University College London Medical School
Dept of Academic Haematology
UCL Medical School (Royal Free Campus)
Rowland Hill St,
London   NW32PF

Dear Dr Hughes

Study title:	**Laboratory investigation into changes in the coagulation system seen in patients with Gaucher disease and Anderson Fabry disease**
REC reference:	12/LO/0271

The Research Ethics Committee reviewed the above application at the meeting held on 27 February 2012. The committee would like to thank Dr Alison Thomas for attending to discuss the study.

Ethical issues discussed by committee before inviting researcher:

1. It was noted that factor XI deficiency was mentioned in Q A22 of the IRAS application; the committee suggested this to be added to the Participant Information Sheet.
2. The committee also suggested adding "patients who are treatment naïve and initiated on treatment during the study period will have repeat samples taken at specific time points after initiation of treatment" (page 8 of 15 in the Protocol) to the Participant Information Sheet.
3. Was it necessary to look at medical records of patients?
4. It was discussed and suggested to remove reference to Research Ethics Committee would look at medical notes, if required, from the Participant Information Sheet and Consent form.
5. The committee would like assurance that the Questionnaire included with the study application was the correct one used for the study.

Dr Alison Thomas was invited to join the meeting. During her attendance the following discussion took place.

A. It was asked whether the samples for research purpose would be anonymised or non-anonymised. Clarification was given the samples would be pseudo-anonymised for this study.

B. It was suggested and agreed to add explanation on factor XI to the Participant Information Sheet.

C. It was also suggested and agreed to remove reference to Ethics committees looking at medical notes from the Participant Information Sheet and Consent form.

A Research Ethics Committee established by the Health Research Authority

D.   Would extra blood tests be done on participants if required? It was confirmed this would be the case if there was a problem with collection of blood sample at the routine visit. The committee suggested adding this to the Patient Information Sheet. This was agreed.

Ethical opinion

Decision – FAVOURABLE (WITH CONDITIONS)

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

- **Revised Participant Information Sheet – removing reference to "Ethics committee looking at medical notes" (from the Consent form as well), details of factor XI and addition of extra blood sample as agreed.**
- **Assurance the Questionnaire is the right one for this study.**

Ethical review of research sites

NHS Sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. Confirmation should also be provided to host organisations together with relevant documentation

Health Research Authority

NRES Committee London - Camden & Islington
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28 March 2012

Dr Alison Thomas
Lysosomal Storage Disorders Unit
Royal Free Hospital NHS Trust
Pond Street
London   NW3 2QG

Dear Alison,

Full title of study:	**Laboratory investigation into changes in the coagulation system seen in patients with Gaucher disease and Anderson Fabry disease**
REC reference number:	**12/LO/0271**

Thank you for your letter of 22nd March 2012. I can confirm the REC has received the documents listed below as evidence of compliance with the approval conditions detailed in our letter dated 27 February 2012. Please note these documents are for information only and have not been reviewed by the committee.

Documents received

The documents received were as follows:

Document	Version	Date
Covering Letter	Response to Further Information	22 March 2012
Participant Consent Form	1.3	21 March 2012
Participant Information Sheet	1.4	21 March 2012

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to R&D offices at all participating sites.

12/LO/0271	Please quote this number on all correspondence

Yours sincerely

Alison Winter
Assistant Co-ordinator

E-mail: alisonwinter2@nhs.net

Patient Information Leaflet

Laboratory Investigation into changes in the coagulation system seen in patients with Gaucher disease and Anderson Fabry disease

- Participant Information

You are being invited to contribute to a clinical research study being co-ordinated by the Lysosomal Storage Disorders Unit and the Haemophilia Centre at the Royal Free Hospital. Please take time to read the following information carefully and take time to decide whether or not you wish to take part. Please do not hesitate ask us if there is anything that is not clear or if you would like more information.

What is blood coagulation?

Blood coagulation is a complex process by which blood forms clots, necessary to prevent excessive blood loss after injury. The bleeding and thrombotic disorders are due to under or over active coagulation factors and platelets.

What is the purpose of this Study?

The aim of this study is to investigate problems with bleeding or blood clotting in patients with Fabry and Gaucher Disease. It is known that patients with Gaucher disease may have a tendency to bleeding. Conversely, patients with Fabry disease may have a tendency towards clotting, such as strokes. In both disorders, the extent of bleeding and clinical problems and the factors that contribute to these remain unclear.

This study is in the form of:

a) A questionnaire about bleeding problems and clotting problems you may have had and also asks about some factors which may be associated with blood clots in the general adult population

b) Taking a blood sample for further tests to look at the proteins and platelets involved in the process of forming a blood clot and white blood cells that can activate clotting processes. This will include looking for changes in the DNA of two proteins involved in the clotting process that can affect how well they function.

Why have I been chosen?

You have been invited to participate in this study because you have Gauchers disease or Fabry's disease. You may or may not be on treatment for these disorders.

Do I have to take part?

It is up to you to decide 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, and if, at a later date you wish to withdraw, you are still free to do so anytime and without giving a reason. This will not affect the care you receive.

What will happen to me if I take part?

If you do agree to take part, we do not anticipate any extra hospital visits .This study will NOT influence the care and the standard of treatment you receive. If you agree to participate a member of the medical or nursing staff will go through the questionnaire with you when you next visit. Two tablespoonfuls of extra blood will be taken when blood samples are being taken for your routine investigations or treatment. Occasionally, it make not be possible to get an adequate blood sample on the first visit. If this is the case, we would invite you for a repeat blood test when you next attend an appointment.

As a part of the research, we may also need to review your medical notes to correlate your medical condition with the laboratory test results. The notes will be only be reviewed by members of the Lysosomal Storage Disorders Unit.

What will happen to the leftover samples?

If there is any leftover blood samples, if agreeable to yourself, they will be transferred to the Katharine Dormandy Coagulation Research Plasma bank after completion of this study. This bank stores plasma samples from patients with a wide variety of possible bleeding or clotting disorders to enable future research in this area. Otherwise, samples will be destroyed at the end of the study.

What are the possible benefits of taking part?
We cannot promise the study will help you, but the information we get might help advance the treatment of people with coagulation abnormalities due to lysosomal storage disorders. Low levels of a clotting protein called Factor XI have been identified in some patients with Gaucher and can be associated with an increased risk of bleeding. Should this be detected, you will be offered an appointment at the Haemophilia Centre at the Royal Free for review by a clotting specialist.

Who has reviewed the study?

This research programme has been reviewed by the Research Ethics Committee.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. If you consent to take part in the research, your data will be handled in a manner in accordance with the Data Protection Act 1998 and the rights you have under this act. The results will be published in various journals and international meetings in a way that you will not be identified.

Representatives of regulatory authorities, research and development team at the Royal Free Hampstead NHS Trust or ethics committee will be allowed to see your medical notes as required to ensure the research is being properly conducted and that the data collected is accurate. Your privacy will be respected at all times.

What happens if there is a problem?

We would not expect you to suffer any harm or injury because of your participation in this study. If you are harmed by taking part in the study there is no special compensation arrangement. If you are harmed due to someone's negligence then you may have grounds for legal action, but you may have to pay your legal cost. Regardless of this, if you wish to complain or have any concerns about any aspect of the way you have been approached or treated during the course of this consent process, the normal National Health Service Complaints mechanism is available to you. If you have any concerns regarding the care you have received or as an initial point of contact if you have a complaint, please contact the Patient Advice and Liaison Service (PALS) at the address given below;

PALS office: 020 7830 2174 , rfh.pals@nhs.net

PALS, Royal Free Hospital, Pond Street, London, NW3 2QG

Contact for further information?

If you require any further information please do not hesitate to contact the Lysosomal Storage Disorders team on 0207 472 6409 Monday to Friday from 0900hrs to 1700hrs.

Thank you for your patience in reading this leaflet.

Appendix 2 Solutions & Buffers

General solutions

Solutions used for tissue culture were autoclaved prior to use.

TBE buffer

Constituents: 1.1M Tris, 900mM borate, 25mM EDTA, pH 8.3

A 5X stock solution was made by dissolving 54g of Tris base, 27.5g of boric acid and 4.7g of disodium EDTA in 1L of distilled water. Stored at room temperature.

Solutions for enzyme assays

Red Cell lysis solution:

To make 1L of x10 stock solution: 82.9g ammonium chloride (NH_4Cl), 10g $KHCO_3$, 380mg EDTA (disodium), distilled water. Stored at room temperature. 1:10 dilution to make x1 solution.

4-methylumbelliferone standard

A 1mM stock solution of 4-methylumbelliferone standard was made by dissolving 176mg of 4 methylumbelliferone (Sigma) in 1L of distilled water. A working solution of standard was made by diluting 100µl of stock solution in 19.9ml of distilled water to give a working standard concentration of 1nM per 200µl. Stock and working solution stored at -20°C.

Mcilvaine's buffer

Mcilvaine's buffer comprises a mixture of 0.1M sodium citrate solution and 0.2 disodium phosphate solution with the quantities of each determined by the required pH. To make 20ml of buffer the following volumes were added:

pH	0.2M NaHPO₄	0.1M citrate
4.4	8.82	11.18
4.7	9.6	10.4
5.1	12.2	7.8

Glycine stopping buffer:

1M glycine, pH 10.4.

75g glycine and 58g sodium chloride dissolved in 1L of distilled water. To 557ml of glycine-NaCl solution add 443ml of 1M sodium hydroxide. The resultant solution in 1M glycine at pH 10.4.

Solutions for Western blotting

Whole cell lysis solution:

Constituents: 20mM HEPES, 50mM NaCl, 2% nonyl phenoxypolyethoxylethanol (NP40), 0.5% sodium deoxycholate, 0.2% sodium dodecyl sulphate (SDS), 1mM sodium orthovanadate, 1mM EGTA, 10mM sodium fluoride, 2.5mM sodium pyrophosphate, 1mM β glycerophosphate.

100mM sodium orthovanadate: dissolve 18.4g of sodium orthovanadate in 950ml of water. Adjust pH to 10.0 with concentrated hydrochloric acid. Boil yellow solution until colourless, adjusting pH until there is a colourless solution with a pH of 10.0. Make total volume up to 1 litre with water. Aliquoted and stored at -20C.

To make 500ml whole cell lysis solution, add together: 2.4g HEPES, 1.5g NaCl, 10g NP40, 2.5g Na dooxycholate, 1g SDS, 5ml 100mM Na orthovanadate, 190mg EGTA, 210mg Na fluoride, 558mg Na pyrophosphate, 108mg β glycerophosphate and make up to 500ml in distilled water. Aliquoted and stored at -20°C.

20X Transfer buffer

Constituents: 500mM Bicine (Sigma), 500mM Bis Tris (Sigma), 20.5mM EDTA (BDH)

To make 1L, 81.6g Bicine, 104.6g Bis Tris and 6g EDTA were dissolved in distilled water and the volume made up to 1L. The solution was stored at 4°C.

TBS-Tween

20x stock TBS solution: 20mM Tris, 137mM sodium chloride, pH 7.4-7.8. To make 1L, 48.4g of Tris (Sigma) and 160g of sodium chloride (BDH) were dissolved in 1L of distilled water and the pH adjusted to 7.4-7.8 with hydrochloric acid. Stored at 4°C

1XTBS-Tween solution made by adding 2g of Tween 20 (Sigma) and 50ml of 20X TBS stock and making up to 1L in distilled water. The solution was stored at 4°C

Blocking solution

5% polyvinylpyrolidine in TBS-Tween. 25g of polyvinylpyrolidine (Sigma) was added to 500ml TBS-Tween and dissolved overnight using a magnetic stirrer. The solution was stored at 4°C

Solutions for coagulation assays

TrisHCl-NaCl buffer

Constituents: 0.05M Tris-hydrochloride and 0.15M sodium chloride, pH 7.4

7.88g of Tris-HCl (Sigma) and 8.76g NaCl (BDH) were added to 950ml of distilled water. The pH was adjusted to 7.4 with sodium hydroxide. The volume was made up to 1L with distilled water. The solution was stored at 4°C.

To make Tris-HCl-1%BSA buffer, 200mg of bovine serum albumin was added to 20ml of Tris-HCl and dissolved with gentle agitation. The solution was stored at 4°C and discarded after 2 weeks.

HEPES buffer for PRP thrombin generation

Constituents: 20mM HEPES, 6% bovine serum albumin, pH 7.35

476mg of HEPES and 6g of bovine serum albumin were dissolved in 100ml of distilled water. The solution was aliquoted and stored at -80°C.

Krebs Ringer buffer

Constituents: 4mM potassium chloride, 107mM sodium chloride, 20mM sodium bicarbonate, 2mM sodium sulphate, pH 5.0.

To make 1L: 298mg KCl, 6.25g NaCl, 1.68g $NaHCO_3$ and 284mg $NaSO_4$ were dissolved in 950ml of distilled water. The pH was adjusted to 5.0 with hydrochloric acid and the volume made up to 1L with distilled water. The solution was stored at 4°C.

Krebs Ringer buffer 0.9g/L glucose, pH 6.0. 500ml of Krebs Ringer buffer was supplemented with 450mg of glucose (Sigma) and the pH adjusted to 6.0. The solution was stored at 4°C and discarded after 1 month.

Stopping buffer for prothrombinase assay

Constituents: 0.05M Tris-HCl, 0.12M sodium chloride, 2mM EDTA, pH 7.5

To make 1L, 7.88g of Tris-HCl, 7.008g of NaCl and 2.33g of EDTA were dissolved in a final volume of 1L distilled water. The solution was stored at 4°C.

High salt wash buffer for VWF ELISA

1L of 10X stock solution was made by dissolving 3.9g sodium dihydrogen orthophosphate dehydrate, 26.8g disodium hydrogen orthophosphate 12 hydrate and 282.2g of sodium chloride in 800ml of distilled water. The volume was made up to a total of 1L with distilled water. The stock solution was stored at room temperature.

Working strength wash buffer was made by adding 100ml of 10X stock and 2ml of Tween 20 to 900ml of distilled water. The working solution was stored at 4°C for 1 month

ELISA coating buffer

500ml of coating buffer was made by dissolving 790mg of sodium carbonate and 1.47g of sodium hydrogen carbonate in distilled water. The pH of the resultant solution should be pH9.6 and the solution was re-made if outside the range of pH 9.4-9.8. The solution was stored at 4°C

ELISA dilution buffer

Dilution buffer was made by adding 3% polyethylene glycol (PEG) to working strength wash buffer. 15g of PEG 8000 was added to 500ml of wash buffer. The solution was stored at 4°C.

ELISA substrate buffer

500ml of substrate buffer was made by dissolving 3.65g of citric acid and 11.94g of disodium hydrogen orthophosphate.12 hydrate in 500ml of distilled water. The resultant solution should be pH 5.0. The solution was stored at 4°C.

Appendix 3 GM approval

UCL SAFETY SERVICES

Dr Derralynn Hughes
Cancer Instiute
Royal Free Campus

26 June 2013

Our ref: GM/1556 RF

Dear Dr Hughes,

Experiment number 1556 RF

We are pleased to inform you that the Genetic Modification Safety Committee has approved your experiment entitled *In vitro cellular models of lysosomal disorders*, as an Activity Class 1, Containment Level 1 project.

Any enquiries regarding your project should be addressed to Jillian Deans, extension 41814, email j.deans@ucl.ac.uk

Thank you for your co-operation with the procedure.

Yours faithfully,

Jon Blackman

cc GMSO, Adele Fielding

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252

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