

**THE USE OF GLOBOTRIAOSYLSPHINGOSINE TO
DETECT AND MONITOR FABRY DISEASE**

By

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ABSTRACT

Fabry disease (FD) is an *X-linked* lysosomal storage disorder caused by a deficiency of the α -galactosidase-A (α -gal-A) enzyme. The lack of enzymatic activity results in the accumulation of glycosphingolipids (GSLs) in the lysosomes of various tissues and organs. Globotriaosylceramide (Gb3) and Globotriaosylsphingosine (Lyso-Gb3) and their isoforms/analogues have been identified and quantified as potential biomarkers.

This study aimed to develop an HPLC-MS based method for the quantitation of plasma and urinary Lyso-Gb3 and its analogues in Fabry patients to evaluate its utility in diagnosis and monitoring FD.

The results showed that plasma Lyso-Gb3 as a reliable diagnostic biomarker for FD, as plasma Lyso-Gb3 levels could easily discern classical Fabry patients from controls. Moreover, plasma Lyso-Gb3 could also distinguish male cardiac variant Fabry patients from control males. Nevertheless, cardiac variant Fabry females showed an overlap of Lyso-Gb3 levels with controls, hence a positive value in this group would be considered diagnostic but a negative value could not exclude FD.

In a small cohort of our patients on ERT there was a trend towards falling Lyso-Gb3 levels with time suggesting that Lyso-Gb3 has a potential value in monitoring these patients.

Urinary Lyso-Gb3 levels were substantially different between classical and cardiac variant Fabry patients, and the lack of detectable urinary Lyso-Gb3 and analogues in controls allowed us to differentiate between these patients and healthy controls. The total levels of urinary Lyso-Gb3 and its analogues proved particularly useful in differentiating between classical and atypical Fabry patients of both genders.

In the course of the study, a novel rapid MALDI-TOF-MS based Method for measuring urinary Gb3 in Fabry patients has been established. Collectively, the final findings demonstrate that urinary Lyso-Gb3 is superior to urinary Gb3 as a diagnostic biomarker for FD, where the later has been shown to be found in healthy subjects.

Our study subsequently led to development of regional laboratory service for testing Lyso-Gb3 in Queen Elizabeth Hospital, Birmingham, UK. This service is now open to Fabry patients across England.

In conclusion both plasma and urinary Lyso-Gb3 levels are useful diagnostic and monitoring biomarker in classical and cardiac variant males patients, but have questionable utility in cardiac variant females due to overlap with healthy controls.

Although we studied the role of Lyso-Gb3 in diagnosing FD further studies are needed to establish its role in disease severity assessment and a larger study required to test our initial finding related to monitoring disease in patients on treatment.

DECLARATION

I declare that work carried out in this Ph.D. thesis is my own work unless otherwise stated. Some of the data presented in Chapter-6 forms part of a manuscript that has been published in the Journal of The American Society for Mass Spectrometry [1].

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TABLE OF CONTENTS

Chapter-1: Introduction	1
1.1 Inborn Errors of Metabolism	2
1.2 Lysosomal storage diseases	4
1.2.1 Sphingolipidoses	4
1.2.2 Glycosphingolipids	7
1.3 Fabry Disease.....	10
1.3.1 Overview	10
1.3.2 History of Fabry Disease.....	11
1.3.3 Prevalence of Fabry Disease.....	12
1.4 The Genetic Basis of Fabry Disease	14
1.5 The Biochemistry of Fabry Disease.....	17
1.5.1 The Human α -galactosidase-A Enzyme	18
1.6 Pathophysiology of Fabry Disease.....	19
1.7 Clinical Manifestations of Fabry Disease	21
1.7.1 Neurological Manifestations.....	23
1.7.1.1 Acroparesthesia.....	23
1.7.1.2 Cerebrovascular Manifestations	23
1.7.1.3 Sensory neural hearing loss	25
1.7.2 Skin manifestations.....	26
1.7.2.1 Angiokeratomas	26
1.7.2.2 Hypohidrosis/Anhidrosis	26
1.7.3 Gastrointestinal Tract	27
1.7.4 Kidney Manifestations	27
1.7.5 Heart Manifestations	28
1.7.6 Pulmonary Manifestations	30
1.7.7 Musculoskeletal and Growth Abnormalities.....	30
1.8 Diagnosis of Fabry Disease	30
1.8.1 Current methods of Fabry Disease Diagnosis.....	32
1.8.2 Challenges and current issues in diagnosis of Fabry disease	34
1.9 Treatment of Fabry disease	35
1.9.1 Enzyme Replacement Therapy.....	35
1.9.2 Other treatments procedures	39
1.9.2.1 Chaperone.....	39
1.9.2.2 Substrate reduction.....	40
1.9.2.3 Gene therapy.....	40
1.10 Biomarkers of Fabry disease	41
1.10.1 Globotriaosylceramide	42
1.10.1.1 Chemical, Physical and Biological Properties of Gb3.....	42
1.10.1.2 Accumulation of Gb3 in Biological Samples of Fabry Patients:	43
1.10.1.3 Gb3 isoforms and analogues	44

1.10.1.3.1 Gb3 methylated isoforms	44
1.10.1.3.2 Gb3-saturated fatty acids isoforms	45
1.10.1.3.3 Gb3-hydrated sphingosine analogues	45
1.10.1.3.4 Gb3 isoforms/analogues with two double bonds	46
1.10.1.3.5 Gb3 isoforms/analogues with one double bond.....	46
1.10.1.3.6 Short chain-Gb3 isoforms	46
1.10.1.3.7 Methylated short chain-Gb3 isoforms	46
1.10.2 Globotriaosylsphingosine (Lyso-Gb3):	47
1.10.2.1 Biochemical and physical properties of Lyso-Gb3	48
1.10.2.2 Biological effect of Lyso-Gb3	49
1.10.2.3 The mechanism of Lyso-Gb3 catabolism.....	50
1.10.2.4 Accumulation of Lyso-Gb3 in biological samples of Fabry patients	51
1.10.2.5 Lyso-Gb3 analogues.....	52
1.10.2.6 Lyso-Gb3 analysis using Dried Blood Spots	55
1.11 Screening of Fabry Disease	55
1.12 Separation techniques for biological samples	56
1.12.1 High Performance Liquid Chromatography.....	56
1.13 Mass spectrometry	57
1.13.1 Ion sources	57
1.13.1.1 Electrospray ionisation (ESI).....	58
1.13.1.2 Matrix-assisted laser desorption ionisation.....	59
1.13.2 Mass analysers	60
1.13.2.1 MicrOTOF-II Mass Spectrometer	62
1.13.3 Tandem Mass Spectrometry	63
1.13.4 The role of MS in newborn screening for IEM.....	64
1.14 The scientific justification for the research	66
1.15 Research objectives.....	66
Chapter-2 Methodology: Material and Methods	68
2.1 Ethics.....	69
2.2 Subjects	69
2.3 Chemicals and Materials.....	70
2.3.1 General equipment and reagents	70
2.3.1.1 Plastics consumables.....	70
2.3.1.2 Glass consumables	71
2.3.2 Chemicals.....	71
2.3.3 Solutions, standards and reagents	71
2.3.4 HPLC and MS equipment and parts	74
2.4 Extraction of Glycosphingolipids from Clinical Samples	75
2.5 High Performance Liquid Chromatography	76
2.6 Analysis of Glycosphingolipids using Mass Spectrometry	76
2.7 Statistical analysis	78
Chapter-3 Results: Development of Plasma and Urinary Lyso-Gb3 assays	79
3.1 Introduction and objectives.....	80

3.2 Methodology	81
3.2.1 Lipid extraction	81
3.2.1.1 Choice of Internal Standard	82
3.2.1.2 Preparation of Lyso-Gb3 Standard	84
3.2.2 Resolution of Glycosphingolipids using RP-HPLC	84
3.2.2.1 Choice of Tubing Connectors	84
3.2.2.2 Choice of Chromatography Stationary Phase	84
3.2.2.3 Optimisation of Flow Rate	85
3.2.2.4 Optimisation of Binding and Elution Conditions	85
3.2.3 Analysis of Glycosphingolipids using Mass Spectrometry	86
3.3 RESULTS	87
3.3.1 Extraction Protocols.....	87
3.3.1.1 Extraction of Lyso-Gb3 and Analogues	87
3.3.1.2 Extraction Efficiency of Lyso-Gb3 from Urine and Plasma	88
3.3.2 Chromatography	89
3.3.2.1 Choice of Tubing Connectors	90
3.3.2.2 Choice of Chromatography Stationary Phase	92
3.3.2.3 Binding and Elution Conditions.....	95
3.3.2.3.1 Flow Rate	95
3.3.2.3.2 Solvent Composition.....	95
3.3.3 Optimisation of the Mass Spectrometer.....	98
3.3.3.1 Tuning and calibration of MS	98
3.3.3.2 Dry Gas Temperature.....	99
3.3.3.3 Optimum Spectral Rate.....	100
3.3.3.4 Lyso-Gb3 fragmentation profile by MS/MS	101
3.3.3.5 MRM Analysis of Lyso-Gb3 analogues	104
3.3.4 Choice of Internal Standard	108
3.3.5 Role of the Internal Standard	109
3.3.6 Assay Performance Characteristics.....	110
3.3.6.1 Lyso-Gb3 Quality Controls	110
3.3.6.2 Lyso-Gb3 Stability Test.....	111
3.3.6.3 Lyso-Gb3 Intra-assay and Inter-assay	113
3.3.6.4 Limits of Detection and Quantitation	113
3.4 Discussion	114
Chapter-4 Results: Clinical utility of Plasma Lyso-Gb3 and its analogues in the diagnosis and monitoring of Fabry disease	117
4.1 Introduction.....	118
4.2 Sample collection from Fabry patients	119
4.3 Results.....	120
4.3.1 Control Groups.....	120
4.3.2 Plasma Lyso-Gb3 Levels in Classical Fabry Patients.....	121
4.3.3 Plasma Lyso-Gb3 levels in cardiac variant Fabry patients.....	126
4.3.4 Levels of plasma Lyso-Gb3 analogues in classical Fabry patients.....	129
4.3.5 Levels of plasma Lyso-Gb3 analogues in cardiac variant Fabry patients	133

4.3.6 Plasma Lyso-Gb3 levels in classical Fabry patients following ERT	137
4.3.7 Plasma Lyso-Gb3 levels in cardiac variant Fabry patients following ERT	139
4.3.8 Correlation between the levels of plasma Lyso-Gb3 and its various analogues in Fabry patients.....	140
4.3.9 Correlation between the levels of plasma Lyso-Gb3 and the age.....	142
4.4 Discussion.....	143
Chapter-5 Results: Clinical utility of urinary Lyso-Gb3 and its analogues in the diagnosis of Fabry disease	146
5.1 Introduction and objectives (Fabry nephropathy).....	147
5.2 Sample collection from Fabry patients	148
5.3 Results.....	149
5.3.1 Control groups	149
4.3.2 Urinary Lyso-Gb3 levels in classical Fabry patients	151
4.3.3 Urinary Lyso-Gb3 levels in cardiac variant Fabry patients	153
5.3.4 Levels of urinary Lyso-Gb3 analogues in classical Fabry patients	156
5.3.5 Levels of urinary Lyso-Gb3 analogues in cardiac variant Fabry patients	161
5.3.6 Correlation between the levels of urinary Lyso-Gb3 and its various analogues in Fabry patients.....	167
5.3.7 Correlation between the levels of plasma Lyso-Gb3 and urinary Lyso-Gb3 in Fabry patients.....	169
5.4 Discussion.....	171
Chapter-6 Results: Development of a novel method to assay urinary Gb3 and assessment its clinical utility	175
6.1 Introduction.....	176
6.2 Materials and methods	177
6.2.1 Patient samples.....	177
6.2.2 Chemicals.....	178
6.2.3 Total Gb3 standard.....	178
6.2.4 Gb3 internal standard.....	178
6.2.5 Extraction of urinary Gb3	178
6.2.6 Gb3 internal standard and assay calibration	179
6.3 Results.....	182
6.3.1 Optimisation of Gb3 extraction from urine	182
6.3.2 Optimization of MALDI-TOF-MS	184
6.3.3 MS/MS Confirmation of Gb3 peaks in Fabry patient urine	186
6.3.4 Quantitative measurement of urinary Gb3 by MALDI-TOF-MS.....	188
6.3.5 Urinary total Gb3 levels in classical Fabry patients	192
6.3.6 Urinary total Gb3 levels in cardiac variant Fabry patients	194
6.3.7 Correlation between urinary Gb3 levels and Lyso-Gb3 levels in Fabry patients	196
6.4 Discussion.....	197
Chapter-7 General Discussion	200
General Discussion	201
Limitations of the study	213

Contributions to the field	214
Future work.....	214
References.....	216
Appendices.....	231
Appendix-1	232
Appendix-2	234
Appendix-3	235
Appendix-4	236
Appendix-5	237
Appendix-6	244
Appendix-7	245
Appendix-8	246
Appendix-9	248

LIST OF FIGURES

Figure 1.1 Inborn errors of metabolism process	3
Figure 1.2 Metabolic pathways of sphingolipidoses.....	6
Figure 1.3 Chemical structures of a ceramide and three sphingoid bases	9
Figure 1.4 Metabolic pathway of Gb3	11
Figure 1.5 Genetic of Fabry disease	17
Figure 1.6 Clinical manifestations, white matter	25
Figure 1.7 Pacemaker implantation	29
Figure 1.8 Clinical manifestations, left ventricular hypertrophy (LVH).....	29
Figure 1-9 Chemical structure of globotriaosylceramide (Gb3).....	42
Figure 1-10 Chemical structure of globotriaosylsphingosine (Lyso-Gb3).....	49
Figure 1.11 Electrospray ionisation (ESI) mechanism	59
Figure 1-12 MALDI procedure.....	60
Figure 1.13 Components of micrOTOF-II Mass Spectrometer	63
Figure 3.1 Chemical structures of GSLs.....	83
Figure 3.2 Detection of Lyso-Gb3 using MRM mode.....	90
Figure 3.3 Chromatography development	91
Figure 3.4 HPLC valve operation and usage	93
Figure 3.5 HPLC optimization, the final optimized HPLC gradients.....	94
Figure 3.6 HPLC optimisation, stationary phase elution	97
Figure 3.7 MS tuning and calibration	99
Figure 3.8 Optimisation of the MS spectral rate.....	100
Figure 3.9 MS/MS of Lyso-Gb3.....	102
Figure 3.10 Theoretical fragmentation procedure of Lyso-Gb3.....	103
Figure 3.11 Plasma Lyso-Gb3 analogues in untreated Fabry hemizygote	105
Figure 3.12 Urinary Lyso-Gb3 analogues in untreated Fabry hemizygote	106
Figure 3.13 MS/MS fragmentation of Lyso-Gb3 analogues	107
Figure 3.14 Method development, choice of the internal standard.....	108
Figure 3.15 Lyso-Gb3 standard curves using GSG internal standard	110
Figure 3.16 Stability Test.....	112
Figure 4.1 Plasma Lyso-Gb3 Levels.....	121
Figure 4.2 The levels of plasma Lyso-Gb3 in classical Fabry patients	124
Figure 4.3 ROC analysis for diagnostic accuracy of plasma Lyso-Gb3 to predict Fabry disease in classical Fabry patients.....	125
Figure 4.4 The levels of plasma Lyso-Gb3 levels in cardiac variant Fabry patients.	127
Figure 4.5 ROC analysis for diagnostic accuracy of plasma Lyso-Gb3 to predict Fabry disease in cardiac variant Fabry patients	129
Figure 4.6 The levels of plasma Lyso-Gb3 analogues in classical Fabry patients	131
Figure 4-7 The relative distribution of plasma Lyso-Gb3 and its analogues in classical Fabry patients.....	132
Figure 4-8 ROC analysis for diagnostic accuracy of plasma Lyso-Gb3 analogues to predict Fabry disease in classical Fabry patients	132

Figure 4.9 Plasma Lyso-Gb3 analogues levels in cardiac variant Fabry patients	135
Figure 4.10 The relative distribution of plasma Lyso-Gb3 and its analogues in cardiac variant Fabry patients.....	136
Figure 4.11 ROC analysis for diagnostic accuracy of plasma Lyso-Gb3 analogues to predict Fabry disease in cardiac variant Fabry patients	136
Figure 4.12 Following up ERT management in classical Fabry patients	138
Figure 4.13 Plasma Lyso-Gb3 levels through ERT monitoring in classical Fabry patients	138
Figure 4.14 Plasma Lyso-Gb3 levels through ERT monitoring in cardiac variant Fabry patients	139
Figure 4.15 Plasma Lyso-Gb3 levels vs. time in cardiac variant Fabry patients without any ERT management.....	140
Figure 4.16 Correlation between plasma Lyso-Gb3 and its analogues	141
Figure 4.17 Correlation between plasma Lyso-Gb3 and the age.....	142
Figure 5-1 Urinary Lyso-Gb3 levels in Fabry patients and control subjects.....	150
Figure 5.2 Urinary Lyso-Gb3 levels in classical Fabry patients.....	152
Figure 5.3 ROC analysis for diagnostic accuracy of urinary Lyso-Gb3 to predict Fabry disease in classical Fabry patients	153
Figure 5.4 Urinary Lyso-Gb3 levels in cardiac variant Fabry patients.....	154
Figure 5.5 ROC analysis for diagnostic accuracy of urinary Lyso-Gb3 to predict Fabry disease in cardiac variant Fabry patients	155
Figure 5.6 The levels of urinary Lyso-Gb3 analogues in classical Fabry patients	158
Figure 5.7 The relative distribution of urinary Lyso-Gb3 and its analogues in classical Fabry patients.....	159
Figure 5.8 ROC analysis for diagnostic accuracy of urinary Lyso-Gb3 analogues to predict Fabry disease in classical Fabry patients	159
Figure 5.9 The levels of urinary total Lyso-Gb3 and its analogues in classical Fabry patients	160
Figure 5.10 Urinary Lyso-Gb3 analogues levels in cardiac variant Fabry patients...	163
Figure 5.11 The relative distribution of urinary Lyso-Gb3 and its analogues in cardiac variant Fabry patients.....	164
Figure 5.12 ROC analysis for diagnostic accuracy of urinary Lyso-Gb3 analogues to predict Fabry disease in cardiac variant Fabry patients	165
Figure 5.13 The levels of total urinary Lyso-Gb3 and its analogues in cardiac variant Fabry patients.....	166
Figure 5.14 The correlations between urinary Lyso-Gb3 and its analogues in Fabry patients.	168
Figure 5.15 Correlation between Lyso-Gb3 levels in plasma and urine samples in Fabry patients.....	170
Figure 5.16 Correlation between the levels of total Lyso-Gb3 and its analogues in plasma and urine samples in Fabry patients	170
Figure 6.1 Validation of Gb3 depletion	181
Figure 6.2 Calculation of LOD and LOQ of urinary total Gb3	182
Figure 6.3 Validation of liquid-liquid extraction using different solvents	183

Figure 6.4 Validation of liquid-liquid extraction.....	184
Figure 6.5 Evaluation of different matrix compounds.....	185
Figure 6.6 Mass spectra of Gb3 and internal standard.....	186
Figure 6.7 MS/MS Confirmation of Gb3 peaks in Fabry patient urine.....	187
Figure 6.8 Gb3 internal standard in human urine.....	189
Figure 6.9 Urinary Gb3 standard curve.....	190
Figure 6.10 Correlation between urinary Gb3 species.....	191
Figure 6.11 Urinary total Gb3 levels in classical Fabry patients.....	193
Figure 6.12 ROC analysis for urinary total Gb3 in classical Fabry patients.....	194
Figure 6.13 Urinary total Gb3 levels in cardiac variant Fabry patients.....	195
Figure 6.14 ROC analysis for Urinary Gb3 in cardiac variant Fabry patients.....	196
Figure 6.15 Correlation between urinary total Gb3 and urinay-Lyso-Gb3 in Fabry patients.....	197

LIST OF TABLES

Table 1.1 Prevalence of Fabry disease.....	13
Table 1.2 Typical signs and symptoms of Fabry disease	22
Table 1.3 Standard criteria for Fabry disease diagnosis	33
Table 2.1 Anthropometric data of Fabry patients and control subjects.....	70
Table 2.2 Plastic lab-ware.....	70
Table 2.3 Glass lab-ware	71
Table 2.4 Chemicals	72
Table 2.5 Composition of solutions	73
Table 2.6 HPLC and MS equipments and parts.....	74
Table 2.7 HPLC parameters	76
Table 2.8 MS parameters	77
Table 2.9 MRM transitions.....	77
Table 4.1 Control subjects' groups.....	120
Table 4.2 Mutations list for classical Fabry patients	122
Table 4.3 Mutations list for cardiac variant Fabry patients	126
Table 5.1 Anthropometric data of Fabry patients and control subjects	149
Table 6-1 Anthropometric data of Fabry patients and healthy controls	180

ABBREVIATIONS

Abbreviation	Definition
IEM	Inborn errors of metabolism
IMD	Inherited metabolic disorders
LSD	Lysosomal storage diseases
FD	Fabry disease
GSLs	Glycosphingolipids
GM1	Monosialotetrahexosylganglioside
GM2	Disialotetrahexosylganglioside
GM3	Monosialodihexosylganglioside
Neu5AC	N-Acetylneuraminic acid
Glu	Glucose
Gal	Galactose
GalaNAc	N-Acetylgalactosamine
Fuc	Fucose
cDNA	Complementary deoxyribonucleic acid
mRNA	Messenger ribonucleic acid
CpG sites	DNA regions: cytosine-phosphate-guanine
CpG islands	DNA regions with a high frequency of CpG sites
<i>GLA</i>	Alpha-galactosidase A gene
α -gal-A	Alpha-galactosidase A enzyme
LVH	Left ventricular hypertrophy
Gb3	Globotriaosylceramide
Lyso-Gb3	Globotriaosylsphingosine
Ga2	Galabiosaosylceramide
GSG	1- β -D-glocosylsphingosine
IS	Internal standard
ERT	Enzyme replacement Therapy
MRI	Magnetic resonance imaging
SPE	Solid phase extraction
MCX	Mixed mode cation extraction
HPLC	High performance liquid chromatography
RP-HPLC	Reverse Phase High performance liquid chromatography
ESI	Electrospray ionisation
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MRM	Multiple reaction monitoring
AUC	Area under the curve
STD	Standard
CNS	Central nervous system
ECG	Electrocardiography
T wave	repolarization (recovery) of the ventricles in ECG
ST wave	The period when the ventricles are depolarized in ECG
FOS	Fabry outcome survey
HGT	Human Genetic Therapies
CTH	Ceramide trihexoside

UPLC	Ultra Performance Liquid Chromatography
OPA	o-Phthalaldehyde or ortho-phthalaldehyde
eGFR	estimated Glomerular Filtration Rate
DBS	Dried blood spots
TOF	Time of flight
rf	Radio frequency
DC	direct current
<i>m/z</i>	Mass to charge ratio
UV	Ultraviolet
Q-TOF	Quadrupole time of flight
QQQ	Triple quadrupole instrument
CID	Collision-induced dissociation
FWHM	Full width at half maximum
HCD	High energy collisional-trap dissociation
FAB	Fast Atom Bombardment ionisation
FIB	Fast Ion Bombardment ionisation

CHAPTER - 1
INTRODUCTION

1.1 INBORN ERRORS OF METABOLISM

Inborn errors of metabolism (IEM) also known as inherited metabolic disorders (IMD) constitute a large group of heterogeneous inherited disorders. Individually, IEM are very rare, however collectively numerous. Each disease results from a defect in a single gene encoding a regulatory enzyme, co-factor, or catalytic enzyme. These catalytic enzymes are responsible for the degradation of various metabolic substrates such as carbohydrates, proteins, fats and other metabolites (Figure 1.1). Consequently, the defective metabolic pathway results, primarily, in the accumulation of a metabolic substrate. Subsequently, this accumulation may be directly toxic to the cell or lead to a blockade in the catabolic pathway or production of abnormal metabolic intermediates through alternative metabolic pathways [1-5]. Additionally, IEM can result from defects in metabolite transportation into cells or energy production and utilization. All of these metabolic abnormalities are considered to be the main reasons for the various clinical effects of the EIM [5].

IEM vary from being progressive, degenerative diseases to acute life threatening diseases and can be divided into different types according to the type of the accumulated metabolite substrate such as disorders of carbohydrate metabolism, disorders of protein metabolism, trace metal disorders and lipid metabolism disorders or according to the location (the part of the living cell) its metabolism such as lysosomal storage diseases (LSD) and mitochondrial disorders [6].

Sanderson and co-workers [7] reported the incidence of some selected IEM in the West Midlands-UK in the period between 1999 and 2003. The prevalence of phenylketonuria was 0.8 per 10,000, other amino acid disorders excluding phenylketonuria 1.9 per 10,000, peroxisomal diseases 0.7 per 10,000, glycogen

storage disorders 0.7 per 10,000, mitochondrial disorders 2.0 per 10,000, urea cycle disorders 0.5 per 10,000, organic acidemias 1.3 per 10,000, carbohydrates defects 0.6 per 10,000, lipids and steroids disorders 0.6 per 10,000 and LSD 1.9 per 10,000. The reported figures were similar to the published incidence of IEM in the other geographic locations in the world [7].

The diagnosis of IEM is challenging due to the rarity of individual IEM cases, and their low prevalence, variable clinical signs and manifestations, which overlap with a large number of common diseases [5, 8]. It has been reported that the early diagnosis of IEM is fundamental to prevent progression of the metabolic error and, ultimately, to treat the disorder. Currently, newborn screening is applied in most developed countries worldwide - see section (1.5.3.6).

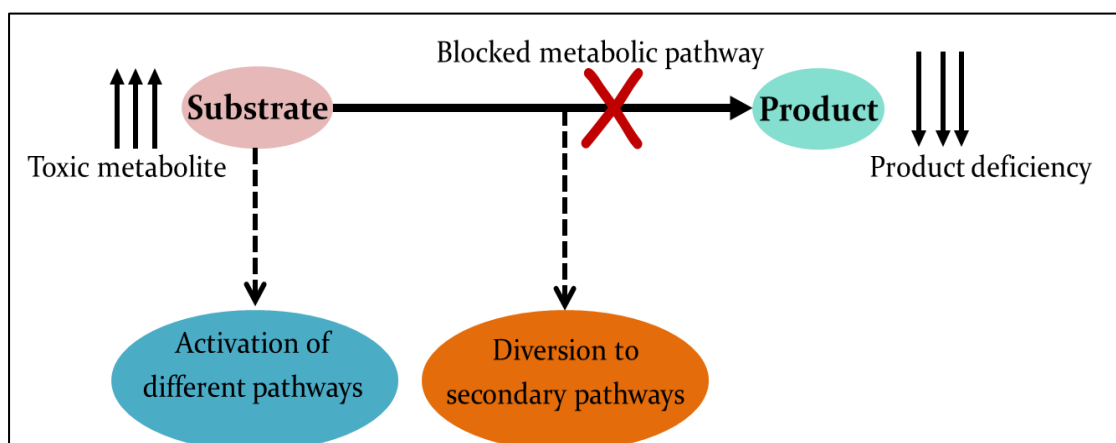


Figure 1.1 Inborn errors of metabolism process: a schematic enlightenment shows the process of inborn errors of metabolisms (IEM) in the living cells: The chemical substrate (metabolite) is transported into the living cell. So, IEM could be resulted from the deficiency of the transporter. In the normal condition, substrate is catalysed to a product metabolite, so any deficiency in the enzyme or the co-factor could lead to IEM. Substrate accumulation resulting from the enzyme deficiency causes IEM. In some cases the accumulated substrate follow alternative metabolism pathway producing toxic metabolites and leading to IEM (metabolic disorders).

1.2 LYSOSOMAL STORAGE DISEASES

Lysosomes are sub-cellular membrane-bound vesicles containing hydrolytic enzymes. These enzymes are produced in the endoplasmic reticulum and are responsible for degradation of carbohydrates, lipids proteins and nucleic acids. Lysosomal storage diseases (LSD) are a large group of rare hereditary disorders that result primarily from an accumulation of un-catabolised macromolecules, largely due to the absence or deficiency of lysosomal enzymes which are responsible for the degradation of metabolites in lysosomes. More than 50 LSD have been described worldwide and according to the substrate that accumulates, LSD can be classified into different types including sphingolipidoses, oligosaccharidoses, mucopolidoses, lipoprotein storage disorders, mucopolysaccharidoses, neuronal ceroid lipofuscinoses, lysosomal transport defects, and others [9-13].

1.2.1 Sphingolipidoses

Sphingolipidoses are a subclass of LSD caused by defects in sphingolipid metabolism. Normally, the synthesis and catabolism of sphingolipids are balanced yielding relatively constant amounts of these molecules in membranes. However, if the activity of a sphingolipid hydrolase is lost or deficient, an accumulation of the substrate molecules can occur. Subsequently, the elevated levels of the accumulated lipids leads to their accumulation in different organs, tissues and body fluids, causing symptoms that characterise the particular sphingolipidosis. Sphingolipidoses include disorders such as Fabry disease (FD), Gaucher disease, Niemann-Pick disease, Sandhoff disease, Gangliosidosis Krabbe Disease (Globoid cell leukodystrophy), Metachromatic leukodystrophy Farber disease and Tay-Sachs disease. All sphingolipidoses can be

fatal at different stages in life. All are autosomal recessive diseases except FD, which is *X-linked* [13-15].

Acid ceramidase (*N*-acylsphingosine deacylase, EC 3.5.1.23; AC) is an abundant enzyme responsible for sphingolipids hydrolysis in cellular lysosomes. AC breaks down the ceramide backbone into the sphingolipid base (sphingosine) and fatty acid chain. AC plays a role in sphingolipid metabolism, mainly in controlling the levels of ceramide and sphingosine in cells. Numerous advances have been made in studying the cellular function and diseases associations of AC. Farber disease, extremely rare autosomal recessive LSD, is associated with AC deficiency. The first “knock-out” mouse model of AC deficiency has been developed, which phenocopies Farber disease in most aspects. The gene encoding AC has been cloned and numerous mutations have been identified, most, if not all of which are associated with Farber disease. Gene cloning has allowed the production of large quantities of the recombinant enzyme and certain properties of the enzyme have been characterised *in vitro*. Several anti-AC antibodies are also available and have been used in studies to investigate the cell biology of this important protein. However, despite these advances, much more information is required fully to understand the role of AC in sphingolipid-mediated signal transduction and human disease [16].

Guce and Garman [17] have posited that FD can be used as a model to study the whole family of LSD. Firstly, it is well known that the mutations observed in FD offer a wide spectrum of structural, clinical and genetic information. Secondly, broad database of mutations have been identified in FD patients. In addition, the three-dimensional structure of the glycoprotein has been solved, which has facilitated a better understanding of the molecular basis of the disease. Thirdly, due to the similarities between FD and other LSD, a better understanding of molecular defects in

FD can facilitate a better understanding of the whole family of LSD. Lastly, FD is characteristically a protein misfolding disease allowing it to be used as a model for other misfolding disorders such as Parkinson's disease. Figure 1.2 shows the metabolic pathways in sphingolipidoses disorders.

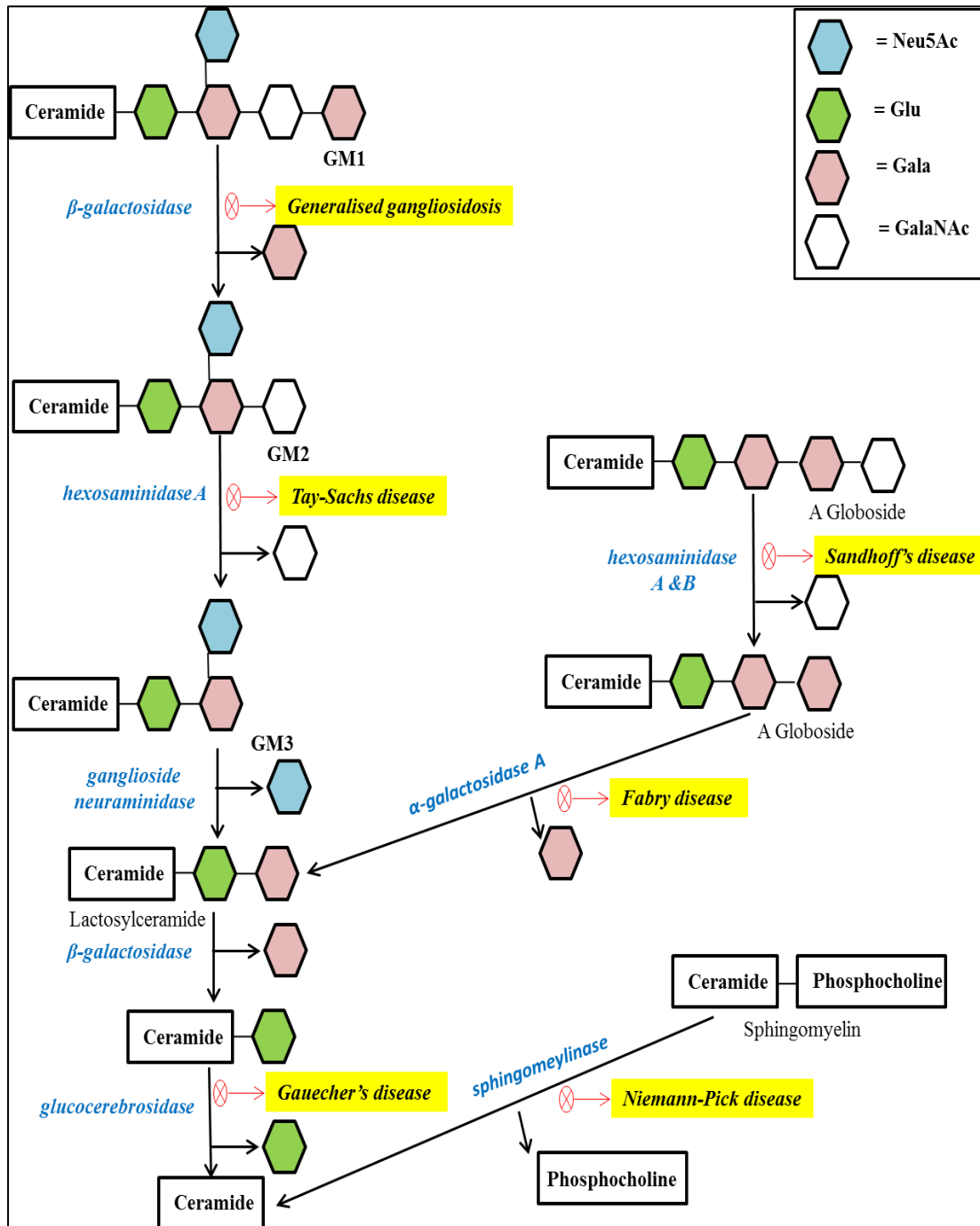


Figure 1.2 Metabolic pathways of sphingolipidoses: a schematic illustration shows the metabolic pathways in sphingolipidoses. Diseases appear with a yellow background and enzymes with italic blue colour.

1.2.2 Glycosphingolipids

Glycosphingolipids (GSLs) are a complex heterogeneous class of lipids. GSLs constitute a large component of the cellular membrane [18, 19]. This class of lipid is characterised biochemically as having two main parts: a hydrophobic ceramide backbone and hydrophilic carbohydrate head group portion. The ceramide backbone is composed from sphingoide base and fatty acid. GSLs are characterised by their diversity which relates to the various structures either in the main ceramide backbone or sugar head group [18-20]. Degroote et al [18] reported that more than 300 carbohydrate chains and 60 sphingoid bases have been identified. These various types of sphingoid bases and carbohydrates chains can combine to form a large diversity of GSLs structures.

The structure of sphingoid bases varies according to their length, saturation, branching and hydroxylation (Figure 1.3). 3-ketosphinganine is considered to be the precursor of other sphingoid bases. The main sphingoid base in humans is sphingosine (also known as trans-4-sphingosine, D-erthro-1dihydroxy or 2-aminooctadec-4-ene), which contains 18 carbon atoms with a single and/or double bond. It has been reported that sphingosine represents approximately 80% of the sphingoid bases in the kidney of mammals such as cow [18-21]. Other examples of the main sphingoid bases in human include sphinganine which is a sphingosine lacking C4-C5 double bonds.

The fatty acid is linked to the amino group of the sphingoid base via an amide bond. The variety of these fatty acids contributes to the heterogeneity of GSLs. The fatty acids vary in the length, saturation, and hydroxylation based on the host cell type. They are typically more than 16 carbon atoms and fully saturated. In certain conditions, they are unsaturated at carbon 15 or hydroxylated at carbon 2 (Figure 1.3).

The carbohydrate head group in GSLs are categorised into two types according to the oligosaccharide linked to the ceramide backbone. These two types are Glucose (Glu β 1-) and galactose (Gal β 1-). However, although rare, other types of sugars can link to the ceramide backbone, as has been observed in human colon cancer cells such as Fucose [Fuc α 1-] [18]. The synthesis of GSLs comprises several steps, starting by the condensation of serine and palmitoyl-CoA to 3-ketosphinganine.

In human living cells, sphingolipids are categorised into two main types' sphingomyelin and GSLs. GSLs synthesis is essentially initiated from the hydrophobic ceramide backbone. Subsequently, a glucosylation process of ceramide is achieved generating glucosylceramide. The latter is considered the source of about 400 various species of GSLs.

Based on the features of the sugar head groups, GSLs can be categorised as follows [22]:

- a- According to the structure of sugar head groups, GSLs can be categorised into: gal-series glycolipids, muco-series glycolipids, ganglio-series glycolipids, lacto-series glycolipids and globo-series glycosphingolipids.
- b- According to the number of sugar head groups, GSLs can be categorised into: poly-glycosylceramide, penta-glycosylceramide, tetra-glycosylceramide, tri-glycosylceramide, di-glycosylceramide and mono-glycosylceramide.
- c- According to the substitution of sugar head groups, GSLs can be categorised into: phosphoinositido-glycolipids, gangliosides-glycolipids, sulfatides-glycolipids and neutral glycolipids.

Despite the fact that GSLs are chemically well characterised, the biological functions of GSLs and their synthesis through ceramide glycosylation process were not fully

characterised. Nevertheless, GSLs-deficient cell lines and the isolation of a cDNA clone for glucosylceramide synthase, has revealed clearer insights into the biological functions of these complex GSLs molecules [23].

GSLs play vital roles in the living cell. Many functions are provided by GSLs in the cytosolic surface of the cellular membranes. GSLs play significant roles in the sorting of proteins, allowing selective intracellular membrane transport. GSLs are involved in the recognition and signalling on the cell surface.

It has been reported that some GSLs are present in tumour cells at levels that exceed those in normal cells, findings suggestive of a role of GSLs as tumour-associated antigens [24, 25]. Moreover, it has been postulated that GSLs play a role as cell surface antigens in the differentiation of different cells during embryonic development [22, 24]. In addition, other biological roles for GSLs have been suggested, such as acting as receptors for bacterial protein toxins, adhesion roles in the cell surface and immunomodulatory and roles in signal transduction [26].

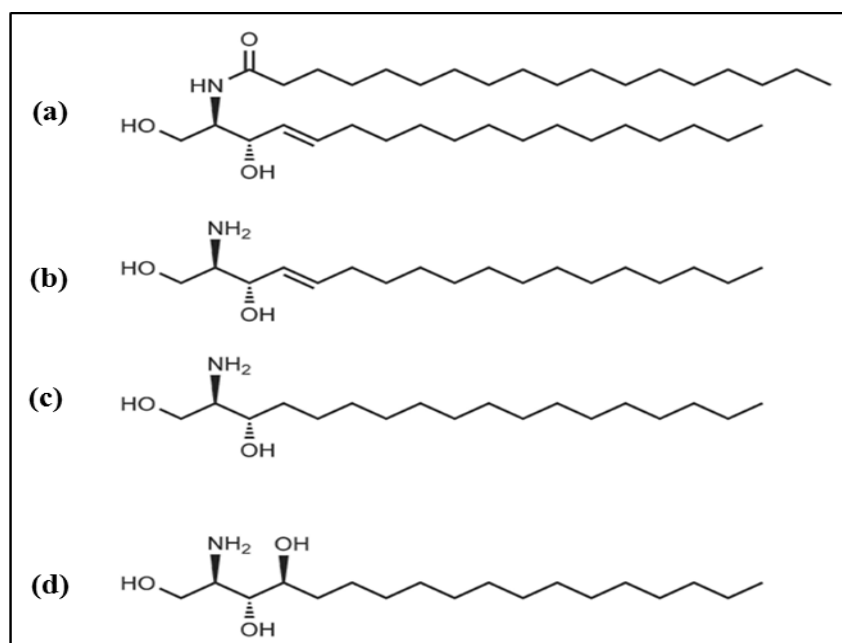


Figure 1.3 Chemical structures of ceramide and sphingoid bases: (a) Ceramide consist a sphingosine moiety (18 Carbon + a single double bond) linked by an amide bond to a fatty acid (16 Carbon). The chemical structures of sphingoid bases: (b) Sphingosine. (c) Sphinganine. (d) Phytosphingosine (Figure adapted from [20]).

1.3 FABRY DISEASE

1.3.1 Overview

Fabry disease (FD, OMIM#301500) is inherited multi-systemic *X-linked* LSD. The defect in the key enzyme results from a mutation in α -galactosidase-A (*GLA*) gene which results in the expression of a defective enzyme [17, 25, 27-30]. This inborn error of GSLs metabolism results from the absence or deficiency of alpha-galactosidase-A (α -gal-A, also known as ceramide trihexosidase, EC 3.2.1.22) which is responsible for the hydrolytic cleavage between the galactose residues in galactosylgalactosylglucosylceramide also known as globotriaosylceramide (Gb3), galactosylgalactosylglucosylsphingosine also known as globotriaosylsphingosine (Lyso-Gb3) (Figure 1.4) and their related isoforms and analogues. Moreover, it has been reported that other GSLs such as galabiosaosylceramide (Ga2) and blood group B glycolipids can be affected by this enzymatic defect. The none-metabolised GSLs accumulate in the lysosomes of various cells, biological fluids, and various body organs and tissues such as skin, eye, unmyelinated nerves, small blood vessels, vascular endothelium, heart and kidney. GSLs accumulation results in a range of symptoms that affect the kidneys, heart, brain, eye, and skin [17, 25, 27-30]. The disease process is based on accumulation of the un-catalysed GSLs in lysosomes. Every cell except red blood cells has lysosomes. The lysosome in the affected organs become enlarged with storage and eventually may burst or leak and trigger secondary reaction such as inflammation.

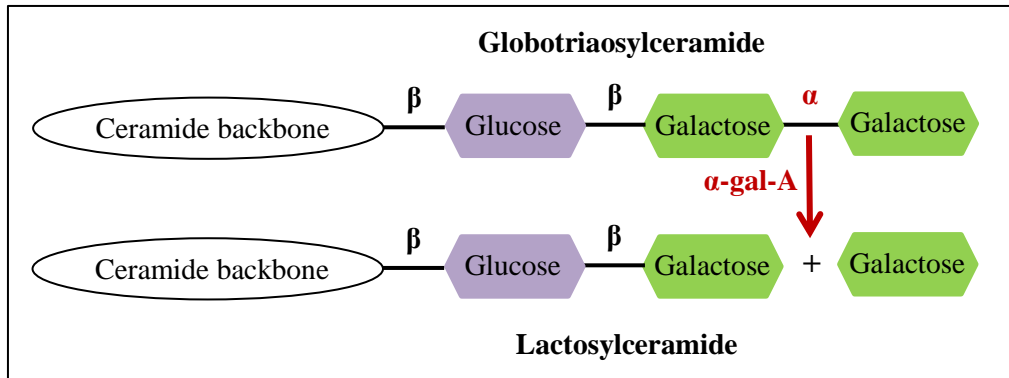


Figure 1.4 Metabolic pathway of Gb3: a schematic illustration for the degradation pathway of globotriaosylceramide (Gb3), where the hydrolytic enzyme alpha galactosidase A (α -gal-A) is responsible for the hydrolytic cleavage between the galactose residues forming lactosylceramide. In the same manner the deacylated form globotriaosylsphingosine (Lyso-Gb3) is degraded in presence of α -gal-A forming lactosylsphingosine.

1.3.2 History of Fabry Disease

Fabry disease (FD, also known as Anderson-Fabry disease) was first described in 1898 by two dermatologists working independently in England (William Anderson) and Germany (Johannes Fabry). The disease was termed ‘angiokeratoma corporis diffusum’ due to its dermatological manifestation [31-33]. In 1947, Ruitter and collaborators described the disease as a vascular disease [34]. Later, in 1953, the disease was classified as a lipid storage disorder [35]. In 1955, lysosomes and their role in living cells were described by the scientist Christian de Duve (Belgium) [36]. The concept of LSD was established by Henri-Gery Hers in 1965 after his discovery of the aetiology of Pompe disease where he found that the responsible deficient enzyme could work better at an acidic pH suggesting that this enzyme being localized in lysosomes [37, 38]. In 1963, Sweeley and Klionsky demonstrated that the glycolipids ceramide trihexoside (now known as Gb3) accumulate in different organs of affected individuals [39]. However, the *X-linked* nature of FD inheritance was first

recognised in 1965 [40]. In 1967, Brady and colleagues linked aetiology of the disease with deficiency of Ceramide-trihexosidase enzyme [41]. Later, Kint identified this lysosomal enzyme to be known as α -galactosidase-A (α -gal-A) [42]. Hamers and co-workers [32] determined that α -gal-A gene is located at Xq22. In 1972, the first trial of treatment was achieved by Desnick using kidney transplantation [43]. The treatment of FD is further developed in the recent decades through enzyme replacement therapy (ERT) [37, 44, 45]. In 2008, Lyso-Gb3 has been introduced for the first time by Johannes Aerts and colleagues [46].

1.3.3 Prevalence of Fabry Disease

FD is the commonest LSD. However, the reported incidence varies considerably. For example, it has been reported that the incidence of FD is very rare, approximately 1:40,000 to 1:117,000 of male-live births in certain parts of the world [47-50] while recent newborn screening data suggest a much higher figure, with frequencies of the order of up to 1:3,100 [48]. Spada and co-workers [48] reported that newborn screening programs revealed a high incidence of late-onset variants phenotype in Fabry patients making the importance and the ideal time of applying the screening for Fabry patients to be questionable. Moreover, the reported statistics among males and females suggest that there are a lot of asymptomatic or undiagnosed cases in females [49, 50]. Due to the *x-linked* nature of the disease, it is predicted that FD prevalence can be twice higher in females compared to Fabry males [51, 52]. The reported global prevalence is shown in Table 1.1 but as many other rare diseases the actual prevalence of FD is likely to be underestimated.

Interestingly, a high prevalence of FD has been shown in previous high risk targeted screening subjects for instance, 2-5% of patients having left ventricular hypertrophy (LVH) showed very low α -gal-A activity, typical of FD [53, 54]. Moreover, other screening work showed that 2-5% of males under 55 years of age suffering from a stroke were confirmed as Fabry patients. However, previous screening studies in chronic hemodialysis patients showed prevalence of FD of 1.2% in males and 0.34% in females [17, 55-62].

Study place (period)	Source	Method	cases	No / 1×10^6	Reference
UK (1980-1995)	The UK FD register - Family history	Prevalence of heterozygous females	60	2.9	[63]
UK (1980-1995)	Local genetic units /Enzyme reference laboratories/Individual doctors' records	Prevalence of Hemizygous males	98	2.7	[64]
Netherlands (1970-1996)	Prenatal and postnatal LSD diagnoses labs	Birth prevalence = number of cases born in a certain period/total number of live births in the same period	27	2.1	[65]
Australia (1980-1996)	2 centres for enzymatic analyses	Birth prevalence = (postnatal + prenatal enzymatic diagnoses) / number of births	36	8.5	[47]
North Portugal (1982-2001)	Prenatal and postnatal LSDs diagnoses centre	Birth prevalence = (postnatal + prenatal enzymatic diagnoses) / number of live births	1	1.2	[66]
Turkey (1997-2002)	Two main reference centres for diagnosis of sphingolipidoses by enzyme analysis of patients under 5 years suspected of LSD	Birth prevalence = number of cases born within a certain period / total number of live births in the same period	1	0.15	[67]
North Italy (2004-2006)	Northern Italy	Neonatal screening	12	300	[48]
Taiwan (2006-2008)	Taiwan	Neonatal screening	73	800	[68]

Table 1.1 Prevalence of FD: A table shows the frequencies of FD in different geographic locations in the world [27].

1.4 THE GENETIC BASIS OF FABRY DISEASE

The deficiency of α -gal-A enzyme activity is caused by mutation in the *GLA* gene. This gene is located in Xq22.1 on the long arm of the X chromosome, which spans approximately 13kb of genomic sequence and contains a total of 7 exons spanning 92 to 291 base pairs in length; the spliced full-length mRNA generates a cDNA that is 1290 base pairs in length. The gene encodes a polypeptide of 429 amino acids including a 31-amino acid signal peptide [17].

The genetic basis of FD is well established. The α -galactosidase-A gene has been sequenced and hundreds of mutations identified [69]. According to the Human Gene Mutation Database (last accessed at www.hgmd.cf.ac.uk, May, 23, 2016), more than 800 mutations in the *GLA* gene have been described. Cebada and colleagues [70] suggested that more than one modification can cause FD, recommending that the whole gene should be sequenced to confirm the presence of FD-related mutations.

Mutations can be of various types. In classical FD, affected males possess any one of a number of pathogenic allelic defects of the *GLA* gene [69, 71]. The most common and frequent ones are point mutations including missense or nonsense nucleotide substitution (approximately 70%), other defects such as small deletions, insertions, polymorphism, large and small gene rearrangements and genetic changes that impact on mRNA splicing [17, 70, 72, 73]. The missense mutations have one of three effects:

- 1- It causes an alteration to the active site of the enzyme.
- 2- It can affect correct folding and hence stability of the resultant protein.
- 3- It can negatively affect the enzyme function.

Most of the identified mutations in the *GLA* gene are family specific and, as such, occur in single-family pedigrees [69, 71, 74-77]. Unlike mutations that impact on the

protein, mutations involving CpG dinucleotides upstream of the promotor region have been found in unrelated families of different ethnicities and geographic backgrounds. Haplotype analysis of mutant or variant alleles that have been identified in several families has revealed that those families carrying rare alleles are probably related, while those identified with mutations involving the CpG islands are not [78]. To date, the identification of novel mutations; data is not available regarding the frequency of germline mosaicism.

On the other hand, a number of *GLA* variants have been identified in atypical late onset Fabry patients, such as D313Y, R118C, S126G, IVS0-10C>T, IVS4-16A>G and IVS6-22C>T. The variant D313Y is a common variant identified within exon 6 of the *GLA* gene. Compared to wild-type *GLA*, this variant displays decreased enzymatic activity in vitro and reduced activity at neutral pH, the net result being low plasma α -gal-A activity. This variant has been identified in approximately 0.45% of normal individuals (n = 800 alleles). As this variant possesses approximately 60 % of the activity of the wild type protein, D313Y, like many of the aforementioned variants, is a variant that does not cause typical presentations of FD [79]. Classical phenotypes of the disease associated with mutations resulting in complete loss of the gene product's function while the late onset variants phenotypes of the disease potentially associated with mutations resulting in amino acid substitutions [80].

In Fabry patients carrying cardiac variants, virtually all individuals possess missense mutations or mutations that affect mRNA splicing. Unlike mutated forms of *GLA* observed in classical Fabry patients, these so-called cardiac variant proteins possess residual enzymatic activity [55]. Representative mutations include I91T, R112H, F113L, N215S, M296I, R301Q, and G328R. Interestingly, the N215S mutation has been identified in several unrelated individuals carrying the cardiac variant. Unlike

cardiac variants, virtually all renal variants identified to date are associated with missense mutations [60].

Despite extensive efforts, attempts to establish a correlation between genotype and a particular phenotype have proved challenging. This appears to be due to the variation observed in the age of onset, the rate of disease progression and organ involvement making the clinical presentations variable even in the same family [81].

The heterogeneity of FD suggests that other independent factors such as other genetic modifiers and the environmental factors may play an important role in establishing disease progression in both genders. Nonetheless, it is not clear yet if there are specific factors can have a role in the modification of the disease course. The role of X inactivation in females has been suggested, but this alone cannot explain the clinical variability. It has been reported that typical Fabry females can have skewed X inactivation but inconsistently [80, 82-84]. There are certain mutations which have been noted in both classical and cardiac phenotypes such as A112H, A301G, and G328A suggesting that other modifying factors are involved in dictating disease severity and course. Figure 1.5 shows the chance of transmitting FD from affected parents to their new children.

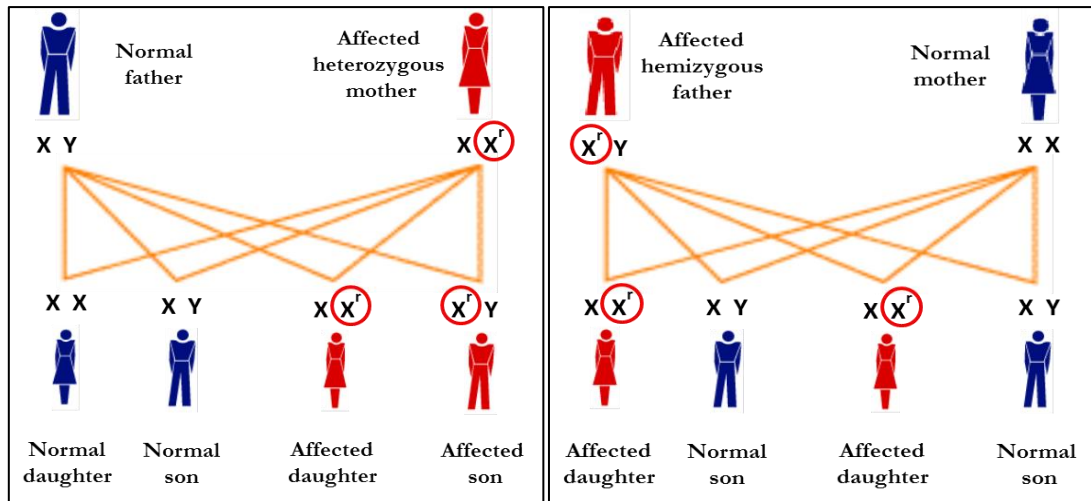


Figure 1.5 Genetic of Fabry disease: The heterozygous Fabry females have a 50% chance during each pregnancy of transmitting the gene to their children (**left panel**). Affected hemizygous Fabry males transmit the disease to all of their daughters and none of their sons (**right panel**).

1.5 THE BIOCHEMISTRY OF FABRY DISEASE

FD results from an error in the metabolic pathway affecting GSLs degradation; mainly Gb3 and its related derivatives. This is due to insufficient/deficient expression and/or activity of the hydrolase key enzyme (α -gal-A). Subsequently, GSLs accumulate in various tissues and organs. The main substrate GSLs is Gb3, which can be substituted into various related derivatives. This is achieved due to either modifications in the sphingoid base, generating various analogues of Gb3, or, additionally due to variations in the fatty acid chain moieties that form various Gb3 isoforms. On the other hand, Lyso-Gb3, which can be generated as described previously by a deacylation process of the major accumulated substrate, Gb3. In the same manner, Lyso-Gb3 can be substituted into various related derivatives due to modifications in the sphingoid base, generating various derivatives of Lyso-Gb3 (termed as Lyso-Gb3 analogues). Therefore, Lyso-Gb3 and its analogues play a role

in the diversity of FD. The causative role of several Lyso-Gb3 analogues has been postulated to be involved in the aetiology and pathogenesis of FD [85].

1.5.1.1 The Human α -Galactosidase-A Enzyme

The human body contains two types of α -Galactosidase enzymes: A and B. The α -gal-A enzyme is a lysosomal exoglycohydrolase enzyme which is responsible for degradation of GSLs; mainly Gb3 and its derivatives such as Lyso-Gb3. Any defect in the activity of α -gal-A enzyme will block the degradation pathway and the subsequent accumulation of un-catabolised metabolites in various locations in the body. The structure of the key-enzyme α -gal-A can be studied either by three-dimensional X-ray crystallography [86] or by gel electrophoresis [87]. The α -gal-A enzyme functions as a homodimer, comprising two 50 kDa glycosylated subunits. It contains three functional N-glycosylation sites. Specifically, α -gal-A enzyme recognizes the GSLs substrates in the terminal α -galactose. Therefore, this key enzyme is responsible for the hydrolytic cleavage between the galactose residues in GSLs. This enzyme cannot act on the other portions of GSLs such as the terminal β -galactose or GSLs containing α -N-GalaNAc.

One of the physical properties of α -gal-A is the requirement of an acidic pH to function optimally. It has been demonstrated that GSLs hydrolysed by α -gal-A in human lysosomes is achieved at an acidic pH. This finding has been proven by Aerts and co-workers *in vitro*, who showed that the degradation of Lyso-Gb3 was low at neutral pH [46]. Moreover, Dean and co-workers [87] have shown that *in vitro*, the catabolism efficiency of Lyso-Gb3 by α -gal-A is 50 fold less compared to Gb3.

It has been shown that the activity of α -gal-A is commonly absent in symptomatic hemizygous Fabry males. Nonetheless, a various residual activity of α -gal-A ranging between normal and nearly completely absent, has been shown in both Fabry females

and atypical late onset Fabry males[88]. This residual enzyme activity cannot be used as a diagnostic marker or as a strong tool to study the clinical severity of FD in females [89].

1.6 PATHOPHYSIOLOGY OF FABRY DISEASE

FD is characterised by the complete or partial loss of α -gal-A activity. The loss of enzymatic activity, results in the accumulation of neutral GSLs mainly Gb3 and its derivatives in the lysosomes of cells. Smooth muscle and endothelial cells of target organs are primarily affected resulting to the predominance of vascular complications seen.

The GSLs accumulation compromises cellular function and may lead to cell death. This, in turn, can trigger inflammation and the generation of fibrotic responses [80]. It has been estimated that *GLA* variants or mutants that still retain an average of 7.5% of residual enzyme activity is sufficient to prevent the accumulation of GSLs and the ensuing clinical manifestations [29, 80]. Unlike Fabry hemizygotes, Fabry heterozygotes can have significant amounts of residual α -Gal-A activity. Despite this they still display a spectrum of clinical disease extending from being asymptomatic to severe organ damage. Hence residual α -gal-A activity is a poor predictor of the clinical course [29, 80].

Over time, the aberrant accumulation of GSLs leads to organ dysfunction and the clinical manifestation of FD. While the mechanisms involved in mediating tissue damage are not fully resolved, they are thought to arise through poor perfusion of

oxygen, caused by the accumulation of GSLs in the vascular endothelium lining key organs such as the kidneys, heart, nervous system, and skin [29, 80, 90].

The accumulation of GSLs in the endothelial cells of the renal vasculature, podocytes leads to vascular occlusion, glomerulosclerosis and ultimately renal failure [91, 92]. While accumulation of GSLs in mesangial cells has been documented, its contribution to renal failure is unclear [93].

The accumulated GSLs in all major cell types' cellular components of the heart (endothelial cells, vascular smooth-muscle cells, cardiomyocytes, valvular fibroblasts and conduction system cells) play a role in the pathophysiology of cardiac disease in Fabry patients. However, other mechanisms such as hypertrophy, apoptosis, necrosis, and fibrosis may contribute to cardiac disease as well [80, 94].

Fabry patients are at increased risk of developing strokes. This may occur through cardiogenic embolism or aberrant activation of the coagulation pathway in vessels due to heightened reactivity [95]. These factors may be exacerbated in Fabry patients who have other underlying prothrombotic risk factors [96]. Abnormalities in the cerebrovasculature and its associated effects on cerebral circulation with substantial cerebral hyperperfusion in the posterior cerebral circulation, can all contribute to neurovascular disease [97, 98].

The acute pain and acroparesthesia frequently observed in Fabry patients are believed to occur due to the accumulated GSLs in neurons, spinal cord and dorsal root ganglia, which can lead to atrophy of small unmyelinated nerves [29, 90]. Moreover, poor perfusion of peripheral nerves has a role in the pathophysiology of these disease manifestations [90].

1.7 CLINICAL MANIFESTATIONS OF FABRY DISEASE

The clinical manifestation of FD are heterogeneous with multi-systemic manifestations including a wide spectrum of symptoms that are manifest in the skin, eye, nervous system, heart, kidneys and other body organs and tissues [29]. Onset of organ involvement for classical Fabry patients is age dependent (Table 1.2). Generally, in classical *X-linked* disorders, females act as carriers of a mutation but do not show symptoms (e.g. red-green blindness in males). Nonetheless, heterozygous Fabry females can present in the same way as their male counterpart perhaps at a later age [29, 99-101].

Male sufferers show symptoms at an early age, while females display symptoms when older. Both male and female Fabry patients covered in a study by Mehta [50], showed symptoms of the disease at different ages. The study showed that disease severity in the affected organs increase considerably with age for both genders. .

The typical onset of FD manifestations in childhood include angiokeratomas, hypohidrosis, corneal opacities and painful acroparesthesias [30, 102].

It has been reported that Fabry patients with renal and a cardiac variants that have splicing and missense mutations don't show the classical onset of the disease in childhood such as angikeratoma. Fabry patients with the cardiac variant develop arrhythmias, LVH and cardiomyopathy. All these manifestations can appear from 40 years of age onwards. Moreover, Fabry patients with the renal variant who don't develop the typical early symptoms can develop proteinuria and subsequently end-stage renal disease in the fifth decade of life [30, 48, 53, 60, 103-105]. MacDermok [64] has stated that the life span of Fabry patients is reduced on average by 20 years compared to normal healthy individuals. On the other hand, previous studies

suggested that within an average of a decade later, Fabry heterozygous females can develop similar manifestations exactly like Fabry hemizygous males, regarding the severity and the expanded spectrum of various manifestations [106, 107].

Symptoms	Age at onset
<ul style="list-style-type: none"> - Neuropathic pain - Ophthalmological abnormalities (cornea verticillata and tortuous retinal blood vessels) - Hearing impairment - Dyshidrosis (hypohidrosis and hyperhidrosis) - Hypersensitivity to heat and cold - Gastrointestinal disturbances and abdominal pain - Lethargy and tiredness - Angiokeratomas - Onset of renal and cardiac signs, e.g. micro-albuminuria, proteinuria, abnormal heart rate variability 	Childhood and adolescence (0-16 years)
<ul style="list-style-type: none"> - Extension of any of the above - Proteinuria and progressive renal failure - Cardiomyopathy - Transient ischaemic attacks, strokes - Facial dysmorphism 	Early adulthood (17-30 years)
<ul style="list-style-type: none"> - Worsening of any of the above - Heart disease (e.g. LVH, angina, arrhythmia and dyspnoea) - Stroke and transient ischaemic attacks - Osteopenia and osteoporosis 	Later adulthood (>30 years)

Table 1.2 Typical signs and symptoms of FD [108]: a table shows the various signs shown by Fabry patients with the expected age of the onset of these signs.

The clinical manifestations of FD can vary by the type and extent of the organ involvement. The clinical manifestations can be classified according to the organ in which the damage occurs. These are considered below in more detail:

1.7.1 Neurological manifestations

1.7.1.1 Acroparesthesia

This painful small fiber neuropathy presents with intermittent bouts of burning and tingling pain in the hands and feet. It is the earliest manifestation and most disabling. Acroparesthesia can be triggered by factors such as exercise, stress, fever, fatigue and changes in the weather or temperature [80]. As with other affected tissues, the accumulation of Gb3 in Schwann cells and dorsal root ganglia together with deposits in neurons of the CNS disrupt neuronal cell function. However, the effect of Gb3 accumulation in neuronal cells appears to have little.

1.7.1.2 Cerebrovascular manifestations

Cerebrovascular manifestations in FD were not recognized in early studies because renal and cardiac events were more evident and computerized tomography and magnetic resonance imaging (MRI) were not readily available [109, 110]. However, in recent years, many cerebrovascular abnormalities, including early onset stroke, transient ischemic attacks, hemiparesis, diplopia, dizziness, nystagmus, dysarthria, dementia and ataxia have been identified in Fabry patients [71, 111, 112]. Of particular concern is the vulnerability of Fabry patients to early ischemic stroke, due in part to a thickening of small arteries; an effect which is due to the accumulation of associated lipids mainly Gb3 [113]. The formation of such thrombi may be enhanced due to inflammation and adhesion of neutrophils and monocytes to endothelial cells lining the vessels [114]. In a retrospective study performed by Grewal, ischemic strokes were noted in 8 of 33 Fabry patients (24%) [113].

The risk of haemorrhagic stroke is increased in Fabry patients because of hypertension due to renal failure [113]. Recently, MRI employed in order to quantify cerebral vasculopathy in 50 Fabry patients with an average age of 33 [115]. However, only 38% of Fabry patients showed a correlation between the presence of cerebral lesions and neurologic symptoms, neurological involvement increased with age, with all patients over the age of 54 years displaying some form of cerebrovascular involvement [115]. In a study by Mehta and colleagues, who studied 366 Fabry patients, cerebrovascular events appeared to be more prevalent in females than males, with 27% of 165 Fabry females and 12% of 201 Fabry males experiencing a stroke, transient ischemic attack, or prolonged ischemic neurologic deficit [50]. A study of white matter lesion (Figure 1.6) in 27 Fabry patients showed a comparable incidence (36% of 14 Fabry females and 31% of 13 Fabry males) and the severity level in both genders [116].

The main involvement of the CNS seems to be attributed to effects on endothelial cells within the vasculature which results in Cerebrovasculopathy and an increased likelihood of a stroke or transient ischemic attack. Unlike many other LSD effects on the CNS appears to be less prominent in FD. However, like other sphingolipid disorders, neurological abnormalities are common [71, 117, 118]. Using Proton magnetic resonance spectroscopy the presence of diffuse central neuronal involvement in cortical and subcortical locations has been identified in Fabry patients [119]. The degeneration of both small myelinated and unmyelinated nerve fibres has been documented in Fabry patients, a finding which may account for the pain and hypohidrosis associated with FD [71, 118]. In a similar vein, the loss of small peripheral sensory neurons in the dorsal root ganglia [118] and small fiber

dysfunction [120] may be related to autonomic neuropathy, pain and temperature sensitivity.

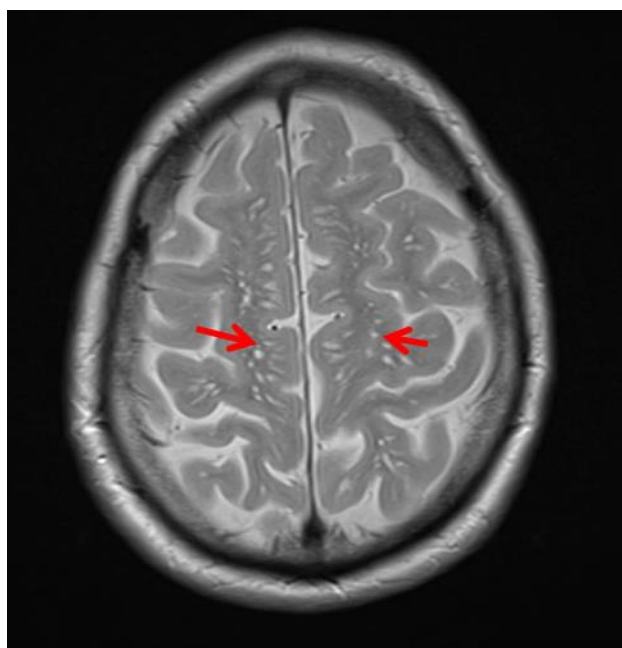


Figure 1.6 Clinical manifestations, White matter: MRI section for a classical Fabry patient enrolled in the current study. Brain MRI section shows white matter spots. There are several multiple small bright spots shown bilaterally within peritrigonal white matter.

1.7.1.3 Sensory neural hearing loss

While the clinical manifestations of FD in classical patients involve the kidney, heart, and brain, several recent studies have reported that FD patients also experienced some form of hearing loss [121-123]. Sakurai and colleagues reported that sudden sensorineural hearing loss was common in both male and female patients, although the incidence was always higher in affected Fabry males. Furthermore, all patients with renal dysfunction also displayed some form of hearing impairment [123]. While the exact mechanisms involved are still unclear, studies in the mouse model of Fabry disease have identified the presence of densely-stained material in cells of the inner ear stria vascularis [124]. Studies performed in Europe and North America, have

reported a beneficial effect of ERT on hearing impairment in Fabry patients [122, 125].

1.7.2 Skin manifestation

1.7.2.1 Angiokeratomas

One distinct clinical feature of Fabry patients, is the presence of cutaneous vascular lesions termed angiokeratomas [126]. These lesions are found almost universally among affected Fabry males and in more than half (55%) of Fabry females[106]. These clusters of reddish purple, non-blanching lesions are usually distributed predominantly on the buttocks, groin, umbilicus, and upper thighs. Their size ranges from pinpoint to several millimetres. Their shape can be flat or marginally raised. Angiokeratomas lesions are very rare in childhood but they become more common starting from adolescence and early adulthood; with age, they become larger and more numerous.

1.7.2.2 Hypohidrosis/Anhidrosis

Hypohidrosis or anhidrosis is another classic feature of FD, with affected individuals displaying an impaired ability to sweat or perspire. This condition leads to dry skin and heat intolerance (hot or cold temperature extremes); individuals also frequently exhibit exercise intolerance. In classical Fabry patients, this symptom normally presents in childhood or adolescence. This condition may result from selective peripheral nerve damage [71, 127] or the formation of cytosolic lipid deposits in capillaries surrounding sebaceous glands [128]. In almost 50% of cases, the production of tears and saliva are reduced [129].

1.7.3 Gastrointestinal Tract

Gastrointestinal disturbances manifest themselves as episodes of diarrhoea, vomiting, nausea, weight loss, irritable bowel syndromes and post-prandial bloating and pain [49, 130]. The improvement in the baseline diarrhoea in some Fabry patients has been reported with a decrease in the severity and frequency abdominal pain after ERT administration [131, 132].

1.7.4 Kidney Manifestations

Renal pathology is a hallmark of classical FD and appears to be one of the most frequent causes of death. The effects are progressive and, in most instances occur during the third to fifth decade of life. Isosthenuria due to concentration defects is among the first manifestations of kidney malfunction; however, in most cases, this does not prompt testing for FD diagnosis [133]. During late adolescence, Fabry patients develop proteinuria, indicating glomerular dysfunction due to Gb3 accumulation in the podocytes, progressive renal failure [71].

Azotemia usually occurs by the third to fifth decade of life [71] while renal failure has also been reported in younger Fabry patients [134]. Azotemia and progressive proteinuria reflect deterioration in renal function. Uremia usually ensues and heralds end-stage renal disease. According to data from the Fabry Registry, 430 of 1158 males and 507 of 1077 females (Genzyme-sponsored Fabry Registry) exhibited chronic kidney disease.

In one study, performed on young hemizygous Fabry males, diffuse lipid accumulation was observed in glomerular, vascular, and interstitial cells, and in distal tubules and loops of Henle. With increased age, intimal thickening and degenerative changes were also apparent [135].

Collectively, the effects on the kidney manifest themselves as progressive renal Insufficiency (proteinuria, isothermia, azotemia, high plasma creatinine levels) and an advanced stage of renal failure; the latter is the main cause of high mortality in FD.

1.7.5 Heart Manifestations

The frequency of Cardiac involvement in Fabry patients is variable and increases in severity with age like most aspects of FD. The most common cardiac anomalies include LVH (Figures 1.7 and 1.8), coronary artery disease, regional systolic dysfunction, diastolic dysfunction [136] and conduction abnormalities. These disorders precipitate congestive heart failure, arrhythmias and myocardial infarction [71, 137, 138].

Some of the cardiac structural changes associated with FD may not be related to significant loss of cardiac function, but may serve as useful markers of disease progression and severity [139]. Defects identified by electrocardiography include LVH, ST-T wave changes, conduction abnormalities and arrhythmias [71]. Echocardiographic (ultrasound) abnormalities include concentric LVH, aortic root dilatation, dilated cardiac chambers, myocardial dysfunction, and valvular disease (usually involving the mitral valve) [134, 140, 141]. Aortic root dimension and Left ventricular wall thickness usually increase with age [142, 143]. Contrast-enhanced MRI shows delayed gadolinium enhancement indicating myocardial fibrosis [144].

Cardiac events were reported to occur in 23% of both males and females enrolled in the Fabry Registry as of December 31, 2007. These cardiac-related events were occurred at median ages of 42 years in males and 48 years in females [Genzyme Fabry Database]. A prospective study performed by Kampmann and colleagues [145] identified a high correlation between the appearance of LVH and patient age in a

small cohort of females diagnosed with FD (mean patient age of 39.6 years with a range of 6.1 to 70.8 years). In this study, 25 of the women over age 45 were diagnosed with LVH [145]. In another study from the same group, 39% of women and 30% of men were identified as having right ventricular hypertrophy [146].

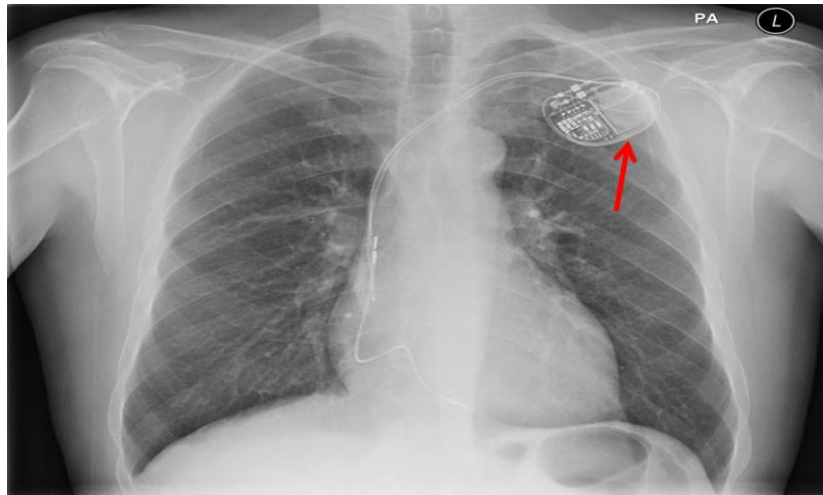


Figure 1.7 Chest X-Ray showing a Permanent Pacemaker Implantation in patient with FD: It is inserted for Bradyarrhythmia which is a known cardiac complication in this group of patients.

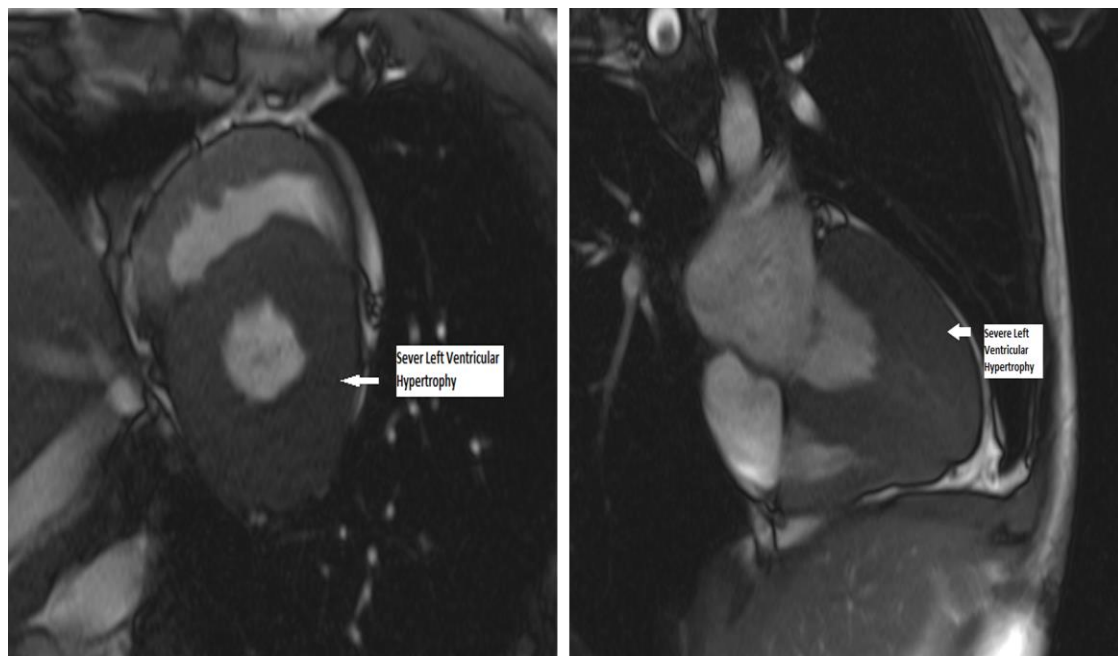


Figure 1.8 Clinical manifestations, left ventricular hypertrophy (LVH): two MRI sections from a male classical Fabry patient enrolled in the current study. MRI sections show LVH. It has been reported that cardiac mass may increase in Fabry patients.

1.7.6 Pulmonary Manifestations

Hemizygous Fabry patients may develop airway and pulmonary defects that can result in bronchitis and a shortness of breath (dyspnea) [71]. Although not considered as a principle feature of FD, patients can experience airway obstruction, an effect that is due to airway narrowing due to GSLs deposits in epithelial cells lining the airways. In a recent study, 36% of Fabry males were found to have some form of airway obstruction [147]. The extent of respiratory symptoms and obstructive impairment has been found to correlate with increased age. Interestingly, pulmonary manifestations were only observed in patients with either frameshift mutations or the presence of the D264V missense mutation, suggesting a possible genotype/phenotype correlation [147].

1.7.7 Musculoskeletal and Growth Abnormalities

Although somewhat less common, Fabry hemizygous males can develop permanent deformity in their hands. This usually involves the distal interphalangeal joints of the fingers, limiting extension of the terminal joints. Other manifestations include a general retardation in growth, a delayed onset of puberty, and sparse, fine facial hair [71].

1.8 DIAGNOSIS OF FABRY DISEASE

Due to the non-specific nature of FD, diagnosis can be delayed for years if not decades [49, 107, 148]. Classical Fabry hemizygous males normally have the disease onset in early childhood or adolescence. The most common signs and symptoms include bouts of severe pain in the extremities (acroparesthesias), the appearance of

cutaneous vascular lesions (angiokeratomas), and an inability to sweat (anhidrosis, hypohidrosis). Affected adults also suffer from unexplained fatigue and intolerance to extremes of temperature. Additional symptoms include corneal and lenticular opacities and the excretion of protein in the urine (proteinuria). Deterioration of renal function progresses during life to complete renal failure and death (end-stage renal disease).

Due to residual α -gal-A activity, Fabry heterozygous females typically have milder symptoms and present at a later age of onset than classical Fabry males. While affected Fabry females are rarely asymptomatic, they may have symptoms that are less severe than those observed in classical Fabry males. Fabry males who possess residual α -gal-A activity may display cardiac variant or renal variant phenotypes. Affected individuals usually present in the sixth to the eighth decade of life with LVH, mitral valve defects or cardiomyopathy. While such patients may show proteinuria, there is no evidence to suggest this will progress to end stage renal disease. Alternatively, they may display a renal variant phenotype. This is associated with end stage renal disease yet rarely experience symptoms such as acroparesthesias and angiokeratomas). Hoigne and others [149] have reported that the combination of four criteria in patients presenting with cardiac involvement (LVH), acroparesthesia or polyneuropathy, anhidrosis, the absence of hypertension, and the presence of Sokolow criteria for LVH, proved useful in distinguishing FD from other disorders. Pieroni and others [150] noted that Fabry's cardiomyopathy could be identified by ECG and the appearance of a hypoechogenic line described as a subendocardial with a binary appearance, which was absent in controls and patients with hypertrophic cardiomyopathy.

Once the clinician suspects that the patient may have FD, a diagnosis can be made in men by screening plasma or purified leukocytes for α -gal-A activity, or by gene sequencing [151]. Although the concentration of Gb3 is often raised in the plasma or urine of patients with the classical form of FD, a broad range of values and plasma concentrations exist that might be normal in Fabry females. The concentration of Gb3 is also frequently used to monitor the effectiveness of ERT in Fabry patients with low sensitivity. However, the levels of plasma or urinary Gb3 are only of diagnostic use if they are sufficiently high before the start of ERT [152]. Unlike the situation in males, the diagnosis in Fabry females can only be achieved by gene sequencing, and the identification of a disease-associated mutation.

While increased levels of protein, the presence of red blood cells, desquamated epithelial cells, and characteristic Maltese crosses are observed in urinary sediments obtained from adolescents, significant signs of deterioration in kidney function are not manifest until the patient is older.

Evidence of renal damage can be obtained using polarization microscopy of urinary sediment, which may reveal the presence of birefringent lipid globules with the characteristic Maltese cross configuration; so-called “Maltese crosses.” The presence of birefringent inclusions in the urinary sediment (i.e. fat-laden epithelial cells or mulberry cells) may also be noted on closer examination.

1.8.1 Current methods of Fabry Disease Diagnosis

The diagnosis of FD is mainly based on the individual's clinical presentation. Currently, the diagnosis is made through a variety of methods, and includes:

- Measurement of the level of α -gal-A activity by an enzyme assays; usually performed on isolated leukocytes from blood. However, this enzyme assay is not

reliable for the diagnosis of classical FD in females due to the random nature of X-inactivation.

- Molecular genetic analysis of the *GLA* gene is the most accurate method of diagnosis in females, particularly if the mutations have already been identified in male family members. Almost 800 mutations have been identified and those which predispose to classical or non-classical FD have been recognised.
- Measurement of plasma or urinary GSLs levels such as Gb3 or Lyso-Gb3 and their isoforms/analogues by HPLC or LC-MS/MS.
- Tissue biopsy taken from the kidney or the heart to confirm the build-up of lipid deposits.
- Various investigations such as kidney function test, ECG and MRI.

Zarate and co-workers [80] suggested standard criteria for FD diagnosis as shown in Table 1.3.

Group	Diagnosis
Prenatal diagnosis	<ul style="list-style-type: none"> • The <i>GLA</i> gene sequencing assessing for a known familial mutation
Males	<ul style="list-style-type: none"> • The α-gal-A enzymatic activity in peripheral leucocytes or plasma • The <i>GLA</i> gene sequencing and identification of disease-causing mutation or testing for all known familial mutations
Females	<ul style="list-style-type: none"> • The <i>GLA</i> gene sequencing and identification of disease-causing mutation or testing for a known familial mutation (α-gal-A enzymatic activity in peripheral leucocytes is not preferred and plasma levels are unreliable)

Table 1.3 Standard criteria for Fabry disease diagnosis [80].

1.8.2 Challenges and current issues in diagnosis of Fabry disease

The non-specific nature of the symptoms in FD, coupled with its clinical manifestations make the diagnosis of FD difficult [80, 153]. In addition, other factors hamper the diagnosis, such as the rarity of FD, the *X-linked* nature in heterozygous Fabry females, the general lack of awareness of the disease, and some early signs of FD being shared with other more general pathological conditions.

Measuring the activity of α -gal-A in leukocytes can be useful in the diagnosis of adult hemizygous Fabry males [153, 154]. However, the method is inadequate in case of heterozygous Fabry females and late onset Fabry patients such as those with cardiac variants, where the patients can show residual levels of the enzyme activity similar to those in healthy individuals [153, 154]. The molecular analysis procedures could be useful as a diagnostic tool for the individuals having FD history in family members. On the other hand, all the diagnosis procedures mentioned above are of no use in follow up of disease progression and assaying the response to treatment.

Recently, it has been suggested that the accumulation of Gb3 and Lyso-Gb3 and their related isoforms/analogues in biological samples such as plasma and urine can be used as diagnostic biomarkers with inconsistent sensitivity due to the heterogenotypic and heterophenotypic specificities of FD [26, 155]. Despite the biological roles of Lyso-Gb3 in deactivation of the key enzyme and stimulation of the proliferation of cardiomyocytes and other smooth muscle cells, some roles of Lyso-Gb3 in the pathogenesis of the disease are still missing or, at least, unclear [46].

1.9 TREATMENT OF FABRY DISEASE

1.9.1 Enzyme Replacement Therapy

Enzyme replacement therapy (ERT), using intravenous infusion of recombinant α -gal-A, has become the standard treatment for symptomatic Fabry patients since 2001 [35]. Despite its clinical benefit being limited, mainly slowing progression and requires a long time to appreciate the benefit, previous studies showed the importance of ERT in FD administration. It has been reported early initiation of ERT minimizes most of the complications and stabilizes FD progression. It may well be true that damaged tissues may not be reversed by ERT [108].

Two different types of recombinant α -gal-A (genetically-engineered of the human enzyme) have been approved as treatment for symptomatic Fabry patients. The first treatment is agalsidase- α (Replagal from Shire HGT) with a dose of (0.2 mg / kg body weight / 2 weeks). The second treatment is agalsidase- β (Fabrazyme from Genzyme Corp) with a dose of (1.0 mg / kg body weight / 2 weeks) [44, 45, 89]. The consensus is that ERT has a limited impact on the long-term outcome of patients with FD. While the results from long-term registry studies e.g. Fabry outcome survey (FOS) [50] have shown encouraging results, others have shown that the effects of ERT on cardiac, renal, and cerebrovascular outcomes are comparable between treated and untreated cohorts [156, 157].

A recent Cochrane review has also highlighted the poor quality of evidence in favour of ERT for the treatment of Fabry patients. Despite this ambiguity, Desnick and co-workers advocate the use of ERT in all affected males, in Fabry females with significant disease, and those with end stage renal disease undergoing dialysis [151, 158] given that all are at high risk for of developing cardiac, cerebrovascular, and neurologic complications such as strokes.

Aerts's team [46] studied the response of plasma Lyso-Gb3 in Fabry patients, to elongated ERT using agalsidase- α treatment. The study illustrated a significant decrease in the levels of Lyso-Gb3 after ERT with recombinant α -gal-A in the first year, but this decrease did not reach the reported levels of healthy individuals. Aerts and co-workers [46] suggested that the observed drop in Lyso-Gb3 levels after ERT could be a result of intra-lysosomal direct degradation of Lyso-Gb3 or caused by the complete clearance of the stored origin source Gb3.

On the other hand, it has been reported that some hemizygous Fabry patients receiving ERT showed a Lyso-Gb3 levels raise following the early drop; which may result from treatment resistance due to development of α -gal-A antibodies [46, 159-162].

There are many challenges face applying ERT as an effective treatment for Fabry patients. Firstly, ERT is extremely costly [156, 163] for example previous studies showed that the average cost of ERT for a single typical Fabry patient can exceed £8 million per patients life time [156]. Other challenges should be considered such as being ERT an invasive procedure, burdensome for the patients where the dose is repeated each 2 weeks and it can cause infusion reaction. Moreover, production of α -gal-A antibodies has been reported in some Fabry patients under ERT course, which may cause reverse increasing levels for un-catabolised GSLs substrates [159, 162]. Finally, other challenges related to difficulties in the treatment preparation and storage should be taken in the consideration.

The initiation age of ERT in Fabry patients is still debatable. It has been suggested that initiation of ERT at early stage of the disease could enhance the efficacy of the treatment, secure better outcomes and increase lifespan of the patients. Nonetheless, both genders with atypical late onset FD and heterozygous females with typical FD

show residual activity of the key-enzyme α -gal-A [88] and the ERT effectiveness in these groups of Fabry patients is questionable.

Some ERT guidelines suggest different criteria for the treatment initiation in Fabry hemizygous male and Fabry heterozygous females [158]. Nevertheless, a study by Hughes and colleagues [52] suggested that the assessment criteria of ERT in both Fabry genders should be similar.

The levels of Gb3 in biological samples have been measured to monitor the efficacy of ERT with limited sensitivity. Gb3 levels in biological samples decrease considerably within two weeks after starting ERT but may develop α -gal-A antibodies hence Gb3 level may increase again [44, 45, 47, 164]. On the other hand, Lyso-Gb3 levels in biological samples from Fabry patients markedly decreased after starting ERT [165] while the complete normalisation of Lyso-Gb3 levels was not achieved.

Togawa and co-workers [166] studied the levels of Gb3 and Lyso-Gb3 in Fabry mice monitored with recombinant α -gal-A. Considerably, tissue Lyso-Gb3 levels decreased more than Gb3 levels especially in kidney suggesting that Lyso-Gb3 could be more accurate biomarker compared with Gb3 to monitor ERT.

Smid et al. [167] reported that male Fabry patients on ERT with agalsidase- β showed elevated concentrations of plasma Lyso-Gb3 after decreasing the dosage of the enzyme or switching to agalsidase- α ; however, this elevation proved not to be statistically significant. The interpretation of their findings is confounded by the fact that only a small numbers of patients were enrolled in the study, The inclusion of two apparent outliers with very high plasma Lyso-Gb3 concentrations and the multiple changes in the dosage of agalsidase- β made necessary by an unexpected interruption in the supply of the medication also had a negative impact on the study.

Treatment with ERT is very expensive, and cost-effectiveness of ERT is arguable [156, 163] given the high cost of ERT and the efficacy of the treatment in some cases such as heterozygous Fabry females and late onset FD patients (e.g. cardiac variants). Moreover, the time and age where ERT should be administered usually is not known and the effect of production of anti- recombinant α -gal-A antibodies should be administrated [162]. Hence, a reliable biomarker for FD that aids diagnosis and disease monitoring is fundamental to counter the above challenges and to avoid the use of other invasive diagnostic procedures such as cardiac and kidney biopsy.

In order to prove the efficiency of ERT in FD monitoring, many challenges confirm the importance of having a reliable biomarker for FD. These challenges are either related to the treatment system or related the diagnostic procedures [168]. Examples of these challenges are as follows:

- a- Cost-effectiveness of ERT: the reported cost of ERT is very expensive. Moreover, it has been suggested that the efficiency of ERT in typical Fabry hemizygoties is limited in terms of economic evaluation of the treatment in one side comparing with quality of the life, organ necrosis and life expectancy in the other side [156, 163].
- b- Efficacy of various suggested doses of ERT [169-171].
- c- Influence of production of α -gal-A antibodies especially in hemizygoties Fabry males who show typical manifestations of the disease [162].
- d- It is well-known that the other diagnostic procedures for FD may be invasive such as cardiac biopsy and kidney biopsy.

1.9.2 Other treatment Strategies for Fabry disease:

Other therapeutic options of FD are under study including pharmacological chaperone, substrate reduction and gene therapy [172-174].

1.9.2.1 Chaperone

While large mutations within the *GLA* gene lead to the absence of protein or expression of a catalytically inactive protein, missense mutations often produce an aberrant misfolded protein that possesses residual enzymatic activity. Misfolded proteins are either degraded, retained in the endoplasmic reticulum or partitioned to other intracellular organelles [175, 176]. Pharmacological chaperones have been shown to bind and stabilize certain *GLA* mutants. Chaperone binding promotes correct protein folding and trafficking to the lysosome, where the mutant protein can function, albeit less efficiently [177].

Chaperone therapy has been proposed as a feasible strategy for treating certain LSD [177, 178]. Migalastat hydrochloride (1-deoxygalactonojirimycin HCl) has been shown to bind and stabilize wild-type and mutant forms of *GLA* [179, 180].

In pre-clinical studies, Migalastat HCl has been shown to reduce the storage of Gb3 *in vitro* and *in vivo* in a mouse model of FD [181-183]. Recent phase 2 clinical trials in patients with amenable *GLA* mutations have shown that Migalastat HCl increases enzymatic activity and reduces the accumulation of Gb3 in the blood, skin, and kidneys [184, 185]. Migalastat HCl is currently being evaluated in phase 3 clinical trials as a potential treatment for FD.

In contrast to ERT, pharmacological chaperones are non-invasive, orally available, and have a broad tissue distribution, including access to the CNS [186]. While

chaperone therapy is genotype-specific, it is estimated that one-third to one-half of mutations of the 630 gene mutations identified, may be amenable to currently available chaperone therapy [178]. Pre-clinical studies have shown that pharmacological chaperones and ERT may be beneficial in the management of FD [187], as the chaperones may also enhance the stability, and efficacy of the recombinant enzymes.

1.9.2.2 Substrate reduction

Substrate reduction therapy is a novel therapeutic approach that aims to decrease the synthesis of the substrate Gb3, by targeting enzymes involved in its biosynthesis [188, 189]. One such agent, Miglustat, reduces the biosynthesis of GSLs [190]. While Miglustat has shown positive results in the mouse model of FD [191], its lack of specificity for glucosylceramide synthase, results in a variety of side effects [192, 193]. Another glucosylceramide synthase inhibitor, Genz-682452, appears to be more specific than Miglustat, where, in pre-clinical studies, Genz-682452 lowered the levels of tissue Gb3 in mice. Since the majority of Fabry patients are null for a-gal-A activity, using substrate reduction therapy alone is unlikely to be useful. However, it has been demonstrated that combining ERT with SRT is a more efficient alternative to ERT alone in reducing Gb3 levels, especially in the liver, kidneys, and urine [174].

1.9.2.3 Gene therapy

Gene therapy approaches have been investigated in the Fabry mouse model [194-196]. Early studies utilised retroviral vectors to introduce the normal *GLA* gene into bone marrow haematopoietic stem cells derived from Fabry patients [197]. In this

murine model, α -gal-A was able to reduce levels of accumulated Gb3 in various tissues [198]. More long-term enzymatic and functional correction in multiple organs of the Fabry mouse has recently been achieved through the use of adeno-associated viral vectors to deliver the *GLA* gene [196, 199, 200]. Moreover, immune tolerance to *GLA* was observed in this study. These findings argue well for the use of gene therapy in the treatment of FD.

1.10 BIOMARKERS OF FABRY DISEASE

The biomarkers are vital indicators for any disease in order to evaluate the progress of the disease and to study the treatment efficacy. In term of metabolic disorder, the biomarkers can play a significant role in increasing or dramatically decreasing the concentrations of the specific related metabolites. Subsequently, these biomarkers can reveal worthwhile factors including the age of onset of the disease and treatment efficacy. Two essential biomarkers have been reported Gb3 and Lyso-Gb3 as biomarkers for FD diagnosis and treatment efficacy [46, 201-203].

Gb3 and more recently Lyso-Gb3 (the product of an alternative catabolic pathway), have been detected at elevated levels in the plasma and urine of Fabry patients using mass spectrometric (MS) approaches [46, 164, 201, 202, 204]. Furthermore, a series of novel Lyso-Gb3 derivatives (e.g. an oxidised form) have been detected and the possibility that the levels of these derivatives are related to the various symptoms displayed by Fabry patients is intriguing [26, 204-206].

1.10.1 GLOBOTRIAOSYLSCERAMIDE

Globotriaosylceramide (Gb3) also known as ceramide trihexoside (CTH) is the main precursor metabolic substrate which pathologically accumulates within the lysosomes in the various body tissues due to the defective hydrolase enzyme (α -gal-A).

1.10.1.1 Chemical, Physical and Biological Properties of Gb3:

The chemical structure of Gb3 (Figure 1-9) comprised ceramide backbone including sphingoid base (sphingosine moiety) and fatty acid chain which vary in mammals between C16-C26 and a serial of 3 carbohydrates head-groups (galactose-galactose-glucose).

The biological role of Gb3 in the living cells similar to the other GSLs which mainly includes the cell surface functions such as cell surface antigens, receptors for bacterial protein toxins and signal transduction roles [26].

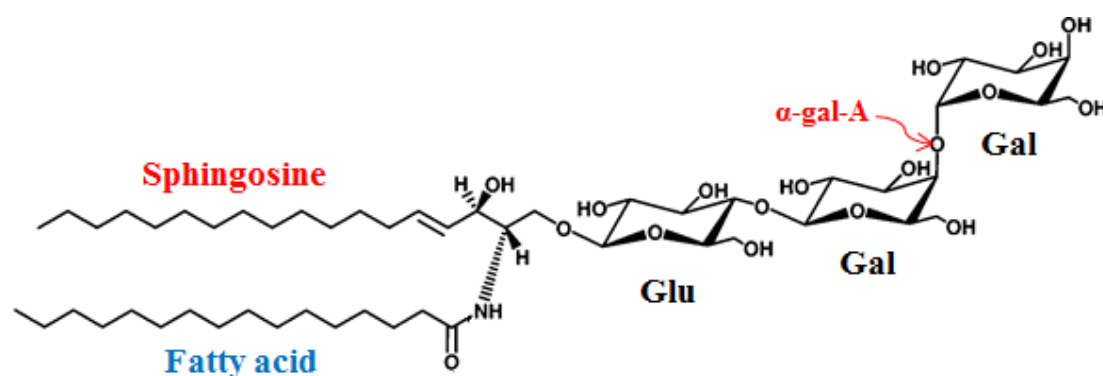


Figure 1-9 Chemical structure of globotriaosylceramide (Gb3): The figure shows the chemical structure of the main accumulated substrate in FD, globotriaosylceramide (Gb3) and the action site of the defective key enzyme α -gal-A. Gb3 is comprised ceramide backbone including sphingoid base and fatty acid chain and a serial of 3 carbohydrates head-groups.

1.10.1.2 Accumulation of Gb3 in Biological Samples of Fabry Patients:

The un-metabolised Gb3 accumulates in plasma, urine and other body tissues of Fabry patients. Therefore, previous studies indicated that Gb3 may be used to monitor the efficacy of ERT and as a diagnostic biomarker although for FD with limited sensitivity.

It has been shown that the excreted urinary Gb3 by both genders of Fabry patients are considerably higher compared with healthy individuals [201, 207]. On the other hand, elevated levels of plasma Gb3 can be detected in hemizygous Fabry males, while female Fabry patients usually show normal plasma Gb3 levels [207].

It has been reported that Gb3 levels in biological samples decrease considerably within two weeks after starting ERT but α -gal-A antibodies may develop therefore Gb3 level may increase again [171, 208-210]. It has been postulated that Gb3 levels correlate with vital factors such as mutation type, ERT outcome and gender of Fabry patients [201]. Whitfield et al [160] suggested that urinary Gb3 is a potential useful biomarker for FD detection and ERT monitoring only in hemizygous males but not in heterozygous females. Nevertheless, Aerts and co-workers [46] suggested that there are many indicators enhance the theory of Gb3 does not cause direct or instantaneous signs of FD for three linked reasons. Firstly, it has been shown that Gb3 accumulates in body tissues before the birth. Secondly, most of hemizygous Fabry males lack to α -gal-A activity. Lastly, it is well-known that the disease manifestations practically are lacking in infantile early childhood [89, 152, 211]. Commonly, the conducted studies on the role of Gb3 in FD showed erratic correlation between the Gb3 levels excreted by Fabry patients in the biological samples and the clinical manifestations of the disease [89, 170, 208, 211]. Additionally, Vedder et al [89] stated that there is no

correlation between the clinical manifestations of FD and excreted Gb3 in plasma and urine of Fabry patients.

1.10.1.3 Gb3 isoforms and analogues

Gb3 isoforms are defined as Gb3 derivatives result from a modification in the fatty acid chain which linked by amide bond with unmodified sphingosine moiety. Nevertheless, Gb3 analogues are defined as Gb3 derivatives result from a modification in their sphingosine moiety [212].

Auray-Blais and co-workers revealed five urinary Gb3 isoforms/analogues which are structurally related to Gb3. These analogues were detected in both gender Fabry patients but none in healthy individuals. These Gb3 isoforms/analogues structurally vary as follows: Gb3-methylated isoforms, Gb3-saturated fatty acid isoforms, Gb3-hydrated sphingosine isoforms, Gb3-two double bonds isoforms and Gb3-one double bond isoforms. These Gb3 isoforms could help in the differentiation between different outcomes of the disease [26]. It has been reported that the levels of Gb3 isoforms/analogous in biological samples from Fabry patients are decreased after ERT providing a positive biological response to the treatment [26, 213].

1.10.1.3.1 Gb3 methylated isoforms

Auray-Blais and colleagues revealed seven methylated isoforms related to Gb3 [26, 212]. The isoforms will be expressed as follows: Gb3(nc:d)(as:e), where n represents sphingosine group; c represents number of carbon atoms in sphingosine moiety; d represents number of double bonds in sphingosine moiety; a represents fatty acid group; s represents number of carbons in the fatty acid moiety and e represents number of double bonds in the fatty acid moiety while Me abbreviation stands for

methylated. Three Gb3 methylated isoforms were detected in both urine and plasma from Fabry patients including Gb3(n18:1)(a22:0)Me; Gb3(n18:1)(a22:1)Me; Gb3(n18:1)(a24:1)Me; Gb3(n18:1)(a16:0)Me; Gb3(n18:1)(a18:0)Me; Gb3(n18:1)(a24:0)Me and Gb3(n18:1)(a24:0)Me [212].

Methylated isoform is not detected in Lyso-Gb3 suggesting a significant role could be played by this isoform to work as an intermediate agent in the metabolic pathway of Gb3 deacylation process to form Lyso-Gb3 [26, 204, 212, 214]. It has been suggested that Gb3 methylated isoforms are possibly promising biomarkers for FD [214].

1.10.1.3.2 Gb3-saturated fatty acids isoforms:

Auray-Blais and Boutin [26] revealed six Gb3 isoforms of saturated fatty acids as follows: Gb3(n18:1)(a16:0); Gb3(n18:1)(a18:0); Gb3(n18:1)(a20:0); Gb3(n18:1)(a22:0); Gb3(n18:1)(a24:0) and Gb3(n18:1)(a26:0). All these isoforms detected in urine and plasma samples from Fabry patients and result from modifications in the fatty acid chain of the ceramide backbone [26, 212].

1.10.1.3.3 Gb3-hydrated sphingosine analogues:

Auray-Blais and co-workers [26] revealed urinary Gb3 hydrated sphingosine analogue, Gb3(n18:0)(a24:1)H₂O which results from a modification in the sphingosine moiety. The fragmentation of this analogue by MS/MS involves the formation of a hydroxyl group by adding H₂O through the double-bond of sphingosine group. The hydrated sphingosine analogue is detected as well in the plasma samples of Fabry patients [26, 212]

1.10.1.3.4 Gb3 isoform/analogue with two double bonds:

Canadian researchers revealed Gb3 isoforms/analogue with two double bonds [26, 212]. These isoforms/analogue were detected in urine and plasma of Fabry patients as follows: Gb3(n18:1)(a24:2) + Gb3(n18:2)(a22:1); Gb3(n18:1)(a22:2) + Gb3(n18:2)(a22:1) and Gb3(n18:1)(a24:2) + Gb3(n18:2)(a24:1).

1.10.1.3.5 Gb3 isoforms/analogue with one double bond:

It has been revealed six Gb3 isoforms/analogue with monounsaturated fatty acid chain as follows: Gb3(n18:1)(a16:1) + Gb3(n18:2)(a16:0); Gb3(n18:1)(a18:1) + Gb3(n18:2)(a18:0); Gb3(n18:1)(a20:1) + Gb3(n18:2)(a22:0); Gb3(n18:1)(a22:1) + Gb3(n18:2)(a22:0); Gb3(n18:1)(a24:1) + Gb3(n18:2)(a24:0) and Gb3(n18:1)(a26:1) + Gb3(n18:2)(a26:0). Gb3 isoforms/analogue with one double bond were detected in urine and plasma samples from Fabry patients [26, 212].

1.10.1.3.6 Short chain-Gb3 isoforms

Manwaring et al [212] revealed a short chain Gb3 related isoform Gb3(n16:1)(a16:0) + Gb3(n18:1)(a14:0). This isoform was detected in plasma samples only.

1.10.1.3.7 Methylated short chain-Gb3 isoforms:

Manwaring et al [212] revealed a methylated short chain Gb3 related isoform Gb3(n16:1)(a16:0)Me + Gb3(n18:1)(a14:0)Me. This isoform was detected in plasma samples only.

1.10.2 GLOBOTRIAOSYLSPHINGOSINE (LYSO-GB3)

Lyso-Gb3 was introduced for the first time as a hallmark biomarker for FD by Aerts et al using plasma samples [46]. Thereafter, Auray-Blais and co-workers showed elevated levels of urinary Lyso-Gb3 in Fabry patients [202]. It has been shown that Lyso-Gb3 is a potential diagnostic biomarker for FD. Rombach et al [215] reported that the plasma Lyso-Gb3 concentration showed significant elevation in all hemizygous Fabry males and adult heterozygous Fabry females compared to healthy individuals. However, young heterozygous Fabry females may show absence or lower elevation in Lyso-Gb3.

Rombach and collaborators showed a correlation between the elevated levels of Lyso-Gb3 and the severity of the clinical manifestations displayed by Fabry patients [215]. Togawa et al [166] suggested that Lyso-Gb3 is expected to be a useful biomarker for monitoring ERT in FD demonstrating that Lyso-Gb3 levels in Fabry mice, are decreased more than Gb3 levels after ERT with recombinant α -gal-A. It has been shown that Lyso-Gb3 is superior to Gb3 as a diagnostic biomarker for FD especially in case of heterozygous females where Gb3 levels in females usually appears as normal [25]. A previous study revealed that plasma Lyso-Gb3 is superior to urinary Lyso-Gb3 as a diagnostic biomarker [25]. The study reported that some typical Fabry males showed Lyso-Gb3 levels in plasma about 500 fold greater than Lyso-Gb3 levels in urine. Alternatively, it has been reported that urinary Lyso-Gb3 concentrations in Fabry patients correlate with clinical severity of FD [201, 202]. Aerts et al [46] introduced plasma Lyso-Gb3 as a promising diagnostic and monitoring biomarker with significantly better sensitivity compared to Gb3.

Various biochemical methods have been developed in order to measure Lyso-Gb3 levels in biological samples in Fabry patients as shown in Chapter 3 (section 3-1).

1.10.2.1 Biochemical and physical properties of Lyso-Gb3

It is crucial to understand and highlighting the biochemical properties of Lyso-Gb3 in order to study its etiologic roles in FD. The chemical structure of Lyso-Gb3 is shown in (Figure 1.10). Lyso-Gb3 results from a deacylation process of Gb3 by a sphingolipid deacylase [17]. As shown in Figures 1.9 and 1.10, the acyl-amide group is replaced by free amine making Lyso-Gb3 a more polar and more water-soluble than its diacyl parent (Gb3). Therefore, this chemical structure of Lyso-Gb3 which characterised by having a free amine group allows applying some extraction procedures such as derivatization process using OPA.

Moreover, the structure of Lyso-Gb3 shows the hydrophilic nature of this compound facilitating its extraction from biological samples using liquid-liquid extraction. On other hand, Gb3 is absolutely totally soluble in lipoprotein [46]. Hence, we believe that applying a single liquid-liquid extraction procedure (e.g. Bligh and Dyer) to extract both Lyso-Gb3 and Gb3 at the same time is attainable where Lyso-Gb3 being harvested from the upper phase while Gb3 is harvested from the lower phase.

Moreover, the free amine reflects the avidity of Lyso-Gb3 to be protonated (H^+ ion) allowing LC-MS analysis on positive mode. Generally, these biochemical and physical characteristics allow HPLC mediated purification linked to mass spectrometric analysis to identify and quantify the Lyso-Gb3 in biological samples.

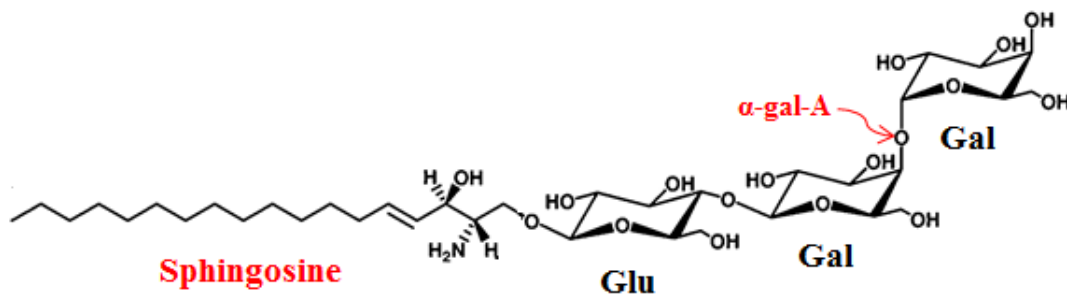


Figure 1-10 Chemical structure of globotriaosylsphingosine (Lyso-Gb3): The figure shows the chemical structure of globotriaosylsphingosine (Lyso-Gb3) and action site of the defective key enzyme α -gal-A. Lyso-Gb3 is resulted from a deacylation process of the precursor compound Gb3. So Lyso-Gb3 consisted from sphingoid base (sphingosine) and 3 carbohydrates head-groups.

1.10.2.2 Biological effect of Lyso-Gb3

Dutch researchers [46] investigated the role of Lyso-Gb3 in vascular remodelling, presenting the biological effect of Lyso-Gb3. The team found that *in vitro*, using levels of Lyso-Gb3 similar to that excreted by symptomatic Fabry patients could significantly stimulate smooth muscle proliferation, which enhances the thickness of carotid-intima media resulting from vascular remodelling [216, 217]. Lyso-Gb3 role in enhancement the proliferation of cardiomyocytes and vascular smooth muscle cells has been postulated in human [215] and *in vitro* [218]. Moreover, the biological role of Lyso-Gb3 has been suggested in glomerular injury [219] and enhancement the toxicity of the viper venom verotoxin 2, a protein which binds to the natural membrane-anchored Gb3 receptor [220].

On the other hand, contrary to Lyso-Gb3, the other GSLs compounds specially the product of Lyso-Gb3 degradation (lactosylsphingosine) and its diacyl-parent (Gb3) which have no influence in the proliferation of small muscle [46].

1.10.2.3 The mechanism of Lyso-Gb3 catabolism

Aerts and co-workers [46] studied the degradation mechanism of Lyso-Gb3 *in vitro*. It has been revealed that Lyso-Gb3 requires the catabolic hydrolase α -gal-A in order to be degraded into lactosylsphingosine. The study showed a weak degradation of Lyso-Gb3 by recombinant α -gal-A *in vitro* at neutral pH due to the optimal acidic pH of hydrolases enzymes in the cellular lysosomes *in vivo*. The study reported that Gb3 degradation efficiency *in vitro* using recombinant α -gal-A is 50 times higher compared with Lyso-Gb3 degradation using the same hydrolysis actors and at the same physical conditions.

Many previous studies showed that Lyso-Gb3 concentrations in biological samples from Fabry subjects are elevated proportionally with the elevation in Gb3 levels and therefore, it is suggested that Lyso-Gb3 plays a significant biological role in inhibition of the key enzyme α -gal-A activity [46]. Aerts and co-workers [46] revealed that the activity of recombinant α -gal-A on Gb3 substrate was inhibited by presence of Lyso-Gb3. A considerable accumulation of Gb3 has been shown in healthy cultured fibroblasts after adding Lyso-Gb3 proving the inhibitory role of Lyso-Gb3 on α -gal-A activity and subsequently promoting Gb3 storage. It has been postulated that the inhibitory capacity *in vitro* was markedly lower than *in vivo* by 200 fold which could be due to the physical properties of Lyso-Gb3 in human lysosomes which characterised by acidic pH and absorptive character of Lyso-Gb3 intra-membranes. On the other hand, Canadian researchers [202] suggested that the cellular elevation of Gb3 possibly due to increase in the Gb3 synthesis and it is not necessary to be due to a catabolism defect.

1.10.2.4 Accumulation of Lyso-Gb3 in biological samples of Fabry patients

The quantitation and potential use of Lyso-Gb3 as a biomarker in plasma [46, 215] and urine [202] for FD detection and monitoring have been demonstrated. It has been reported in a study conducted on Fabry mice that Lyso-Gb3 level is decreased more than Gb3 level after ERT with recombinant α -gal-A [166]. Auray-Blais and co-workers suggested a significant correlation between urinary Lyso-Gb3 and mutation type, ERT outcome and gender of Fabry patients [202]. On the other hand, a considerable correlation has been shown between plasma Lyso-Gb3 concentrations and left ventricular mass in heterozygous Fabry females and cerebrovascular white matter lesions in hemizygous Fabry males [215].

The urinary excretion of Lyso-Gb3 and Gb3 in untreated Fabry patients has been compared by a Canadian study [202]. The study revealed that Fabry patients excrete lower amounts of urinary Lyso-Gb3 compared to urinary Gb3 by 1000 fold less in heterozygous females and by 6000 fold less in hemizygous males.

It has been reported that Fabry patients with late onset cardiac variant mutations and renal variant mutations show normal level or slight increase in their urinary Lyso-Gb3 and Gb3 excretion. For example Fabry patients with mutations R363H and R118C did not excrete urinary Lyso-Gb3 but showed normal urinary Gb3 [202, 221].

Moreover, it has been suggested that the use of urinary Lyso-Gb3 may be not the best predictive biomarker for Fabry patients with late onset renal variant where there is no correlation between urinary Lyso-Gb3 excretion and some kidney functions mainly estimated glomerular filtration rate (eGFR) [89, 202].

It has been reported that both symptomatic hemizygous Fabry males and adult heterozygous Fabry females show elevated levels of plasma Lyso-Gb3 while young

heterozygous females show no or a lesser elevation [71]. Moreover, adult heterozygous females show lower levels of plasma and urinary Lyso-Gb3 compared to hemizygous males [46, 164, 204, 222].

On the other hand, it has been shown that Fabry patients with late onset cardiac variant mutations such as R112H [223] show normal plasma Gb3 and very low level of plasma Lyso-Gb3 [46]. Rombach and co-workers reported that no elevated levels of Lyso-Gb3 are displayed by cardiac variant Fabry patients with R112H and P60L mutations [215]. Moreover, it has been demonstrated that Fabry patients with cardiac variant mutation (N215S) show normal levels or low elevation in plasma Lyso-Gb3 and its analogues with normal levels of Gb3 [206].

Niemann and co-workers reported that some Fabry patients show late onset clinical manifestations in a single organ only such as heart or kidney referring to cardiac variant and renal variant [224]. The researchers suggested that measuring Gb3 and Lyso-Gb3 levels are insufficient for the identification of FD severity and the clinical manifestations in both genders, hypothesizing that assessing a degradation product of the accumulating GSLs can help in the definition of clinically relevant FD [224].

1.10.2.5 Lyso-Gb3 analogues

Lyso-Gb3 analogues are Lyso-Gb3 related molecules result from modifications in the sphingosine moiety [204]. Canadian studies [85, 206, 225, 226] identified eight novel modified derivatives of the sphingosine moiety of Lyso-Gb3 (termed as Lyso-Gb3 analogues) in plasma and urine samples from Fabry patients using an LC-MS approach. These analogues vary in the mass compared to Lyso-Gb3 by the exact amount predicted by modification. Lyso-Gb3 analogues will be expressed as follows: Lyso-Gb3 (n)/(m), where n represents m/z difference between Lyso-Gb3 and its

analogue and m represents the empirical formula of the modification corresponding to the m/z difference between Lyso-Gb3 and its analogue. A total of five analogues were detected in plasma and urine samples from Fabry patients as follows: Lyso-Gb3 (-28 Da)/(-C₂H₄); Lyso-Gb3 (-2 Da)/(-H₂); Lyso-Gb3 (+14Da)/(-H₂+O); Lyso-Gb3 (+16Da)/(+O); Lyso-Gb3(+34Da)/(+H₂O₂) and Lyso-Gb3 (+50 Da)/(+H₂O₃) [85, 226]. The remaining Lyso-Gb3 analogues: Lyso-Gb3 (-12 Da)/(-C₂H₄+O) and Lyso-Gb3 (+14Da)/(-H₂+O) were detected in urine samples only [85, 225] while the analogue Lyso-Gb3 (+18Da)/(-H₂+O) was detected in plasma samples only [206, 226].

The main fragment (daughter ion) of Lyso-Gb3 by MS/MS is at m/z 282. Other Lyso-Gb3 fragments include the following m/z (768, 624, 606, 462, 444, 426, 300 and 264). However, Lyso-Gb3 analogues have the same fragments with the consideration of the mass shifts each time (Chapter-3).

Auray-Blais and co-workers [85] stated that the profiles of three Lyso-Gb3 analogues in urine with mass shifts of (-12 Da, +16 Da and +50 Da) showed split peaks which may indicate the presence of isomers. Some urinary Lyso-Gb3 analogues e.g. Lyso-Gb3 (+16 Da) have higher concentrations than Lyso-Gb3 itself in a hemizygous Fabry male suggesting that these analogues could be used as strong specific and reliable biomarkers for FD in addition to Lyso-Gb3 [225]. However, plasma Lyso-Gb3 concentrations in Fabry patients are higher than its analogues [206].

A considerable correlation has been shown between Fabry patients' gender and urinary concentrations of Lyso-Gb3 analogues. Hemizygous Fabry males had considerably higher concentrations of Lyso-Gb3 analogues, than heterozygous Fabry females [85, 225]. The relative distribution of Lyso-Gb3 analogues vary between Fabry patients. It has been revealed findings suggesting that most Fabry patients with

specific disease symptoms and clinical manifestations showed elevated urinary concentrations of at least one of three main Lyso-Gb3 analogues: Lyso-Gb3 (+16 Da); Lyso-Gb3 (+34 Da) and Lyso-Gb3 (+50 Da). Nevertheless, some Lyso-Gb3 analogues were found with levels below limit of quantitation in Fabry females (e.g. Lyso-Gb3 (-2 Da) and Lyso-Gb3 (-28 Da) analogues). Hence, these findings suggest that the presence and the urinary levels of specific Lyso-Gb3 analogues are associated with clinical features of the disease.

It has been reported that the sphingosine structure could be varied based on its origin tissue [227]. This could help in comparing different Lyso-Gb3 analogues and subsequently matching with variant disease outcomes. Hence, an indicator could lead to the origin tissue of Gb3 [204, 227].

The biological samples from healthy individuals are lacking of Lyso-Gb3 analogues except small traces amounts of one analogue with m/z value of 836 (Lyso-Gb3 + 50 Da) [225]. These findings give indications for the importance of urinary Lyso-Gb3 analogues which could benefit in FD detection and monitoring especially in case of mutation analysis enzymatic activity test results being ambiguous as in heterozygous Fabry females, or in case of Fabry patients with late onset manifestations of FD who show residual enzyme activity.

It has been reported that plasma and urinary Lyso-Gb3 analogues are decreased after ERT providing a positive biological response to the treatment [205, 225].

Lavoie and collaborators [225] studied the urinary levels of Lyso-Gb3 and its analogues in Fabry patients under ERT course. The study reported that hemizygous Fabry males showed a considerable correlation between the response to ERT and male urinary Lyso-Gb3 (-28 Da) and Lyso-Gb3 (+50 Da) analogues. Nevertheless, the remaining urinary Lyso-Gb3 analogues revealed reasonable response to ERT. In

terms of heterozygous Fabry females, only urinary Lyso-Gb3 (-28 Da) analogue showed a significant correlation with ERT. On the other hand, no considerable correlation was given between the response to ERT and urinary Lyso-Gb3 concentrations highlighting the importance of using Lyso-Gb3 analogues which could be more fundamental than using Lyso-Gb3 itself in FD detection and monitoring [225]. It has been suggested that the urinary levels of Lyso-Gb3 analogues in Fabry patients correlate with clinical severity of FD [204, 225].

1.10.2.6 Lyso-Gb3 analysis using Dried Blood Spots

Currently, the dried blood spots (DBS) are used worldwide in the newborn screening for different metabolic disorders. It has been shown that the chance of applying and the use of DBS to measure Lyso-Gb3 in Fabry patients to be applicable [228-230]. This service involves measuring the levels of plasma Lyso-Gb3 where a single blood spot being collected in a filter paper is sufficient determine Lyso-Gb3 concentration. Alternatively, Johanson et al [229] demonstrated that using DBS for Lyso-Gb3 analysis in Fabry patients can be worthwhile in case of symptomatic patients such that in adult hemizygous males and some heterozygous females. However, the procedure is not suitable for newborn screening and late onset heterozygous females.

1.11 SCREENING OF FABRY DISEASE

Commonly, the newborn screening for metabolic disorders is considered a fundamental in order to administrate the disease progression, prevent the damage of affected organ and decrease the advanced various worse manifestations.

Many previous papers demonstrated that newborn screening for LSD and FD is attainable and can reduce the age of diagnosis and subsequently enhance the disease

monitoring [231-234]. Auray-Blais and co-workers suggested a protocol for high risk screening for FD in patients with renal and vascular disease [235]. Maruyama et al [236] revealed plasma Lyso-Gb3 to be a promising secondary screening target. This biomarker can be used to confirm the initial molecular analysis diagnosis or to detect new cases of Fabry patients.

1.12 SEPARATION TECHNIQUES FOR BIOLOGICAL SAMPLES

1.12.1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The complexity of biological samples means that is usually necessary to employ some sort of separation process prior to analysis. A common method is the use of high performance (pressure) liquid chromatography (HPLC) because this method is very adaptable, robust and advances in instrumentation and columns allows for excellent resolution and reproducibility.

Two approaches are possible, normal phase where the column (stationary phase) is more polar than the mobile phase (solvent) and reverse phase where the relative polarities are switched. Unmodified silica is usually the choice of stationary phase in normal phase separation and silica modified with long chain hydrocarbons (typically 8 or 18 carbons long) is used in reverse phase work. HPLC columns are made up small particles or beads (2.7 μ m or less) and the uniformity of the bead size is an important factor in the quality of separation and the pressure required to pump solvent through the column.

Reverse phase HPLC is the most common method. Here, the sample (e.g. Lyso-Gb3) is loaded in a solvent that is more polar than the hydrocarbon-modified silica which leads to analytes binding to the column. The elution of analytes from the column is

often brought about by decreasing the polarity of the solvent in a step-wise or gradient manner. Details of the reverse phase HPLC protocol employed in this work are given in Chapter 3.

1.13 MASS SPECTROMETRY

Mass spectrometry (MS) is one of the most powerful analytical techniques in biological sciences. An upturn in the utilization of MS has occurred in the last 20 years or so because of technological developments in computers and the actual mass spectrometers. Subsequently protocols for the analysis of oligosaccharides, lipids, peptides and proteins in a wide range of sample types have been developed to take advantage of the analytical power offered by MS [237].

Mass spectrometers function by generating gas phase ions from analytes which are then subjected an analysis that determines the mass to charge ratio (m/z) and abundance of the ions [237]. In order to do this all mass spectrometers are made up of three components; the ion generation system (termed the ion source), a mass analyser and a detector [237, 238].

1.13.1 Ion sources:

Ion source is the first part of the MS where the samples are converted to beams of gaseous ions. The most common ionization methods are electro spray ionization (ESI) and matrix assisted laser desorption ionization (MALDI), which are both capable of generating gas phase ions from relatively large, heat labile, non-volatile molecules, which is essential for the analysis of biological samples [237].

1.13.1.1 Electrospray ionisation (ESI):

Electrospray ionisation (ESI) is a soft ionisation procedure which involves transferring ions from the liquid phase to the gas phase, facilitating the analysis and keeping the molecule of interest fully intact [239]. This technique was firstly suggested by Malcolm Dole in the 1960s and applied in practice in the 1980s by John Fenn [240, 241]. A solution of an analyte, containing volatile, polar buffer is pushed into the ion source through a highly-charged capillary, usually of stainless steel, producing a nebula of droplets and solvated ions in the gas phase. These ions pass through a desolvation device under vacuum and are then injected into the analyser. Multiply-charged ions are produced from large molecules through ESI.

As shown in Figure 1.11, there are three main steps in ESI process [239]:

a) Charged droplets are produced at the ES capillary tip by applying a high voltage up to 6 kV at the end of the capillary. Ions travel toward the analyser, pulling the sample along until the charge at the capillary tip is higher than surface tension, resulting in the formation of the droplet.

b) The charged droplets are shrunk by solvent evaporation and repeated droplet disintegrations due to coulomb repulsion, finally resulting in tiny, highly-charged droplets until the Rayleigh limit is reached when coulomb repulsion is equal to the surface tension, resulting in producing of gas phase ions.

c) Gas phase ions are produced from these droplets. The role of establishing the gas phase ion is played by various models including charge residue pattern, through the evaporation of the solvent leaving a bare gas phase ions; ion evaporation pattern as the charges left on the surface of the droplets acquire the required energy, jumping into the gas phase and finally; ion emission pattern, in which the gas phase ion is

formed directly from the capillary due to the applied high voltage. ESI is considerably faster, more sensitive, and offers greater accuracy than previous techniques of mass determination for biological molecules such as gel electrophoresis.

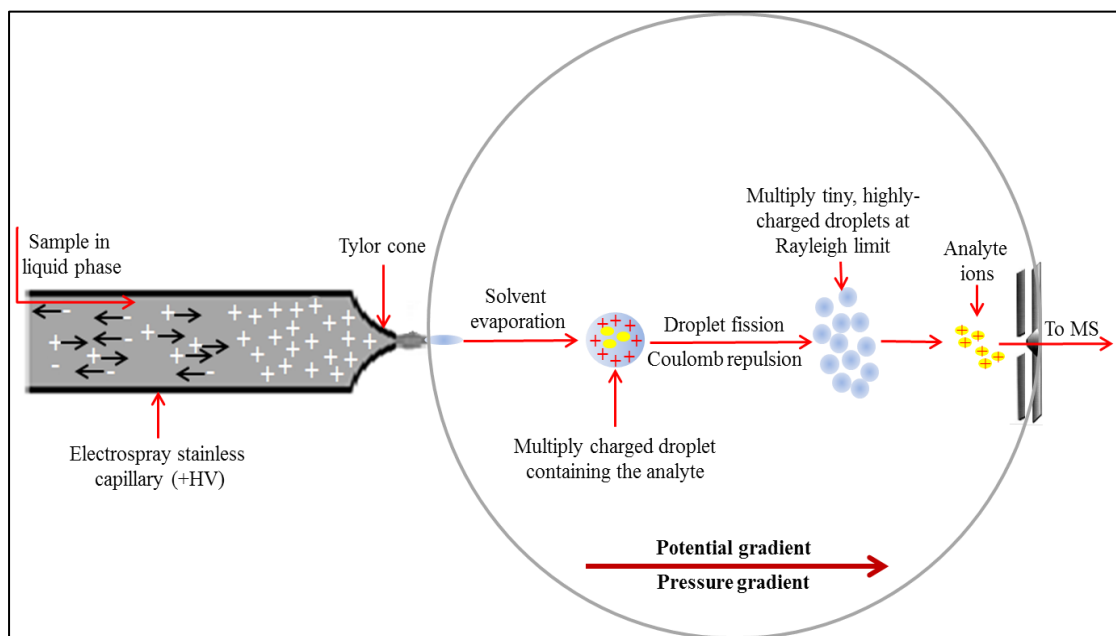


Figure 1.11 Electro spray ionisation (ESI) process: Essential features of the electro spray ionisation and the droplet production in the electro spray interface.

1.13.1.2 Matrix-assisted laser desorption ionisation

Matrix-assisted laser desorption ionisation (MALDI) is an ionisation procedure that involves mixing an analyte with a suitable matrix absorbing at the required laser wavelength. Usually, this is of the order of a 1,000 to 10,000 molar excess of matrix to analyte. After the evaporation of the solvent, this process results in a co-crystallisation of both matrix and the analyte. The combination, of the analyte molecules and the lattice structure of the matrix, enhances the precondition that the laser desorption/ionisation process will work.

Thereafter, the crystallised surface of the sample is exposed to concentrated short waved laser pulse in the high vacuum area in the ion source of MS. The coupling of

the energy is performed on UV radiation by resonant excitation of the matrix molecule (Figure 1.12).

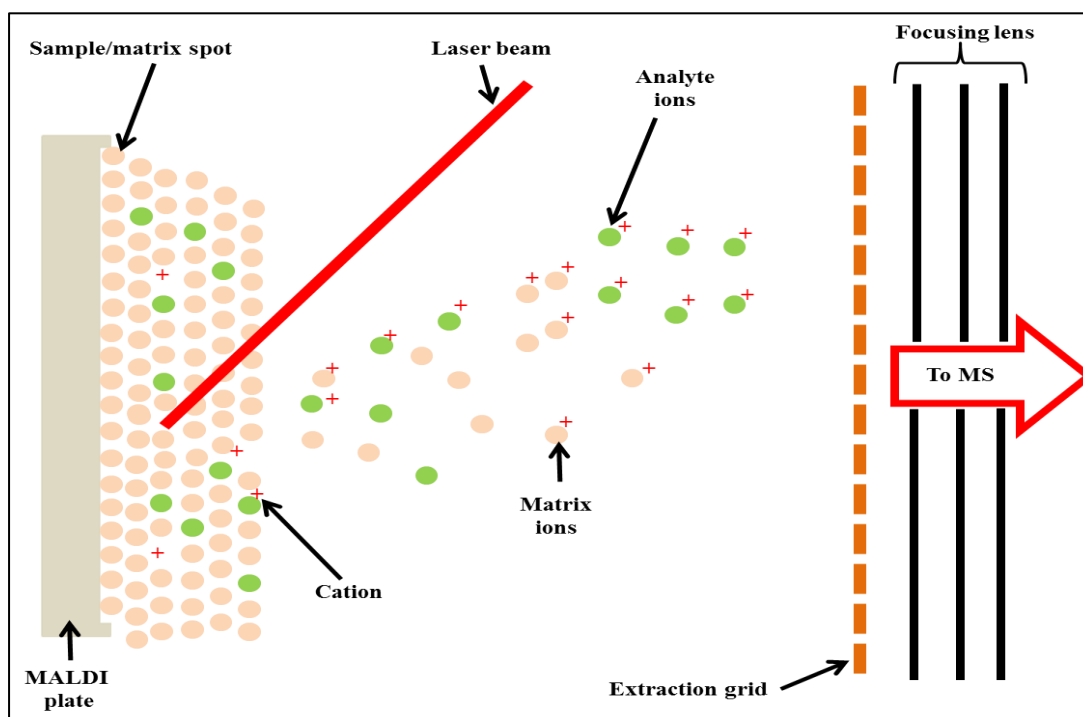


Figure 1-12 MALDI procedure: A schematic highlighting key aspects of the MALDI process. Matrix/analyte crystals are deposited onto a MALDI target plate and are then pulsed with a laser. Matrix molecules absorb energy from the laser and transfer this to the analyte species, facilitating desorption and ionisation. The resultant ionised species are extracted into the mass spectrometer to be analysed.

1.13.2 Mass analysers:

The most common mass analysers used in biological work are time of flight (TOF), quadrupoles and the modified quadrupole, an ion trap [237].

With TOF a package of ions is generated in an ion source (usually by MALDI or ESI) and the whole package is accelerated through a fixed potential at the same time down a flight tube until they reach the detector. As every ion will have the same kinetic energy under these conditions the smaller the ions the faster they will travel. The actual speed attained is related, inversely, to the square root of the m/z . When electrical components that operated quickly enough to detect all of the ions contained

in each pulse of ions were developed in the 1990s TOF use increased considerably. More modern instruments incorporate a reflectron in the flight path which is an electrostatic mirror that improves the reliability of the mass analysis [237], (Figure 1.13). This functions by deflecting the ions such that they travel back down the flight tube. This corrects for small deviations in the kinetic energy dispersions of ions with the same m/z (ions with greater kinetic energy will be travelling faster and therefore, will move further into the reflectron before being deflected thereby travelling further to compensate for the increased speed) and by increasing the length of the flight path considerably.

Quadrupoles are made of four parallel (usually round) rods where each opposing pair, are electrically coupled. A radio frequency (rf) electrical field is applied to the pairs in combination with a direct current (DC) off-set. In this way an m/z specific 2-dimensional stability region along the plane of the poles is established such that ions with the correct trajectory can pass through the quadrupole. By varying the rf (at a constant ratio to the DC voltage) ions over an m/z range can pass, allowing considerable flexibility in the use of the quadrupole. For example, not only it can be used as a mass analyser but it can also act as a filter to allow ions of a specified m/z to pass. This can be useful when a particular molecule is being investigated.

Ion traps are specialized versions of quadrupoles where a 3 dimensional stable zone is established (rather than the 2 dimensional in standard quadrupoles) by having charged end caps where the DC is applied. In this way ions can be trapped and scanned out by changing the rf. This allows the ions to be either analysed conventionally or specific m/z to be isolated within the same space. When specific ions are isolated using conventional quadrupoles a second quadrupole has to be employed to contain the isolated ions [237].

1.13.2.1 MicroTOF-II Mass Spectrometer

The use of a quadrupole orthogonal time-of-flight mass spectrometer (Q-TOF) was first described by Morris *et al.* in 1996 [242]. A schematic illustration of the instrument is depicted in Figure 1.13. The Q-TOF technique was developed as a variation of the triple quadrupole mass spectrometer (QQQ). Using collision-activated decomposition or so called collision-induced dissociation (CID), the Q-TOF technique was applied to sequence a biopolymer, in this case, components of the major histocompatibility (MHC) antigen complex. The substitution of the third quadrupole with a time-of-flight (TOF) mass analyser improved the signal-to-noise ratio of obtained MS/MS spectra, providing mass accuracies in their study of products 'within 0.1 Dalton [242]. This modification enabled the determination of isotope patterns in MS/MS spectra and significantly improved the ability to assign charge states to fragmented ions, a particularly important factor in the analysis of biological analytes [242].

The Q-TOF mass spectrometers have the ability to generate a high signal-to-noise ratio facilitating the accurate prediction of the analysed ion mass. Moreover, these instruments can provide a high resolution which can exceed 3000 (FWHM). Importantly, Q-TOF instruments have the ability to analyse very low concentrations of an analyte in a sample, generating MS/MS spectra of the analysis [242]. While the Q-TOF mass spectrometer was first introduced with ESI as an ion source, currently Q-TOF instruments are available with various ion sources, such as MALDI [243, 244]. A comprehensive review about Q-TOF mass spectrometers has been introduced by Chernushevich and colleagues [245].

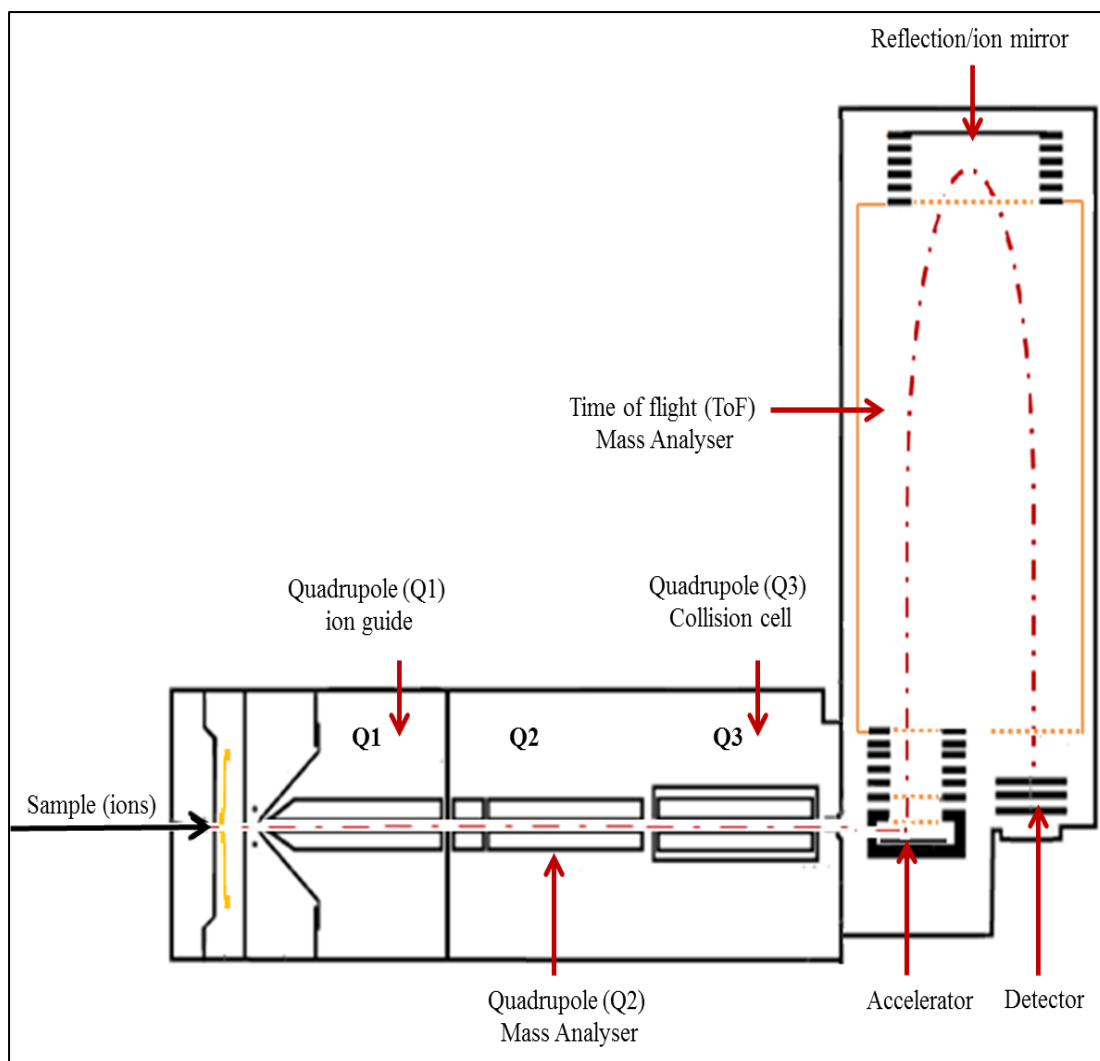


Figure 1.13 Components of microOTOF-II Mass Spectrometer: A schematic illustration shows the various components of microOTOF-II Mass Spectrometer. This instrument was devised in the study in order to measure plasma and urinary metabolites in the participated Fabry patients and healthy volunteers.

1.13.3 Tandem Mass Spectrometry

Tandem mass spectrometry (MS/MS) is the technique in which the ions of interest are fragmented through a dissociation process such as CID, facilitating the study of these fragments in order to identify the parent ion. MS/MS for chemical molecules (e.g. GSLs, amino acids and acylcarnitine) analysis is used in metabolic disorders screening as an excellent tool for use with compound sharing fragment ions or neutral

molecules [246, 247]. It is possible to analyse different chemical families in a single analysis in approximately two minutes. This offers a broad spectrum analysis and high throughput, and is therefore a comparatively inexpensive instrument for analysis. Hence, from a clinical perspective, it is practical to use it to screen for a number of diseases [238].

There are different dissociation methods such as CID and high energy C-trap dissociation (HCD). CID is a tandem mass spectrometry dissociation method in which the parent ions are fragmented as a result of their collision with a neutral gas. An electric field is applied to accelerate these parent ions and to induce bond cleavage through their collision with a neutral gas (e.g. argon, helium or nitrogen) in the collision cell. The collision energy breaks down the parent ions and thereby the daughter ions result [248].

HCD: is a new developed dissociation technique. In this procedure, C-trap is used to store and fragment the ions; thereafter, these ions are transferred to orbitrap [248].

MS/MS is the main tool that used in newborn screening laboratories worldwide [247].

1.13.4 The role of MS in newborn screening for IEM

Newborn screening is a test or investigation for a disorder in the early stages of human life, allowing the management of disease before it becomes advanced [247].

It has been reported that the detection of congenital diseases at an early stage is fundamental for health protection. An example of such diseases are metabolic disorders (inborn errors of metabolism), which were identified by Sir Archibald Garrod at the beginning of the twentieth century [249]. Metabolic disorders comprise disorders of organic acid, amino acids and fatty acid metabolism, urea cycle disorders and lysosomal storage disorders, in addition to other diseases [247].

Tandem mass spectrometry (MS/MS) was first applied to newborn screening in 1990 at Duke University Medical Centre in the USA. Before this, Fast Atom Bombardment (FAB) ionisation MS/MS was used for acylcarnitine analysis of urine and plasma samples for infants with suspected metabolic disorders. FAB was later modified to develop Fast Ion Bombardment (FIB). This modification enhanced the advantages of this version of MS/MS, made it simple to use and improved the sensitivity of the instrument. Moreover, butyl ester derivatisation was selected over methyl ester derivatisation, which helped in decreasing interference, particularly with amino acids [246].

In 1993, blood spot samples were used to develop the analysis of acylcarnitine, together with phenylalanine for phenylketonuria diagnosis [250]. Following this, an expansion occurred in the detection of many amino acids at the same time in a single analysis after discovering the common structural loss (neutral loss of 102) [251, 252]. According to Chace and Naylor [246], routine newborn screening for metabolic disorders using MS/MS started in late 1992 at Magee Women's Hospital in Pittsburgh. By 1999, approximately 25,000 samples were screened. Between 1992 and 1994 different methods were studied to develop the automation of MS/MS to semi-automated sample preparation and dynamic or continuous flow FIB ionisation. One of these studies was carried out by Rashed and co-workers using ESI [253]. These new methods of ionisation were applied to newborn MS/MS analyses. The ESI technique offers considerable advantages, as it is a fully automated system with improved FIB-MS/MS [246].

In the majority of advanced countries, every new-born is screened for many metabolic diseases using MS/MS and a drop of blood is enough to measure multiple metabolites, confirming the prediction of Chace [254]. Chace [254] stated that the application of

MS/MS is crucial to help differentiate between numerous compounds according to their fragmentation pattern, such as identifying a small shoulder from a minor component in a chromatogram.

Currently, metabolic disorders such as phenylketonuria, hypothyroidism, cystic fibrosis and medium-chain acyl-CoA dehydrogenase deficiency are covered by the newborn screening programme in the UK.

1.14 THE SCIENTIFIC JUSTIFICATION FOR THE RESEARCH

The diffuse range of symptoms displayed by Fabry patients makes diagnosis problematic. Whilst the enzyme activity of α -gal-A is useful in this process, this assay does not allow the treatment regime/efficacy to be monitored or help in understanding the disease process. The post/during-treatment monitoring available currently are the patient's symptoms and well-being or the level of Gb3, which is thought to be a poor indicator of patient status. Monitoring the level of Lyso-Gb3 and its related analogues prior to and during treatment will allow us to ask the questions which will be outlined in the research objectives (section 1.15). We hope to develop a post treatment monitoring process that will enhance patient treatment and further our understanding of the disease.

1.15 RESEARCH OBJECTIVES

The aim of this study was to develop an assay to measure Lyso-Gb3 and associated analogues in biological samples taken from Fabry patients and to assess its utility in FD diagnosis and monitoring patient response to therapy. This required the development of methods to extract GSLs from the biological fluid, and the use of a

sensitive HPLC-MS/MS-based assay with sufficient sensitivity to measure the metabolites of interest.

The project aims to measure both plasma and urinary Lyso-Gb3 in a large cohort of Fabry patients (obtained from Birmingham Sheffield and London) and follow changes in their levels of Lyso-Gb3 over a two-year period to determine whether Lyso-Gb3 measurements are useful in monitoring the efficacy of ERT and studying disease progression and/or regression. For the first time, we set out to compare the levels of Lyso-Gb3 in both plasma and urine to determine the best biological fluid used for analyte measurement. With these aims in mind, we have set out to ask the following specific questions:

- Can plasma/urinary levels of Lyso-Gb3 be used to diagnose FD in hemizygous males and heterozygous females?
- Can the levels of plasma Lyso-Gb3 be used to monitor the efficacy of ERT in Fabry patients?
- What is the best biological sample or source for analyte measurement? We will compare Lyso-Gb3 and analogue measurements in both plasma and urine samples from Fabry patients.
- We will examine the significance of the various Lyso-Gb3 analogues with respect to the target organ damage using patients presenting with classical and non-classical (cardiac variants) forms of FD.
- Finally, establish a method for measuring the level of total urinary Gb3 using novel extraction and analysis methodologies.

CHAPTER - 2
MATERIALS AND METHODS

2.1 ETHICS

All human research conducted was in accordance the ethical standards of the Helsinki declaration and was approval by an NHS Research Ethics Committee [North West 5 Research Ethics Committee, Haydock Park, represented by Human Biomaterials Resource Centre (HBRC), University of Birmingham, reference: 09/H1010/75).

2.2 SUBJECTS

FD diagnosis was confirmed in all enrolled Fabry patients by mutation analysis and/or by the α -gal-A enzyme activity test in white blood cells of classical hemizygous Fabry males where the heterozygous Fabry females and Fabry patients with late onset FD (e.g. cardiac variants and renal variants) can show residual enzyme activity within the reference range of healthy control subjects. A total of 100 Fabry patients and 109 age and gender matched control individuals have been enrolled in this project (Subject details are shown in Table 2-1). All Fabry patients and healthy control individuals who enrolled in this study gave written consent before collection of their biological samples. Fabry patients were categorised into two groups' classical Fabry patients (n= 62) and late onset cardiac Fabry patients (38). The classical Fabry are defined as having typical Fabry disease manifestations and known classical mutations with the deficiency of α -gal-A enzyme activity (<1%) while cardiac Fabry patients were confirmed by mutation analysis. Blood samples were collected from the patients and healthy individuals in EDTA tubes, centrifuged and the plasma stored at -80 °C. Urine samples were collected from the patients and healthy individuals in urinary universal plastic tubes and stored at -80 °C until further processing without any centrifugation or filtration.

		Fabry patients				Control subjects	
		classical		cardiac variant		male	female
		male	female	male	female		
Number		23	39	20	18	63	46
Age (y)	mean	42.0±13.5	56.0±16.3	55.7±16.1	46.9±17.8	51.1±20.4	52.8±15.9
	median	49.0±3.1	60.0±2.7	58.6±3.7	41.6±4.2	54.9±2.9	56.1±5.5
	range	19-62	24-82	18-86	25-85	20-82	23-80

Table 2.1 Anthropometric data of 209 Fabry patients and control subjects: a table shows the total number, age and gender of all subgroups of Fabry patients and control subjects.

2.3 CHEMICALS AND MATERIALS

2.3.1 General equipment and reagents

2.3.1.1 Plastics consumables: the plastic consumables used in the study are shown in Table (2.2).

#	Consumable	Supplier
1	Cap w/PTFE/Silicone Seal for 9mm Screw Vial	Thermo Scientific-UK
2	Cryo-Tube Vials	Thermo Scientific-UK
3	Oasis MCX 1cc (30mg) LP extraction cartridges	Waters-USA
4	0.65mL low binding microfuge tubes	Dutscher Scientific-UK
5	1.7mL low binding microfuge tube	Dutscher Scientific-UK

Table 2.2 Plastic lab-ware: the plastic lab-ware used in the project.

2.3.1.2 Glass consumables: the glass consumables used in the current study are shown in Table 2.3.

#	Consumable	Supplier
1	Certified glass inserts mandrel precision point 200 μ L, 6 \times 29mm with plastic spring	Supelco Analytical-USA
2	Screw Top Vials 2 mL clear 9 mm	Thermo Scientific-UK
3	Disposable culture tubes - borosilicate glass (13 \times 100mm)	Fisher Scientific-UK
4	Disposable culture tubes - borosilicate glass (16 \times 150mm)	Fisher Scientific-UK
5	Pasteur pipettes short glass	Sigma Aldrich-UK

Table 2.3 Glass lab-ware: the glass lab-ware used in the project.

2.3.2 Chemicals

HPLC grade water and acetonitrile (ACN) were purchased from VWR international. HPLC grade methanol ($\geq 99.9\%$), formic acid ($\geq 98\%$), hydrochloric acid (HCl, 37%) and ammonium hydroxide (NH₄OH, 28%) were purchased from Sigma Aldrich, UK. 1- β -D-glucosylsphingosine (GSG) internal standard (source: semisynthetic bovine buttermilk), 1- β -D-glucosylsphingosine (GSG) internal standard (source: plant) and globotriaosylsphingosine (Lyso-Gb3) standard (source: semisynthetic porcine RBC) were purchased from Matreya (Pleasant Gap, PA). Human plasma (pooled from 6 mixed-gender individuals, charcoal stripped twice, filtered 0.2 μ m and sodium heparin preservative) was purchased from Sera lab, UK. All the chemicals used in the current study are shown in Table 2.4.

2.3.3 Solutions, standards and reagents

The compositions of the solutions, buffers and reagents that were used in the study are given in table 2.5.

#	Consumable	Supplier
1	HPLC-grade water (H ₂ O)	VWR-UK
2	HPLC-grade methanol (MeOH, ≥99.9%),	Sigma Aldrich-UK
3	Acetonitrile gradient grade	VWR-UK
4	HPLC-grade chloroform (CHCl ₃ , ≥99.9%)	Sigma Aldrich-UK
5	Formic acid puriss (98%)	Sigma Aldrich-UK
6	Hydrochloric acid	Sigma Aldrich-UK
7	Phosphoric acid	Sigma Aldrich-UK
8	Ammonium hydroxide (28% NH ₃ in H ₂ O)	Sigma Aldrich-UK
9	HPLC grade Propan-2-ol	Fisher Scientific-UK
10	Acetone	Fisher Scientific-UK
11	Potassium hydroxide (pellets, 85%)	Sigma Aldrich-UK
12	Ammonium formate	Sigma Aldrich-UK
13	Ammonium acetate	Sigma Aldrich-UK
14	5-chloro-2-mercaptobenzothiazole (5C2M)	Sigma Aldrich-UK
15	2- mercaptobenzothiazole	Sigma Aldrich-UK
16	6-aza-2-thiothymine	Sigma Aldrich-UK
17	2-(4-hydroxyphenylazo) benzoic acid	Sigma Aldrich-UK
18	2,5-dihydroxy benzoic acid (DHB)	Sigma Aldrich-UK
19	Super-DHB	Sigma Aldrich-UK
20	Sinapic acid	Sigma Aldrich-UK
21	2,4,6-trihydroxyacetophenone monohydrate	Sigma Aldrich-UK
22	2,5- dihydroxy acetophenone	Sigma Aldrich-UK
23	α-cyano-4-hydroxycinnamic acid acid	Sigma Aldrich-UK
24	Picolinic acid	Sigma Aldrich-UK
25	9-aminoacridine hemihydrate	Sigma Aldrich-UK
26	Ceramide/sphingoid internal standard mixture I	Avanti polar lipids-USA
27	Lyso-Gb3 standard	Matreya - Pleasant Gap, PA
28	GSG (plant) internal standard	Matreya - Pleasant Gap, PA
29	GSG (bovine) internal standard	Matreya - Pleasant Gap, PA
30	Gb3 standard (source: porcine RBC)	Matreya - Pleasant Gap, PA
31	N-Heptadecanoyl ceramide trihexoside standard	Matreya - Pleasant Gap, PA
32	ESI - Low concentration tuning mixture	Agilent Technologies-USA
33	Human plasma, Sodium heparin, mixed gender, pooled, 0.2µm filtration	Sera lab-UK

Table 2.4 Chemicals: the chemicals used in the project.

#	Solution/reagent/standard	Composition
1	HPLC mobile phase A	0.1%FA / H ₂ O
2	HPLC mobile phase B	0.1%FA / ACN
3	HPLC mobile phase C	20%ACN / H ₂ O
4	Lyso-Gb3 standard stock solution [1272 μM]	1mg Lyso-Gb3+1mL MeOH (stored at -20°C)
5	Lyso-Gb3 standard working solution [20 nM]	Prepared by a serial dilution using (20%ACN / H ₂ O) and stored at -20°C
6	GSG standard stock solution [100 μM]	5mg GSG + 108.45mL of (0.1%FA/50%MeOH) stored at 4 °C
7	GSG standard working solution [0.8 nM]	By a serial dilution using MeOH (stored at -20°C)
8	Gb3 standard stock solution [200 ng/μL]	10mg Gb3+1mL MeOH:CHCl ₃ (2:1, v/v) then adding 49ml MeOH (stored at -20°C).
9	Gb3 standard working solution [80 ng/μL]	By a serial dilution using MeOH (stored at -20°C)
10	Gb3 IS stock solution [50 ng/μL]	0.5mg Gb3+1mL MeOH: CHCl ₃ (2:1) then adding 9ml MeOH (stored at -20°C).
11	Gb3 IS working solution [2 ng/μL]	By a serial dilution using MeOH (stored at -20°C)
12	SPE Conditioning solution 1	MeOH
13	SPE Conditioning solution 2	2% H ₃ PO ₄ in H ₂ O
14	SPE Conditioning solution 3	HCl (1N)
15	SPE wash-1 solution	2% FA in H ₂ O
16	SPE wash-2 solution	2% FA in MeOH
17	SPE elution solvent	2% NH ₃ in MeOH
28	SPE reconstitution solvent	20% ACN in H ₂ O

Table 2.5 Composition of solutions: a table shows the composition of solutions and various reagents used in the project.

2.3.4 HPLC and MS equipment and parts

Table 2.6 shows the parts and columns of HPLC system used in the study.

#	Consumable	Supplier
1	Halo C18 column (2.7µm, 50mm×200µm)	Hichrom Limited-UK
2	Thermo C18 column (3.0µm, 150mm×75µm)	Thermo Scientific-UK
3	Ascentis Express C18 (2.7µm, 50mm×200µm)	Supelco Analytical-UK
4	C18 nano/Capillary (3µm, 250mm×75µm)	Fisher Scientific-UK
5	µ-Precolumn Trap C18 Cartridge (5µm, 5mm×300µm)	Thermo Scientific-UK
6	µ-Precolumn Trap C18 Cartridge (5µm, 5mm×500µm)	Thermo Scientific-UK
7	µ-Precolumn Trap C18 Cartridge (5µm, 15mm×500µm)	Thermo Scientific-UK
8	µ-Precolumn Trap C18 Cartridge (5µm, 5mm×1000µm)	Thermo Scientific-UK
9	HPLC C8 column (5µm, 5mm×300µm)	Fisher Scientific-UK
10	HPLC C4 column (5µm, 5mm×300µm)	Fisher Scientific-UK
11	Dionex™ nano-Viper™ UHPLC Flexible Connection Tubing (50µm×650mm)	Thermo Fisher Scientific-UK
12	Dionex™ nano-Viper™ UHPLC Flexible Connection Tubing (50µm×350mm)	Thermo Fisher Scientific-UK
13	Dionex™ nano-Viper™ UHPLC Flexible Connection Tubing (20µm×350mm)	Thermo Fisher Scientific-UK
14	Dionex™ nano-Viper™ UHPLC Flexible Connection Tubing (30µm×100mm, 1/32inch)	Thermo Fisher Scientific-UK
15	Peek SIL connection tubing (50µm×50mm)	Agilent Technologies-UK
16	Peek SIL connection tubing (50µm×100mm)	Agilent Technologies-UK
17	Peek SIL connection tubing (50µm×200mm)	Agilent Technologies-UK
18	Peek SIL connection tubing (50µm×500mm)	Sigma Aldrich-Germany
19	Ferr Rheodyne	VWR-UK

Table 2.6 HPLC and MS equipment and parts.

2.4 EXTRACTION OF GLYCOSPHINGOLIPIDS FROM CLINICAL SAMPLES

Plasma samples were processed by solid phase extraction (SPE) using mixed-mode cation-exchange cartridges (Oasis, MCX 1 cc, 30 mg, 60 μm , Waters Corp). Each cartridge was conditioned using 1 mL of methanol, then 1 mL of phosphoric acid (2% H_3PO_4 in H_2O). The sample was prepared by mixing 200 μL of plasma, 500 μL of phosphoric acid (2% H_3PO_4 in H_2O) for acidification, 20 μL of 0.1%FA/20%ACN and 500 μL of GSG internal standard (0.8nM) in methanol. When standard curves were prepared the 20 μL of 0.1%FA/20%ACN contained the appropriate amount of lipid required to generate the curve. The sample was vortexed shortly and then transferred to the MCX cartridges using glass pipettes and allowed to drip through the column. Each cartridge was washed twice, firstly with 1 mL of 2%FA/ H_2O and then with 1mL of 0.2% FA/methanol. Thereafter, each cartridge was eluted using 500 μL of basic buffer (2% NH_3 /methanol). The extract was dried and then resuspended using 50 μL of 0.1%FA/20%ACN. Thereafter, 10 μL of this extract was subjected to an MRM mass spectrometric analysis following resolution of the lipids by reverse phase HPLC as set out below.

Urine samples were prepared by mixing 500 μL of urine, 500 μL of GSG (0.8 nM), 100 μL HCl (1.0 M) and 20 μL of 0.1%FA/20%ACN. When standard curves were prepared the 20 μL of 0.1%FA/20%ACN contained the appropriate amount of lipid required to generate the curve. Extraction was performed using the same procedure of plasma extraction except using of 1.0 mL HCl (1.0 N) in the conditioning of MCX cartridges, instead of phosphoric acid (2% H_3PO_4 in H_2O). The resolution of the lipids by RP-HPLC and the subsequent MRM based MS analysis are identical to the plasma samples.

2.5 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

A reverse phase-HPLC system (Ultimate 3000, Dionex) was utilized using mobile phase A (0.1%FA in H₂O), mobile phase B (0.1%FA in ACN) and a Halo C18 column (50mm×200µm, 2.7µm, from Hichrom limited-UK). Samples were loaded at 20% mobile phase B for 3 minutes and then eluted isocratically at 50% mobile phase B for 4 minutes. The column then was washed with 100% mobile phase B for 3 minutes then equilibrated with 100% mobile phase A for 2 minutes. The flow rate was 3µL/min (Table 2.7).

HPLC (Ultimate 3000 – Dionex)	
Mobile phase A	0.1%FA/H ₂ O
Mobile phase B	0.1%FA/ACN
Column	Halo [®] C18
Column (particle size)	2.7µm
Column (length×I.D)	50mm × 200µm
Column (temperature)	30 °C
Auto-sampler temperature	7 °C
Injection volume	10µL
Injection mode	Full loop

Table 2.7 Final optimized HPLC parameters.

2.6 ANALYSIS OF GLYCOSPHINGOLIPIDS USING MASS SPECTROMETRY

A micrOTOF-II mass spectrometer (Bruker-Germany) was used in this study. Positive ion mode electrospray ionisation (ESI) was employed. A multiple reaction monitoring (MRM) acquisition mode, an MS technique, was used to identify and quantify different metabolites with N₂ as the collision gas. Table 2.8 shows the different MS parameters and Table 2.9 shows MRM transitions and the applied collision energies for each metabolite. In order to prevent the system contamination, HPLC divert valve

was programmed to direct the sample solvents toward the MS only between 3-7 minutes of the run.

ESI-microTOF-MS (Bruker)	
Ion source	Electrospray ionisation (ESI)
Polarity	Positive mode
Acquisition mode	Multiple Reaction Monitoring (MRM)
Dry temperature	180 °C
Capillary voltage	3.5 Kv
Nebuliser	0.6Bar
Dry gas	4.0 l/min
Spectra rate	0.5 Hz
Rolling average	3
Mass range	50-1000 <i>m/z</i>

Table 2.8 Final optimised parameters of micro-ToF-II mass spectrometer which was utilised in the study.

MRM acquisitions		
Metabolite	Transition	Collision energy
Lyso-Gb3 (-28 Da)	758.3 >>> 254.3	38
Lyso-Gb3 (-12 Da)	774.3 >>> 252.3	38
Lyso-Gb3 (-02 Da)	784.3 >>> 280.3	38
Lyso-Gb3	786.4 >>> 282.3	38
Lyso-Gb3 (+14 Da)	800.3 >>> 334.3	38
Lyso-Gb3 (+16 Da)	802.3 >>> 280.3	38
Lyso-Gb3 (+34 Da)	820.3 >>> 334.3	38
Lyso-Gb3 (50 Da)	836.3 >>> 350.3	38
GSG (internal standard)	460.3 >>> 280.3	25

Table 2.9 Final optimised MRM transitions and collision energy for each metabolite.

2.7 STATISTICAL ANALYSIS

All data in this thesis were analysed using SPSS software (Version 21, IBM software, UK) or Microsoft Excel 2010 unless otherwise specified. Normality of data distribution was determined by Kolmogorov test and by visual inspection of values in case of small number of data points. The statistical tests used for each experiment are described in the figure legends.

CHAPTER - 3
DEVELOPMENT OF PLASMA AND URINARY
LYSO-GB3 ASSAYS

3.1 INTRODUCTION

Metabolomics is an emerging approach dealing with the scientific study of a large numbers of small molecules less than 1500 Da (metabolites) including the study of their chemical processes and their different profiles leading to identification and quantification of these metabolites in biological fluids [253-257]. The study of metabolome highlights the important details of biochemical pathways and their modifications which associated with various disorders [256]. Hence, Metabolomics has been applied in many clinical researches due to its clinical informative allowing early diagnosis of various disorders and following up the progression and regression of these diseases [258].

Various biochemical methods have been developed in order to measure Lyso-Gb3 levels in biological samples in Fabry patients. Originally, Dutch researchers extracted Lyso-Gb3 by a derivatization process using orthophthaldialdehyde (OPA). Thereafter, the separation and detection of the lipids were achieved using high performance liquid chromatography (HPLC) and fluorescence detection respectively [46, 165, 215]. The method was valuable and fair enough to measure Lyso-Gb3 levels in hemizygous Fabry males. Alternatively, the method lacked the required sensitivity where it can't measure low levels of Lyso-Gb3 such that in case of heterozygous Fabry females, late onset cardiac variant Fabry patients nor healthy controls levels. Moreover, another drawback for this method was interference of the used derivatization agent OPA with other OPA-reactive compounds of plasma.

The same Dutch team developed a new method where the lipids were extracted using liquid-liquid extraction then analyzed using LC-MS/MS. In this method Glycine-Lyso-Gb3 was synthesized and used as an internal standard [164]. This method doesn't require derivatization, decreases the processing time and has low detection

limits allowing measurement Lyso-Gb3 levels in Fabry females, atypical Fabry patients and healthy controls.

Gold et al [25] developed a new method based on using stable isotopic [5,6,7,8,9] $^{13}\text{C}_5$ -labelled Lyso-Gb3 internal standard. The lipids were extracted from the biological samples using liquid-liquid extraction before being separated, fragmented and detected using ultra performance liquid chromatography linked to electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) respectively [25]. The study measured Lyso-Gb3 and another compound was called Lyso-ene-Gb3 which later is known as Lyso-Gb3 (-2 Da) analogue. This method has advantages of being rapid [25].

In the last decade, Auray-Blais and collaborators showed advanced steps in terms of establishment many advanced metabolomics studies facilitating the measurement of chemical biomarkers of FD and revealing novel biomarkers (e.g. various isoforms/analogues of Gb3 and Lyso-Gb3) [26, 201, 202, 204-206, 213].

The worked presented here was performed to establish, optimise and validate an HPLC-MS approach to measure plasma and urinary Lyso-Gb3 and related analogues. The stages examined were the extraction of the lipids from the biological samples, the HPLC separation of the lipids and their analysis and quantitation using mass spectrometry.

3.2 METHODOLOGY

3.2.1 Lipid extraction

A reliable extraction method is an essential aspect of any lipid analysis. Here, the approach developed utilizes the addition of a known amount of a synthetic Lyso-Gb3

standard and 1- β -D-glucosylsphingosine (GSG) to samples. GSG standard is used as an internal standard, in order to quantify the lipid level reliably.

3.2.1.1 Choice of Internal Standard

Two sources of GSG were compared in this work, one derived from bovine buttermilk and the other of plant origin (Figure 3.1). The only difference between the two lipids is the presence of 2 double bonds in the sphingosine moiety of the plant lipid compared to 1 in the bovine lipid, meaning that they differ in mass by 2 Da. In both cases 5 mg of pure, commercially available lipid was dissolved in methanol to give a stock solution of 100 μ M which was stored at -20 °C until used as 0.8 nM working solution.

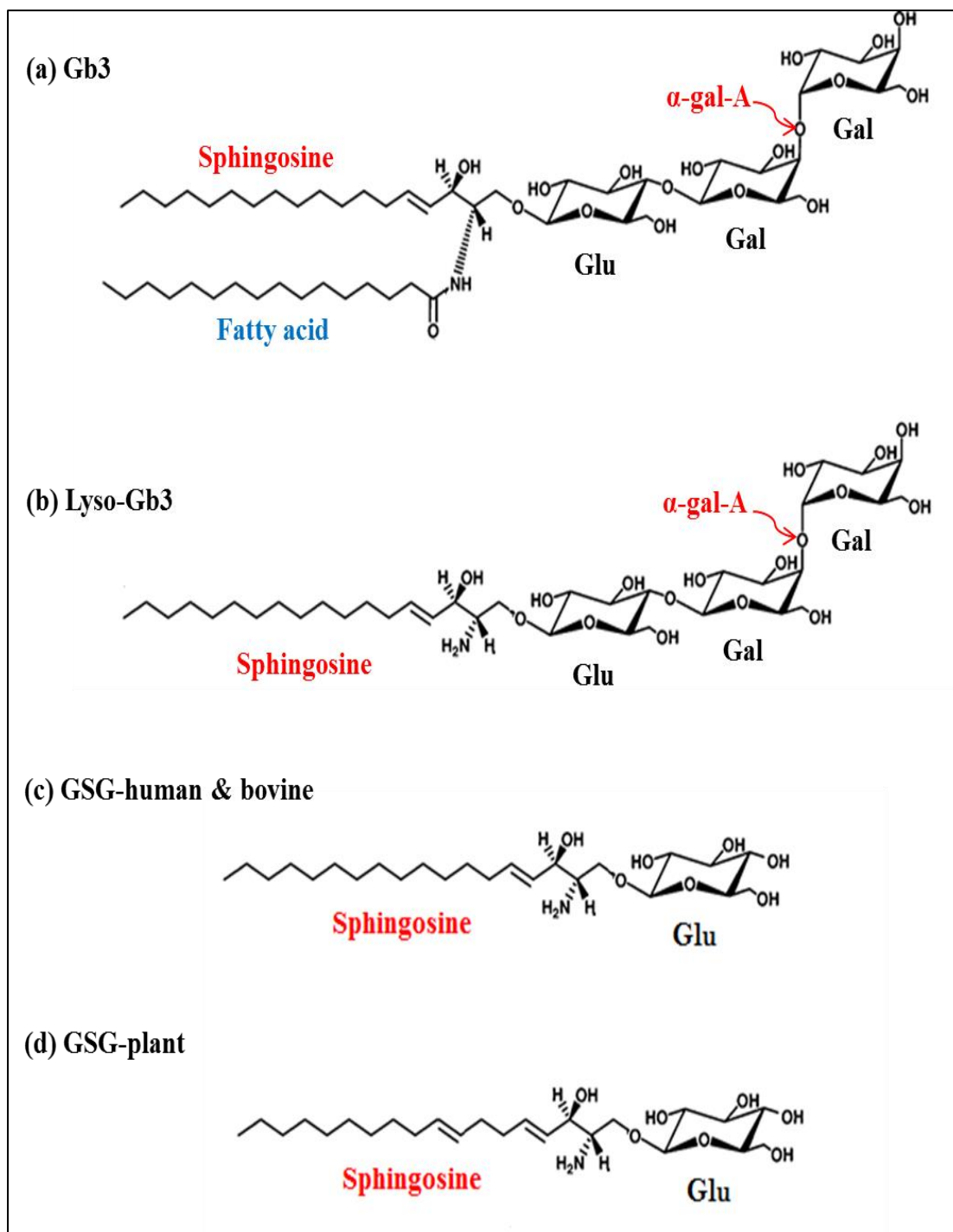


Figure 3.1 Chemical structures of GSLs: The figure shows the chemical structure of four GSLs. (a) Globotriaosylceramide (Gb3), α -gal-A key-enzyme is responsible for the hydrolytic cleavage between the galactose residues. (b) Globotriaosylsphingosine (Lyso-Gb3). (c) 1- β -D-glucosylsphingosine (GSG in human and bovine) which has similar structure of sphingosine moiety of Lyso-Gb3 resulting in similar MS/MS fragmentation pattern. (d) 1- β -D-glucosylsphingosine (GSG- plant source) where sphingosine moiety has two double bonds.

3.2.1.2 Preparation of Lyso-Gb3 Standard

Pure, commercially available Lyso-Gb3 (porcine) was used initially for method development and subsequently to prepare standard curves that were run with each batch of samples. The Lyso-Gb3, 1 mg, was dissolved in methanol to give a stock solution of 1.27 mM, which was stored at -20 °C until used as a working solution (20 µM) following serial dilution with 0.1%FA/20%ACN.

3.2.2 Resolution of Glycosphingolipids using Reverse Phase High Performance Liquid Chromatography

A range of parameters that affect the resolution of lipids by RP-HPLC were examined to establish a robust and reproducible method that is suitable for the analysis of clinical samples. The initial aspects of this work were carried out using commercially available lipids.

3.2.2.1 Choice of Tubing Connectors

The junctions in HPLC systems can have a significant effect on the resolution of compounds. This occurs when “dead space” or gaps are generated which result in a poor peak shape. In this work, the method development aimed at employing the optimal HPLC tubing connectors in order to improve the chromatography. Two different brands of HPLC connection tubing were tested: standard HPLC connection tubing and nano-Viper fittings connection tubing.

3.2.2.2 Choice of Chromatography Stationary Phase

The stationary phase in HPLC is the reverse-phase resin in the trap/guard column and the resolving column. For GSLs separation a silica support with an 18 carbon aliphatic chain is the universal choice. There are many commercially available high resolution columns; here three different brands were tested to compare the separation

of GSLs with respect of area under the curve (AUC), peak intensity, shape and tailing. In addition the background contamination of the MS spectra with each column was compared. These columns tested were: Halo C18 column (2.7 μ m, 50mm \times 200 μ m, Hichrom Limited-UK), Ascentis Express C18 (2.7 μ m, 50mm \times 200 μ m, Supelco Analytical-UK) and Thermo C18 column (3.0 μ m, 150mm \times 75 μ m, Thermo Scientific-UK).

The trap columns employed (recommended by Dionex, the Manufacturer of the HPLC system) were C18 μ -Precolumns (silica support modified with 18 carbon aliphatic chains). These are supplied in cartridges that are fitted into the system with no dead space. Three sizes were (5 μ m, 5mm \times 300 μ m, 5 μ m, 5mm \times 500 μ m and 5 μ m, 15mm \times 500 μ m) compared to ensure sufficient, but not excess capacity was used.

3.2.2.3 Optimisation of Flow Rate

The rate at which the mobile phase flows through the trap and resolving columns is an important parameter that can affect binding and elution characteristics. The flow rate was varied and significant factors such as retention time, peak intensity, AUC and shape were measured.

3.2.2.4 Optimisation of Binding and Elution Conditions

In reverse phase chromatography hydrophobic analytes bind to the stationary phase under relatively polar conditions, so the loading solvent contains a low percentage of organic solvent and a large amount of water. The actual percentage of organic solvent used for binding should be the highest possible that results in complete binding of the analyte. In this way the lowest background contamination binding to the column should occur. The elution of the bound lipids is performed by increasing the

percentage of organic solvent in the liquid that is pumped through the column. This can be done using a gradual increase (termed gradient elution) or by a stepped increase which is then held constant (termed isocratic elution). An appraisal of the elution conditions that are suitable for a reliable analysis of Lyso-Gb3 in Clinical samples was performed.

3.2.3 Analysis of Glycosphingolipids using Mass Spectrometry

A micrOTOF-II mass spectrometer (Bruker-Germany) was selected to be employed in the study. There are many parameters that can affect the accuracy, reliability and reproducibility of mass spectrometers and all of these were optimised in this work. Positive ion mode ESI was employed as an ion source to convert the sample from liquid phase to gas phase. The identity of each metabolite was confirmed using a multiple reaction monitoring (MRM) acquisition mode. MRM is an MS technique used to identify and quantify different metabolites with N₂ as a collision gas. The different MS parameters (Table 2.8), MRM transitions and the applied collision energies for each metabolite (Table 2.9) were tested and optimised. In order to prevent contamination in the HPLC-MS system, the HPLC effluent is discarded to the waste between (0-3 min) and (7-12 min) using the HPLC divert valve.

The identity of each metabolite is confirmed using an MRM mode where the ion of interest, identified by the accurate m/z , is isolated within the collision cell of the MS, where it is subsequently fragmented by collision with an inert gas (N₂) using a specified energy level. This gives rise to characteristic daughter ions where the presence of the daughter ions conclusively identifies the parent ion, and the intensity and AUC of the daughter ion are considered as an accurate measure of the parent ion concentration. This is termed an MRM transition. Here, the intensity of the most

abundant specific daughter ion, the dehydrated sphingosine moiety (m/z 282.3) derived from the Lyso-Gb3 parent ion (m/z 786.4) was recorded and AUC determined using a function available in the manufacturer's software package. Every assay contained the internal standard GSG-plant source (m/z 460.4) or GSG-bovine source (m/z 462.4) which contain the sphingosine moiety, (Figure 3.1, c and d) was also isolated and fragmented in an identical fashion to the lipids under investigation and the intensity and AUC of the m/z 280.3 (for vegetal GSG) or m/z 282.3 (for the animal source GSG) daughter ion derived from the GSG determined. Any losses during any part of the assay procedure, from the point where the GSG was added to the samples, can therefore be accounted for by dividing the measured AUC of the daughter ion derived from the lipid of interest by the recorded AUC of the daughter ion derived from GSG internal standard.

3.3 RESULTS

3.3.1 Extraction Protocols

3.3.1.1 Extraction of Lyso-Gb3 and Analogues

Two approaches for the extraction of Lyso-Gb3 have been developed previously. The SPE protocol used here [204] and a liquid-liquid extraction based on the method developed by Bligh and Dyer [255, 256] where the lyso-lipids partition into an upper water/methanol phase. Initially, a brief comparison of these methods was performed and although the sensitivities of the methods was comparable due to the lower reproducibility, the more complex procedure, and the requirement to use halogenated solvents (chloroform) the Blighe and Dyer method was considered inferior. Data to support this conclusion is not presented as no patient samples were analysed using Bligh and Dyer extraction method.

3.3.1.2 Extraction Efficiency of Lyso-Gb3 from Urine and Plasma

The extraction efficiency of Lyso-Gb3 from urine and plasma using SPE was tested. This was done by performing 2 sets of extractions; the first where 1.0 picomole of the synthetic Lyso-Gb3 standard was added to either urine (500µL) or plasma (200µL) samples prior to addition to the MCX columns (as described previously) or secondly where 1.0 picomole of the synthetic Lyso-Gb3 standard was added to the solvent used to resuspend the extraction residue (post extraction) and will therefore have no extraction related losses. In this way the extraction efficiencies can be calculated by comparing the Lyso-Gb3 levels detected in these 2 sets of samples (Equations 3.1 and 3.2). The efficiency of extraction from urine was found to be more than 90% and for plasma to be 65-70%. The lower extraction efficiency from plasma is probably due to the Lyso-Gb3 binding to (or associating with) the much more abundant proteins and/or lipids in plasma. A range of other factors are thought to affect the amount of analyte that is detected in this type of protocol, especially when the analyte concentrations are low as they are here. For example, non-specific losses due to adsorption to any surface that the analyte is exposed to during the entire analytical process or in the case of an MS-based analysis factors that enhance or suppress ionisation of the analyte will also play a role.

$$\text{Response ratio} = (\text{Lyso-Gb3AUC/GSG AUC}) * 100\% \dots\dots\dots \text{Equation 3.1}$$

$$\text{Extraction recovery} = (\text{Lyso-Gb3 response ratio before extraction} / \text{Lyso-Gb3 response ratio after extraction}) * 100 \dots\dots\dots \text{Equation 3.2}$$

3.3.2 Chromatography

Several of the most important factors that limit the quality of data when using HPLC based methods were optimised in this work. Figure 3.2 shows that the elution peak of Lyso-Gb3 and GSG lasts 30-40 seconds in this work. The flow rate is 3 μ L/min meaning that elution volume is approximately 1.5-2 μ L. This narrow shape leads to a high peak intensity which is a significant help in lowering the limit of detection and quantitation. Anything that causes the peak shape to broaden or tail will diminish the peak intensity and compromise the quality of the assay and anything that helps to narrow the peak will help to improve the analysis. The final different optimisation phases of the HPLC parameters used in current study are shown in Table 2.7.

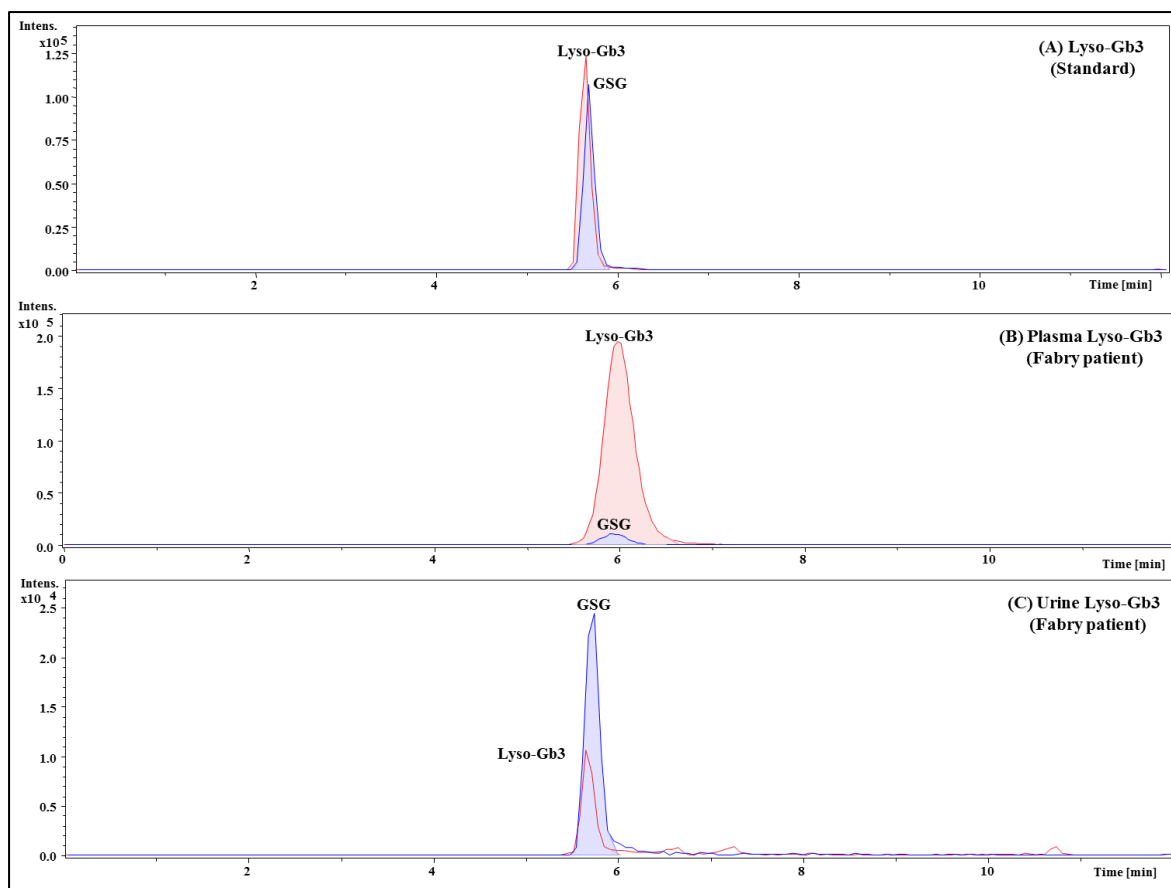


Figure 3.2 Detection of Lyso-Gb3 using MRM mode: (a) Extracted ion chromatogram of Lyso-Gb3 standard (Red) and GSG internal standard (Blue). (b) Extracted ion chromatogram of Lyso-Gb3 in urine sample from a Fabry hemizygous male (Red) and GSG internal standard (Blue).

3.3.2.1 Choice of Tubing Connectors

During the early stages of this project, Dionex, the manufacturer of the HPLC system developed a new zero dead volume tubing connector. Dead volumes at manually prepared conventional unions and junctions are very difficult, if not impossible, to avoid as small imperfections or irregularities will occur when the tubing is cut to form the union/junction. Even small dead spaces can be problematic because the peak volume is only 1.5-2 μ L. A comparison between an analysis where conventional and nano-Viper fittings were used was carried out. There is a minimum of five junctions/unions in the liquid path in the HPLC (from the autosampler to the mass

spectrometer) we employed. Figure 3.3 shows that peaks eluted slightly earlier; were narrower, sharper and more intense when nano-Viper fittings were used. The use of nano-Viper fittings decreased the limit of detection of Lyso-Gb3 from 40 fmols to 10 fmols and was used in all assays of clinical samples presented here.

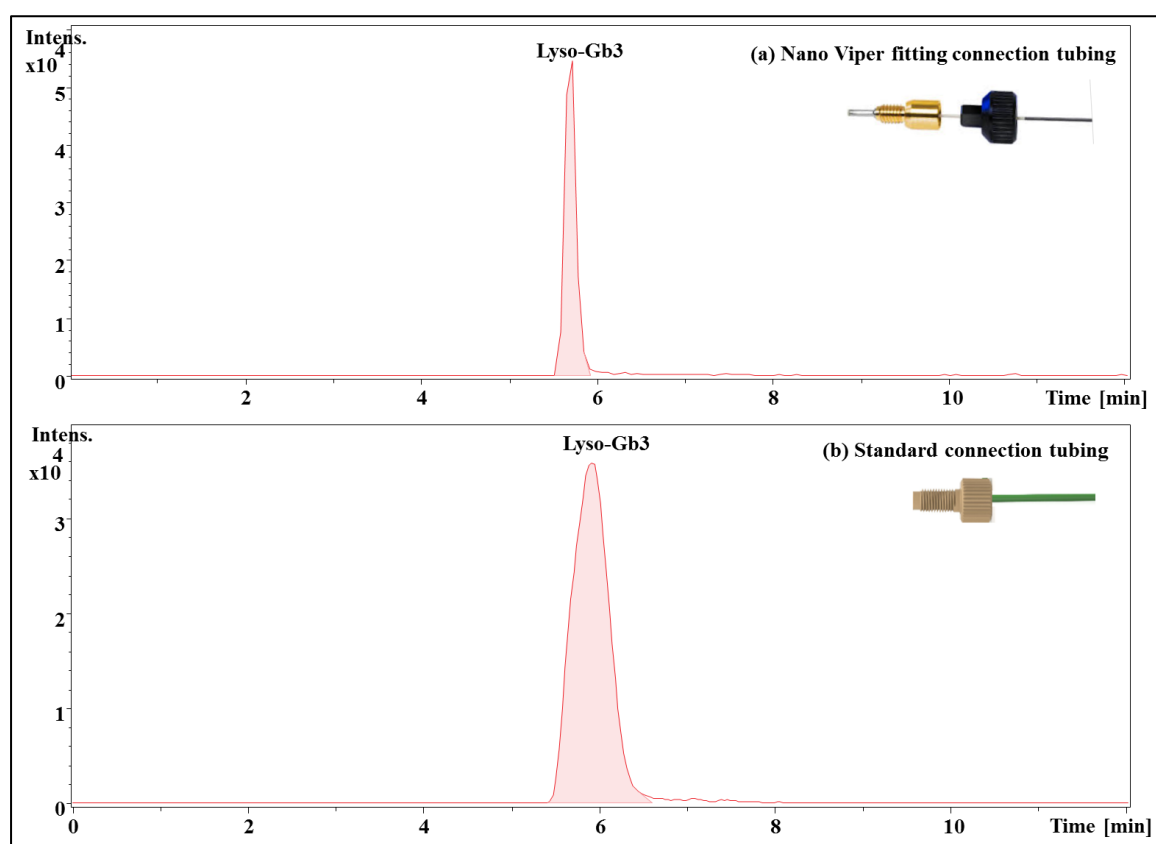


Figure 3.3 Chromatography developments: Extracted ion chromatograms of the main fragment ion (m/z 282.3) derived from Lyso-Gb3 using: (a) nano-Viper connection tubing designed to give zero-dead space displaying sharper peak (lower peak width), higher peak intensity and earlier retention time of detection. (b) Standard connection tubing can cause chromatography dead space due to its end fitting.

3.3.2.2 Choice of Chromatography Stationary Phase

Three different brands of C18 columns were tested for MS background, reproducibility, peak shape and tailing in order to select the most appropriate resolving column. Although all 3 columns were satisfactory the Halo C18 column (2.7 μ m, 50mm \times 200 μ m, Hichrom Limited-UK) was chosen as the background contamination MS peaks were slightly lower in initial tests and was employed for the whole study.

A trap column was employed in this work to protect (guard) the main column. Samples flow through the trap column under conditions where the analyte binds whilst the unbound material passes straight through and is discarded to waste (Figure 3.4). Any particles or poorly dissolved material will collect at the start of the trap column. It is important that the trap column has sufficient capacity to bind all of the analyte under study but an overly large column can lead to long run times, non-specific losses (due to excessive surface area) and peak broadening. My initial studies showed that a trap column with the dimensions of 5 μ m, 5mm \times 300 μ m was optimal. This trap column has 5 μ m particles, compared to 2.7 μ m for the main C18 column and is 5mm in diameter compared to 200 μ m (or 0.2mm which means that the trap column has 25 times greater diameter or 625 times greater cross sectional area) to the main C18 resolving column. This allows the trap column to be loaded with sample at 15 μ L/min. The larger particle size and massively increased surface area means that the trap column can be loaded at higher flow rates to save time, is far less prone to blocking/plugging and acts as a very effective guard, which extends the useful life of the main column, thereby aiding reproducibility (Figure 3.5).

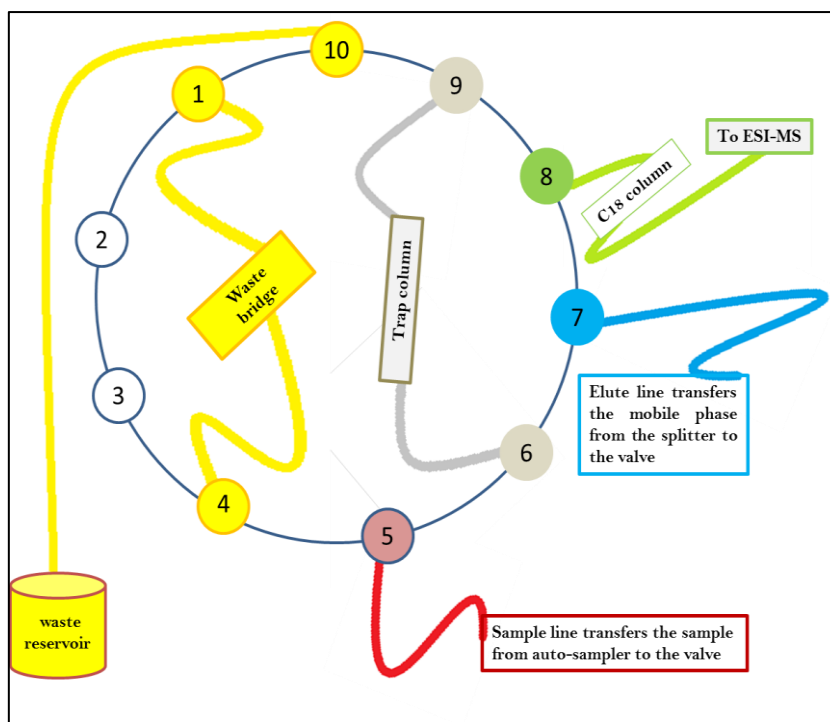


Figure 3.4 HPLC valve operation and usage: The diagram represents the 10 port valve that was fitted to the Dionex 3000 HPLC system used in this work. The valves work by having channels within them that connect each port to one of the two ports on either side of them and when the valve switches the connection is redirected to the port on the other side. For example, if port 5 was connected to port 4 in position A it would change to port 6 after switching to position B and port 4 would then be connected to port 3. In the schematic shown the sample line, from the autosampler, is connected to port 5, and whilst loading this is connected to port 6. The sample (resuspended in 20%ACN/0.1%FA in water) then exits port 6 and flows through the trap column. The GSLs bind to the trap column and the unbound material flows onto port 9, which is connected to port 10, which then directs the flow to waste. During this time the flow into port 7 comes from the HPLC elution line and then onto the resolving column via port 8. During the sample loading the flow through this line contains 20% ACN/0.1%FA in water. After the sample is loaded (3min at 15 μ L/min) and the trap column washed the valve switches so that the flow into port 6, which is connected to the trap column, comes from the solvent elution line. The solvent composition is changed at exactly the same time to 50%ACN/0.1%FA in water to elute the analytes from the trap column. After the valve switch port 9 is connected to port 8 (rather than port 10) so that the eluate from the trap column flows onto the resolving C18 column via port 8. The GSLs are resolved on this column and eventually are sprayed directly into the mass spectrometer for analysis. All the flow rates, valve switches and solvent composition changes/timings etc. are written into the fully automated HPLC program developed in this work.

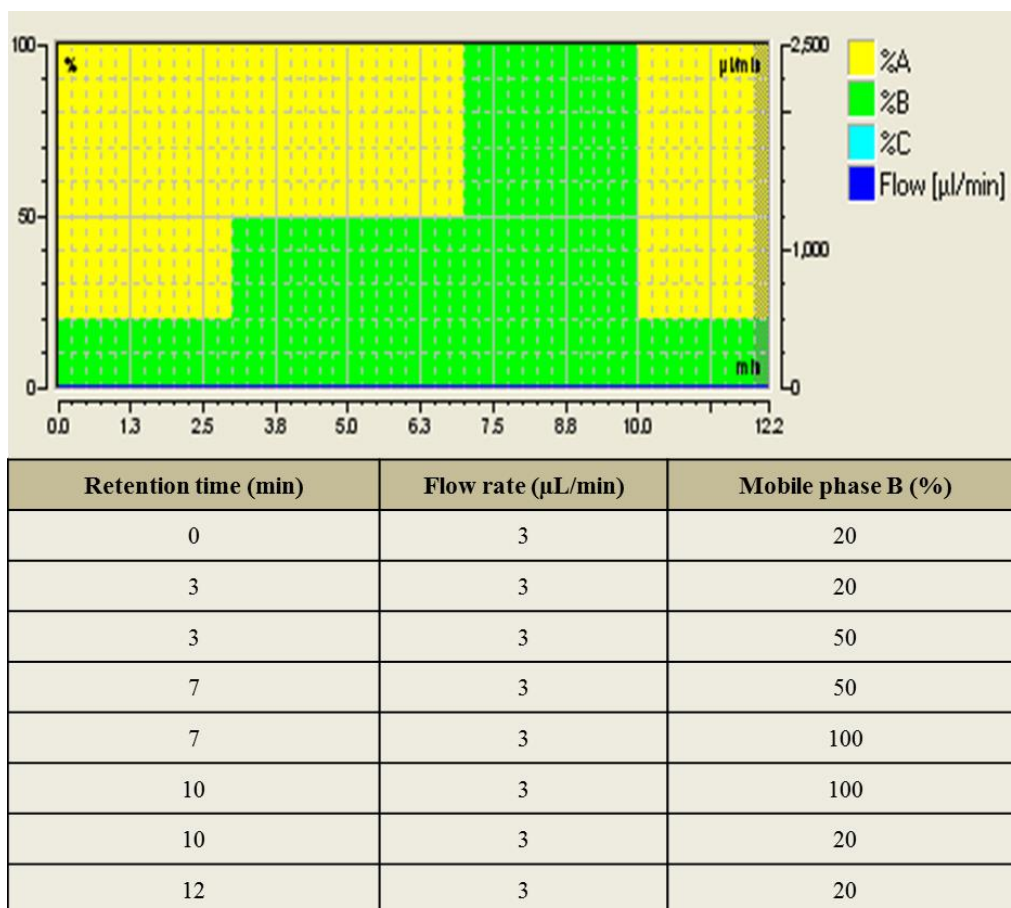


Figure 3.5 HPLC optimization, the final optimized HPLC gradients: The different concentrations of ACN were generated by the HPLC mixing the required proportions of solvent A (0.1% FA in water) and solvent B (0.1% FA in ACN). The sample was resuspended in 20%ACN/0.1%FA in water. Then, the sample was loaded into the trap column using 20% of mobile phase B for 3 min at loading flow rate of 15μL/min and then eluted at flow rate of 3μL/min using 50% of mobile phase B for 4 min. Thereafter, the column was washed with 100% of mobile phase B for 3 min. and finally, the HPLC system was equilibrated using 20% of mobile phase B for 2 min to be ready for the next sample. During the washing and equilibration steps, the eluate was directed to waste to decrease the “dirt” flushed into the mass spectrometer.

3.3.2.3 Binding and Elution Conditions

3.3.2.3.1 Flow Rate

The flow rate used in this type of analysis is, like many parameters, a compromise where the quality of the data, the time it takes to perform the analysis and the back pressure of the HPLC should be considered. For example, higher flow rates lead to shorter analysis times but if it is too high the analyte binding to the trap column may be affected and the back pressure could reach unacceptable levels. A range of flow rates were tested and 15 $\mu\text{L}/\text{min}$ for loading the trap column and 3 $\mu\text{L}/\text{min}$ for elution of the resolving C18 column generated an acceptable back pressures, complete binding of the analytes and good peak shapes and were used for all the subsequent work. Higher flow rates generated too much back pressure.

3.3.2.3.2 Solvent Composition

The conditions used to ensure reliable binding of analyte to the stationary phase are again a compromise. A high polarity (low organic solvent content) acidic solvent would be expected to increase binding of GSLs but would also cause more background lipids/contaminants to bind as well. This may lead to a high background contamination that can lead to poor quality data. Therefore, it is more usual to use a less polar solvent (higher organic content) that results in complete binding of the analyte whilst minimising the binding of other compounds. Another factor to be considered is the solubility of analytes in the solution used to resuspend the residue produced following extraction from the sample. Lipids are often kept in solution in biological samples by being bound to proteins, located in lipoproteins, vesicles, or as a component of micellar type structures. When the samples are extracted these structures will be destroyed and the solubility of many lipids in an aqueous solvent

will be limited. Therefore, a less polar solvent is often used to resuspend extract residues. The lipids of interest here are lyso-lipids (one acyl chain) that are less hydrophobic than lipids that contain 2 acyl chains (like Gb3) so relatively polar solvents can be used. Our initial studies showed that a solvent containing 0.1% FA and 20% ACN in water was suitable to both resuspend the extracted lipids and to ensure complete binding of analytes to the solid phase without increasing background MS contamination to unacceptable levels.

Gradient elution is often used when large numbers of compounds in very complex mixtures are being identified, for example, in a shotgun proteomic protocol but it is quite common to use an isocratic elution when a small number of specific analytes are being quantified. Therefore, a series of isocratic elutions, using Lyso-Gb3 standards, using increasingly percentages (35, 40, 45, 50, 55 and 60% in 0.1% FA in water) of ACN were performed and the elution characteristics of the Lyso-Gb3 recorded. The retention times, peak intensities and AUC were recorded and are shown in Figure 3.6. The peak shapes and the level of background contamination were also monitored. The AUC and peak intensities of the Lyso-Gb3 increased as the proportion of ACN increased, as the extent of elution increased. However, the background contamination also increased at the same time. The retention time decreased as the ACN increased, which allows for a shorter analysis time, which is an important factor when developing a routine assay. As a compromise between increased AUC/peak intensity and background issues all samples were analysed with an isocratic elution using 50% ACN in 0.1% FA in water.

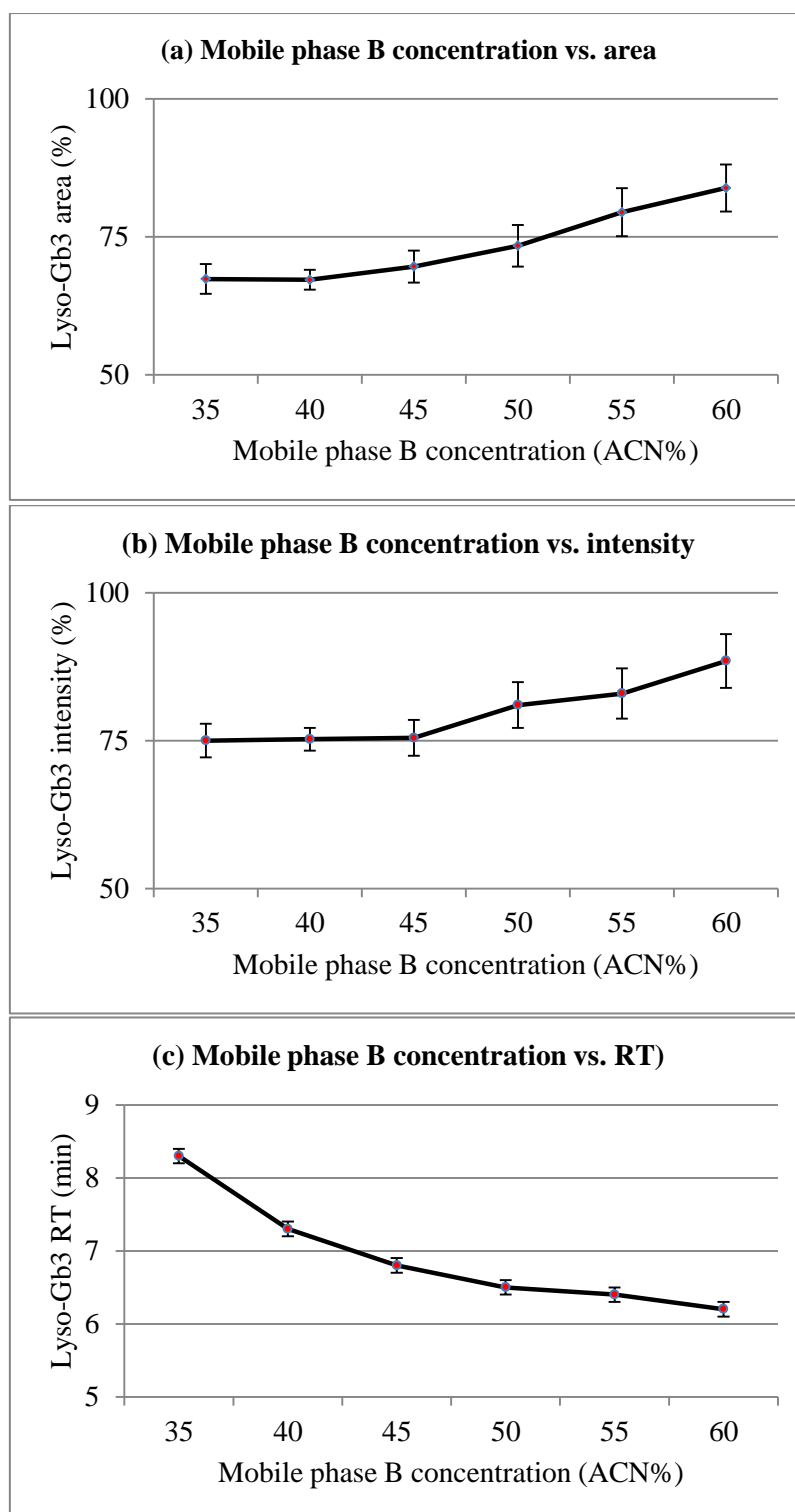


Figure 3.6 HPLC optimisation – the optimised solvent composition used for the stationary phase elution: Isocratic elution of Lyso-Gb3 using a range of mobile phase B (1%FA/ACN) concentrations was performed and (a) AUC, (b) intensity and (c) retention time (RT) recorded and the peak shape and background contamination observed. Increasing the concentration of ACN decreased RT and increased the AUC and intensity but at concentrations above 50% ACN the background was problematic so 50% ACN was used routinely.

3.3.3 Optimisation of the Mass Spectrometer

The sensitivity and stability of the mass spectrometer in this work is of fundamental importance therefore, a great deal of care was taken to monitor, maintain and optimise the instrument performance.

3.3.3.1 Tuning and calibration of MS

The software supplied with the micrOTOF-II mass spectrometer used in this work has an automatic tuning and calibration function. In order to do this a commercially available calibrant (ESI-L low concentration tuning mixture – Agilent Technologies – USA, calibrants at m/z of 118.09, 322.05, 622.03, 922.01, 1221.99, 1521.97, 1821.95, 2121.93, 2421.91 and 2721.89) was infused directly into the MS and a comprehensive optimisation of all variables related to the ion optics and detector performed. Modern mass spectrometers are extremely complex and all instruments are now tuned in this way to ensure that optimal conditions are obtained. The instrument was also calibrated once the tuning was complete (Figure 3.7). This test was done every day and the calibrant mass accuracies and intensities recorded to monitor instrument long term performance. The ion source was optimised manually, prior to and after the tune/calibration procedure was carried out, using calibrant intensity to choose the optimum charge.

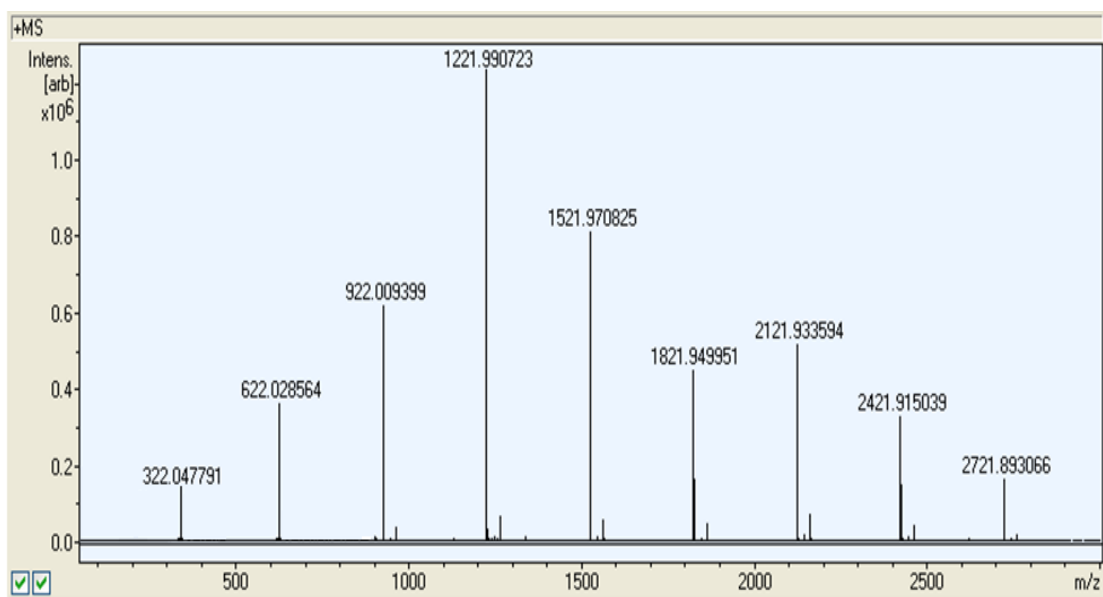


Figure 3.7 MS tuning and calibration: A mass spectrum generated from microToF MS showing the final optimised calibrants to follow the instrument performance. The figure shows 9 peaks of the mixed calibrants at m/z of 322.05, 622.03, 922.01, 1221.99, 1521.97, 1821.95, 2121.93, 2421.92 and 2721.89. The signal intensities and mass accuracies were tested and optimised by MS tuning and calibration before each analytical run. This way allows us to follow the performance of the instrument for long term.

3.3.3.2 Dry Gas Temperature

Another important factor in the ionisation process is the temperature of the ion source. This is important because the hot gas infused into the MS source evaporates the solvent (from the HPLC) that contains the eluted analyte to allow ionisation in the gas phase. Therefore, the gas needs to be hot enough to remove the solvent effectively, but if it is too hot it can lead to premature fragmentation of the analyte, compromising the assay. A range of dry gas temperatures between 50 and 200 °C were tested and 180 °C was chosen as optimum as this gave the highest intensity of the infused calibrants.

3.3.3.3 Optimum Spectral rate

The spectral rate in an MRM based analysis is also a compromise. The spectral rate is essentially; if the rate is low the analyte is allowed to accumulate in the collision cell for longer, increasing the sensitivity of the analysis. However, since the GSLs elute from the resolving column over a narrow 30-40 second peak a low spectral rate will mean that less data points will be collected across the peak which will limit the ability to reliably calculate the AUC. In order to assess the effect of changing the analytical rate different amounts of Lyso-Gb3 were extracted and analysed using the optimised procedures outlined above at spectral rates of 0.5, 1, 2 and 5 Hz. The findings showed that a rate of 0.5 Hz was the optimal condition to be used in the study (Figure 3.8) due to the enhanced sensitivity and the decreased variability.

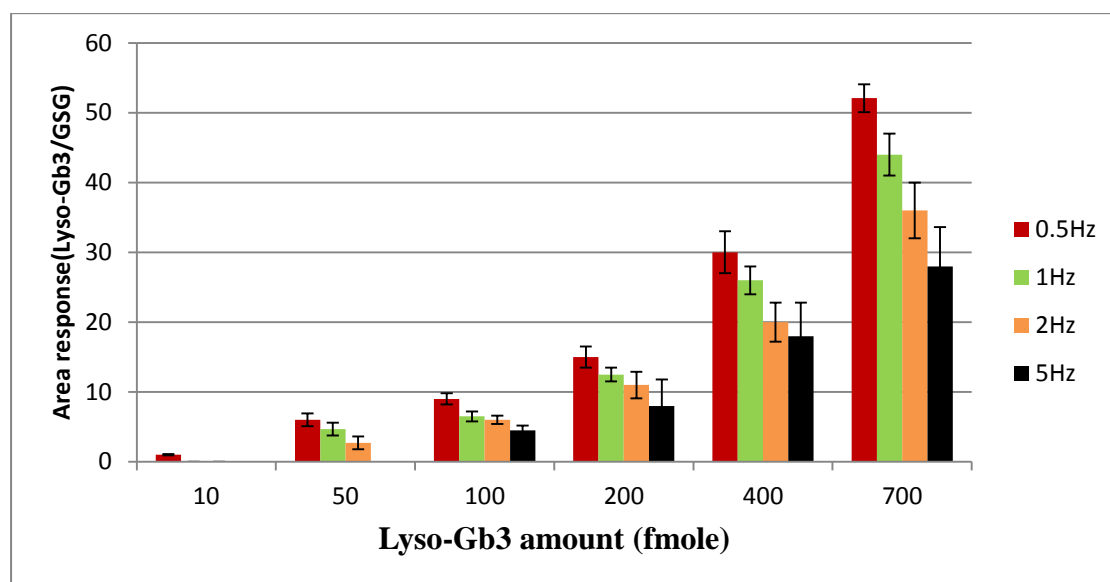


Figure 3.8 Optimisation of the MS spectral rate: MS spectra rate of 0.5Hz gives lower limit of detection, higher intensity and AUC so it has been selected as the optimised MS spectra rate in the study

3.3.3.4 Lyso-Gb3 fragmentation profile by MS/MS

In order to accurately quantify Lyso-Gb3 and other metabolites, MRM mode was used. The metabolites detected at the predicted masses were isolated and fragmented and the masses and intensities of the daughter ions recorded using an MRM based protocol. This procedure eliminates contaminant peaks giving a clean fragmentation spectrum (Figure 3.9). The fragmentation mechanism of Lyso-Gb3 in the collision cell of a mass spectrometer like the micrOTOF-II used here is outlined in Figure 3.10 while Figure 3.9 shows the actual fragmentation spectrum where the protonated Lyso-Gb3 molecule [(a), m/z 786.4] loses the 3 carbohydrates (galactose, galactose and glucose) [(c, e and g), m/z 624.4, 462.3 and 300.3 respectively] represented by losing 162 Da each time. The loss of these 3 carbohydrates results in formation of a sphingosine ion [(g), m/z 300.3]. Moreover, peaks [(b, d, f, h and i), m/z 768.4, 606.4, 444.4, 282.4 and 282.4 respectively] are generated by the loss of a water molecule from the sphingosine moiety at each stage. Subsequently, this mono-dehydrated sphingosine molecule [(h and i), both have the same m/z 282.4] is fragmented by another dehydration process resulting in peak [(j), m/z 264.4]. Therefore the peak of m/z 282.29 is used for identification and quantitation of Lyso-Gb3 by measuring AUC for this ion.

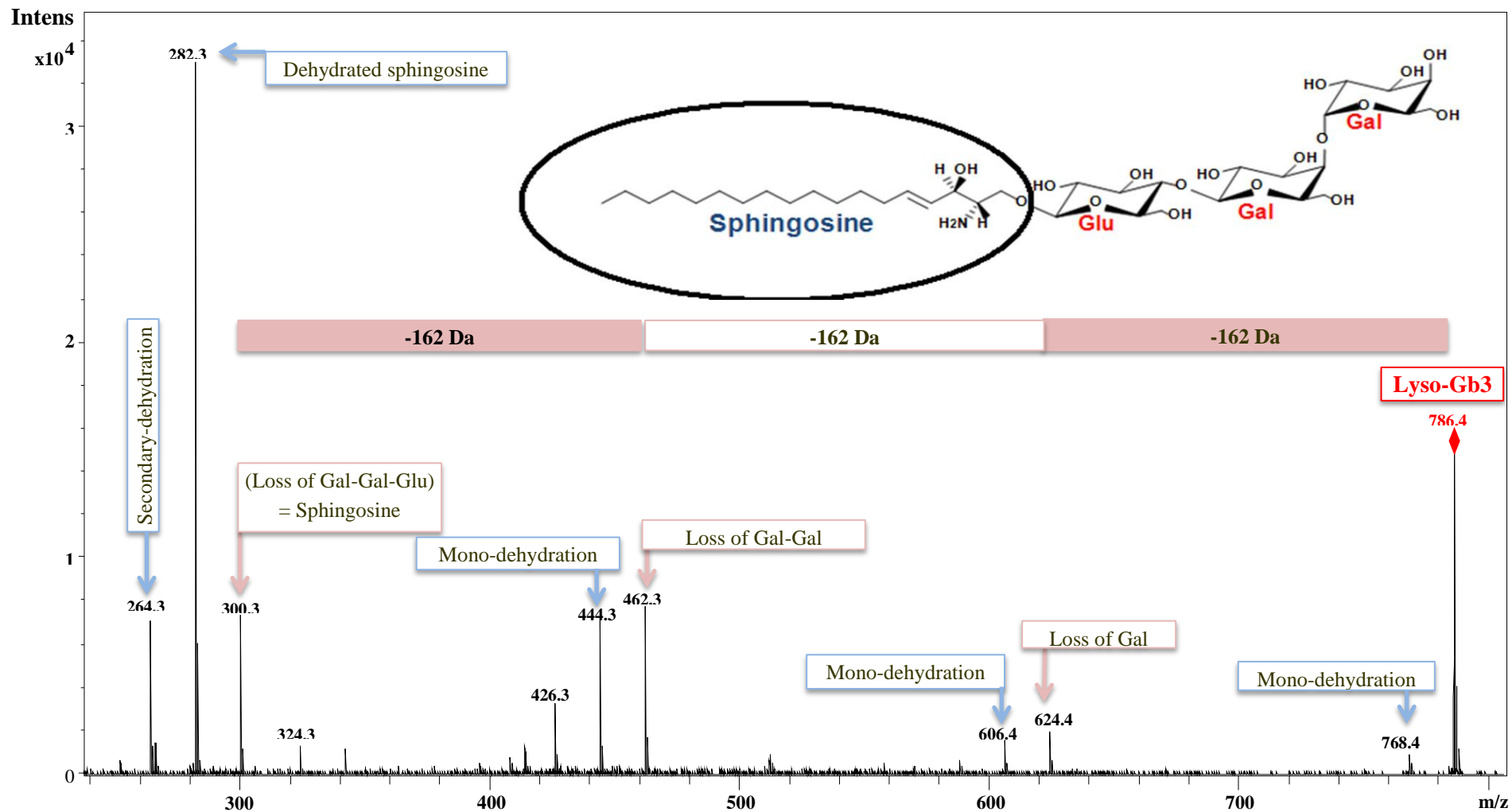


Figure 3.9 MS/MS of Lyso-Gb3: This mass spectrum data was generated using microToF-MS, and shows the fragmentation (MRM transitions) of the Lyso-Gb3 precursor ion (m/z 786.4) into a range of fragments (daughter ions). There are 3 losses of 162 Da represented in the loss of the 3 sugar groups resulting in sphingosine molecule (m/z 300.3). Subsequently, a mono-dehydration process occurs at each stage. The ion m/z 282.3 has two channels (it could result from a mono-dehydration process of sphingosine molecule and/or the loss of glucose molecule from the ion m/z 444.3). The most abundant daughter ion (m/z 282.3) is used to identify and quantify Lyso-Gb3.

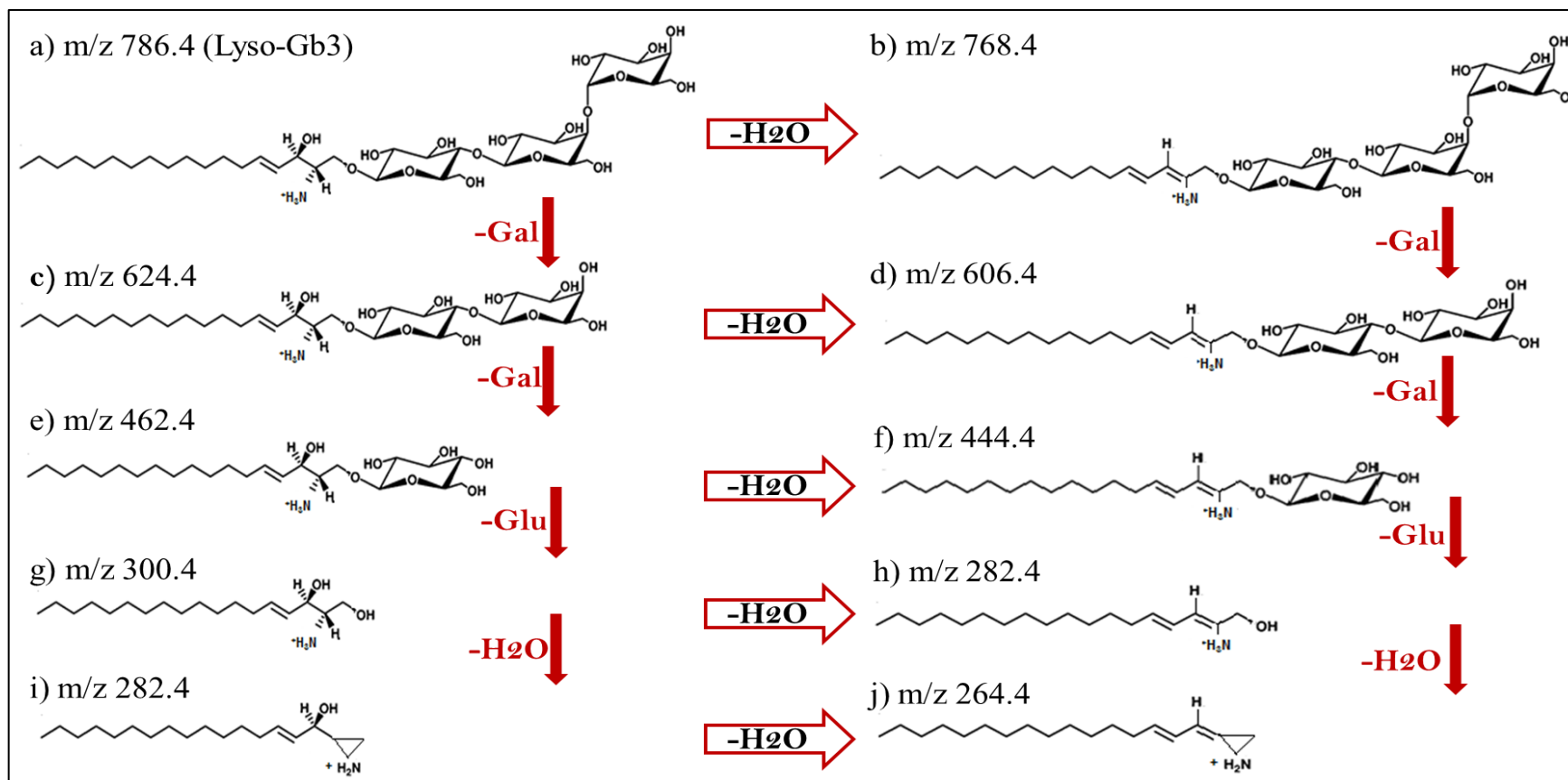


Figure 3.10 Theoretical fragmentation procedure of Lyso-Gb3: The structures of the ions generated in the MRM transition of Lyso-Gb3 precursor ion (m/z 786.4) to the most abundant daughter ion (dehydrated sphingosine, m/z 282.3) are shown along with intermediate structures of all the fragments, explaining the mechanism. A total of 3 losses separated by 162 Da which represent the loss of the 3 carbohydrates (c, e & g) generating a sphingosine molecule (g). At each stage, a mono-dehydration process is achieved resulting in the peaks (b, d, f, h & i). Subsequently, (h & i) are fragmented by another dehydration generating peak (j).

3.3.3.5 MRM Analysis of Lyso-Gb3 Analogues

Recent work by Aurey-Blais et al [85, 206, 225, 226] has identified analogues of Lyso-Gb3 in samples from Fabry patients and there is the potential that these analogues could be clinically relevant biomarkers. Here plasma from an untreated classical Fabry hemizygote (Figure 3-11) was found to contain analogues with m/z 758.4, 784.3, 802.3, 804.4, 820.4 and 836.4, which differ from Lyso-Gb3 by m/z -28, -2, +16, +18, +34 and +50 Da respectively. With urine from a Fabry patient seven analogues were detected with m/z of 758.4, 774.4, 784.3, 800.4, 802.3, 820.3 and 836.3 resulting from a modification in the sphingosine moiety of m/z -28, -12, -2, +14, +16, +34 and +50 Da respectively. Figure 3.12 shows the extracted ion chromatograms for the urine derived analogues from an untreated classical Fabry hemizygote. When these analogues are isolated and fragmented in the collision cell of the mass spectrometer they display a series of daughter ions (fragments) that differ from Lyso-Gb3 fragments, by exactly the same m/z as the parent ion varies from Lyso-Gb3 (Figure 3-13). Therefore, the analogues have the same tri-carbohydrate group as Lyso-Gb3 and a modified sphingosine moiety. The analogues can be quantified in the same way as Lyso-Gb3 using the area response of the analogue to GSG internal standard by calculating the AUC of the modified sphingosine moiety and dividing this by the AUC of the sphingosine derived from the GSG internal standard as shown in the following equation (*Equation 3-3*):

$$\text{Area response} = \text{AUC of Lyso - Gb3 analogue} / \text{AUC of GSG} \quad \dots\dots \text{Equation 3-3}$$

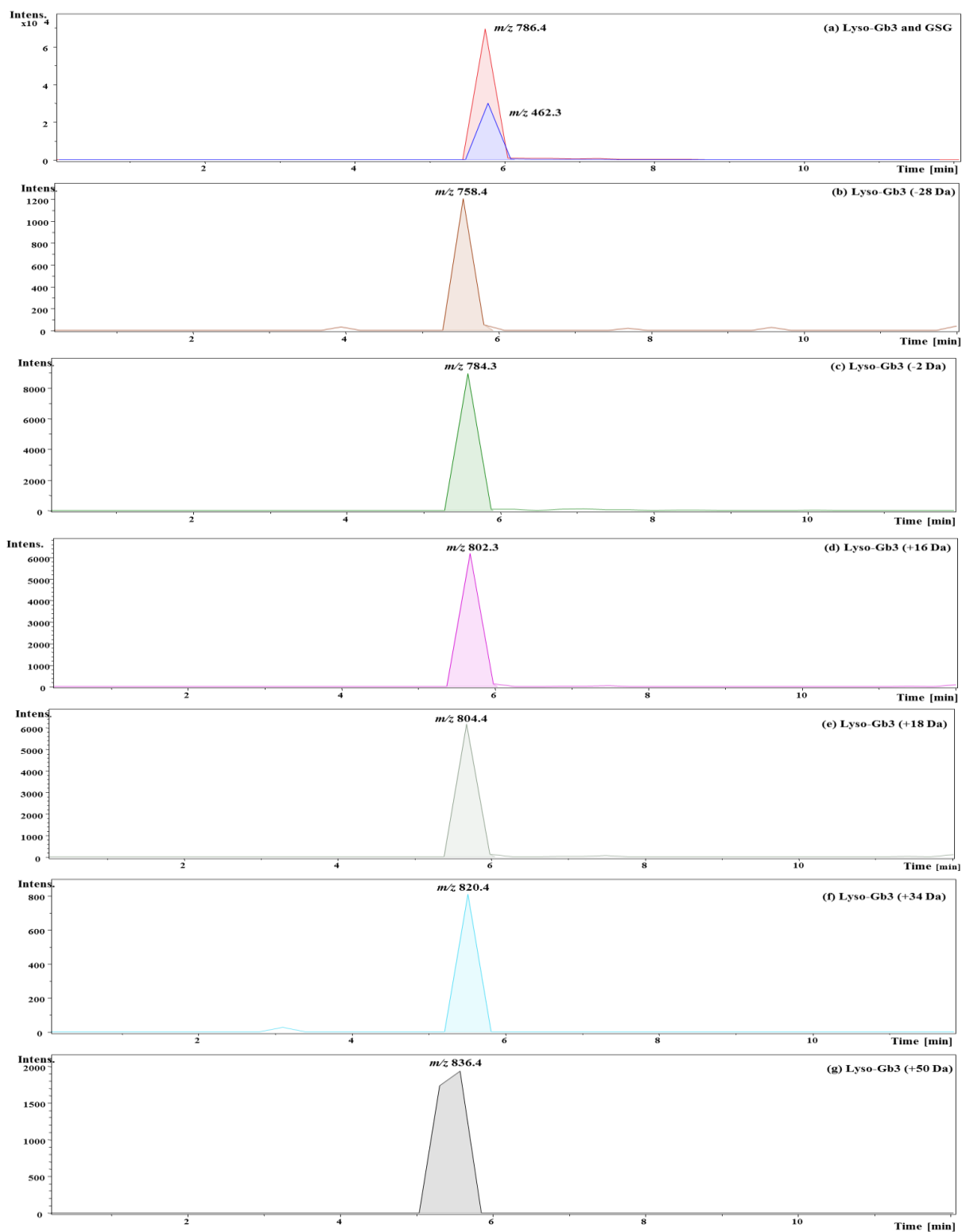


Figure 3.11 Plasma Lyso-Gb3 analogues in untreated Fabry hemizygote: Extracted ion chromatograms of Lyso-Gb3 and its analogues in plasma sample from a hemizygous Fabry male: (a) Lyso-Gb3 (m/z 786.4), (b) Lyso-Gb3 (-28 Da, m/z 758.4), (c) Lyso-Gb3 (-2 Da, m/z 784.3), (d) Lyso-Gb3 (+16 Da, m/z 802.3), (e) Lyso-Gb3 (+18 Da, m/z 804.4), (f) Lyso-Gb3 (+34 Da, m/z 820.3) and (g) Lyso-Gb3 (+50 Da, m/z 836.3).

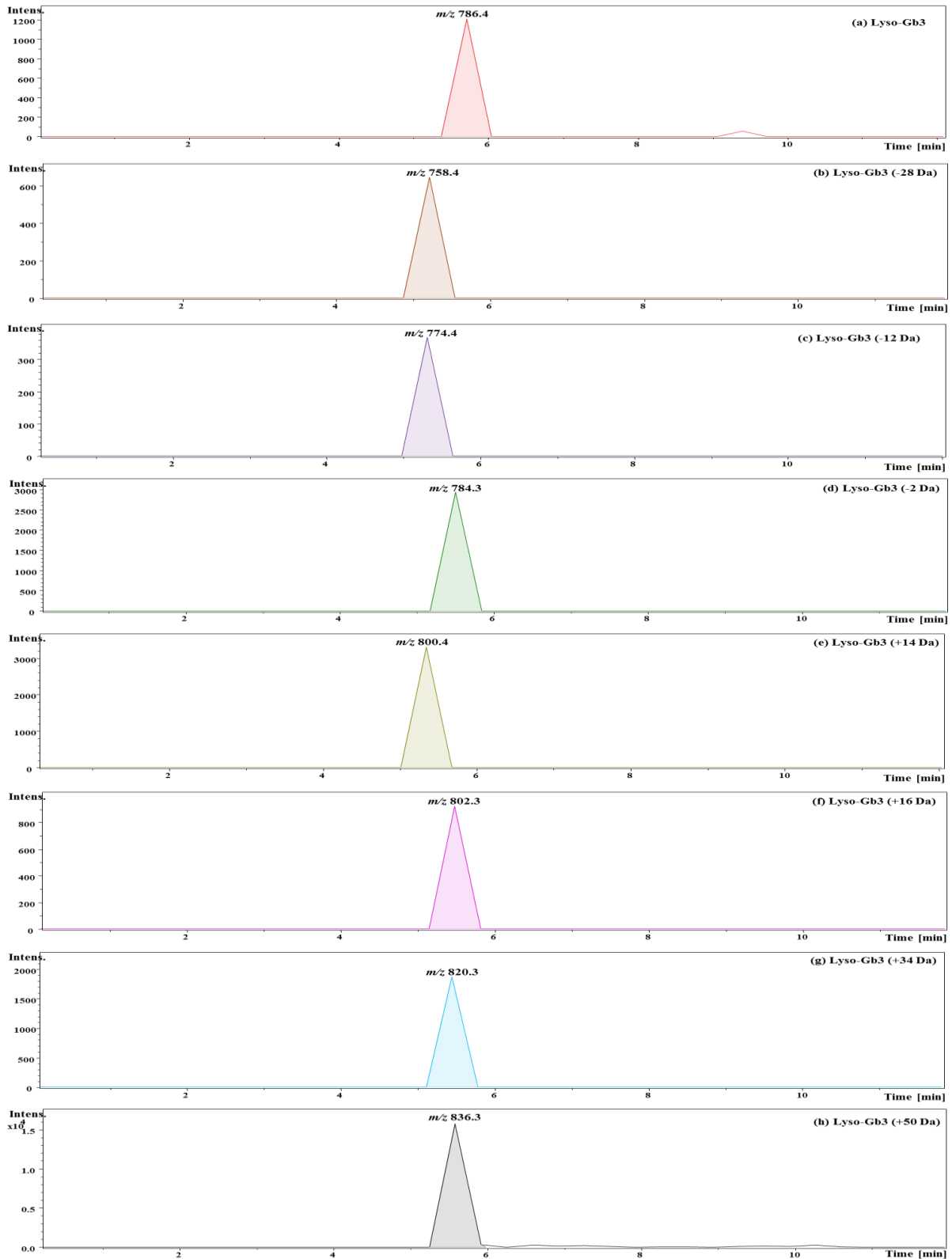


Figure 3.12 Urinary Lyso-Gb3 analogues in untreated Fabry hemizygote: Extracted ion chromatograms of Lyso-Gb3 and its analogues plasma sample from a hemizygous Fabry male: (a) Lyso-Gb3 (m/z 786.4), (b) Lyso-Gb3 (-28 Da, m/z 758.4), (c) Lyso-Gb3 (-12 Da, m/z 774.4), (d) Lyso-Gb3 (-2 Da, m/z 784.3), (e) Lyso-Gb3 (+14 Da, m/z 800.4). (f) Lyso-Gb3 (+16 Da, m/z 802.3). (g) Lyso-Gb3 (+34 Da, m/z 820.3) and (h) Lyso-Gb3 (+50 Da, m/z 836.3).

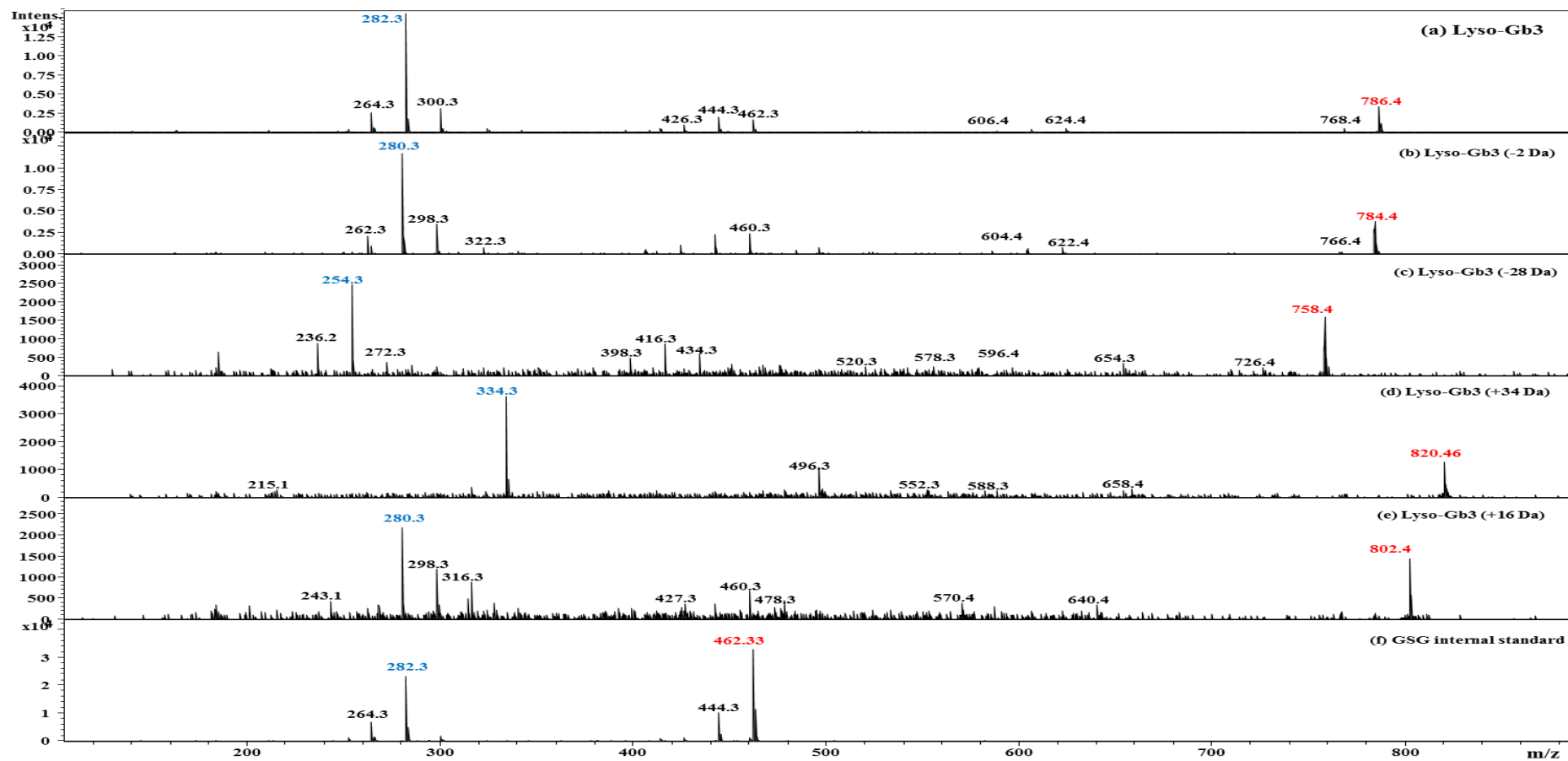


Figure 3.13 MS/MS fragmentation of Lyso-Gb3 analogues: MS spectra show MS/MS fragmentation of (a) Lyso-Gb3; (b) Lyso-Gb3 (-2 Da); (c) Lyso-Gb3 (-28 Da); (d) Lyso-Gb3 (+34 Da); (e) Lyso-Gb3 (+16 Da) and (f) Bovine-GSG internal standard. The modified derivative of the sphingosine moiety of Lyso-Gb3 was detected in plasma of an untreated Fabry male using HPLC-microTOF-MS approach. This analogue fragments differ in m/z compared to the fragments of the parental Lyso-Gb3 by the exact amount predicted by sphingosine modification.

3.3.4 Choice of Internal Standard

GSG derived from bovine, buttermilk was used initially, which has an m/z of 462.4 which is exactly the same as one of the MS/MS fragments of Lyso-Gb3 (Lyso-Gb3 -2 carbohydrates). The structures of MS/MS fragmentation of GSG-bovine origin and Lyso-Gb3 are identical giving the most abundant daughter ion m/z 282.3 (Figure 3.14 a). Alternatively, GSG-plant origin varies from GSG-bovine origin and human-GSG by having an additional double-bond in the sphingosine moiety decreasing the m/z by 2 Da (Figure 3.14 b). This results in variation in MS/MS fragmentation where GSG-plant origin is fragmented giving the most abundant daughter ion m/z 280.30, 2 Da less than human-GSG giving easily distinguishable peaks.

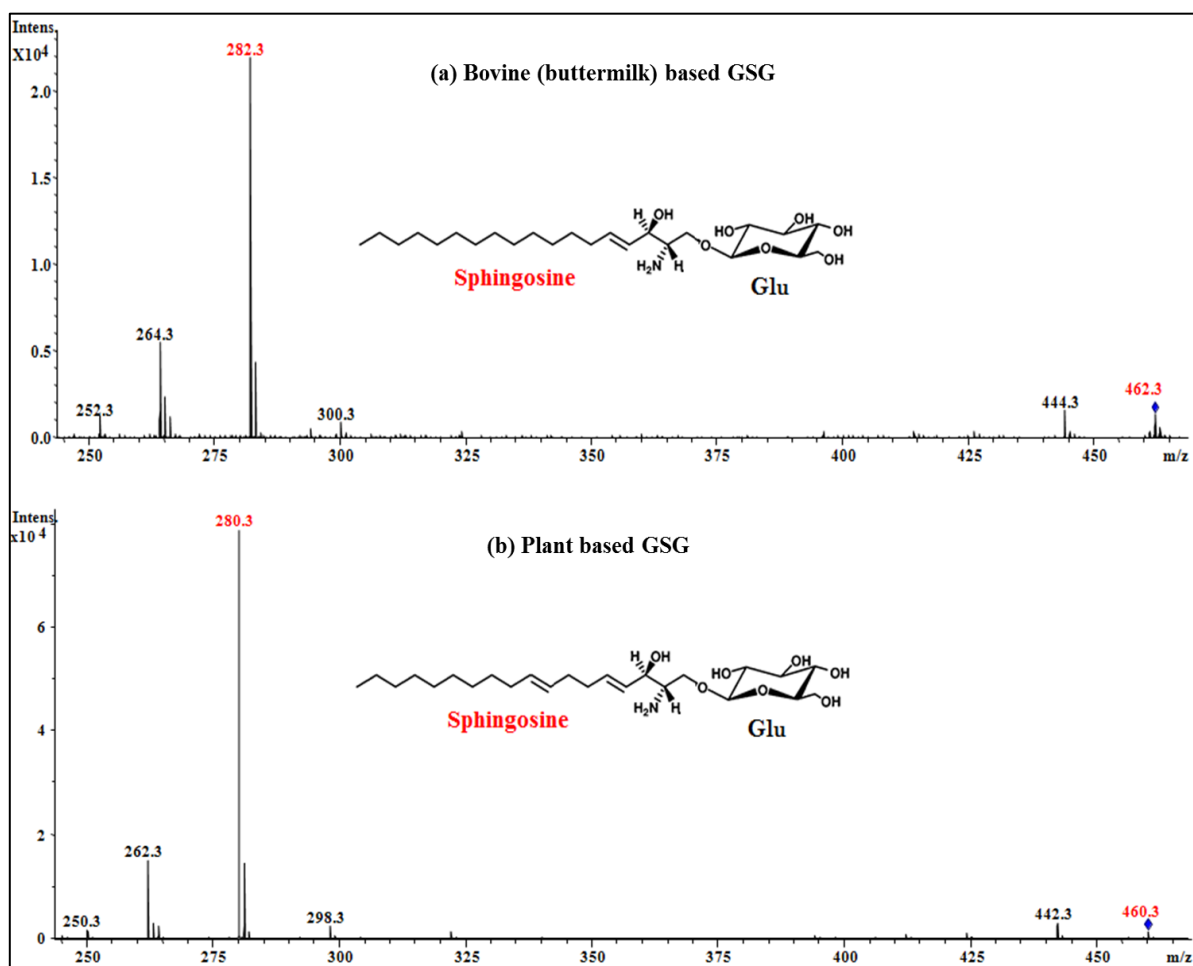


Figure 3-14 Method development- GSG Internal standard: MS spectrum view window shows MRM transitions of the parents and daughters ions of GSG from bovine buttermilk (a) and GSG from plant (b) where the plant origin-GSG has 2Da less in the parent ion and subsequently varies by -2Da in its fragments due to the presence of two double-bonds in the sphingosine moiety.

3.3.5 Role of the Internal Standard

A clinically relevant concentration range of Lyso-Gb3 was added to either control urine (0-8 nM sample concentration or 0-0.8 pmol/MS run) or charcoal stripped plasma (0-200 nM sample concentration or 0-8.0 pmol/MS run) samples along with GSG. The samples were subjected to the optimised extraction and analytical protocols given above. The AUC data of the m/z 282.3 daughter ion derived from the added Lyso-Gb3 for the urine samples and plasma samples. Lyso-Gb3 standard curves were generated using Lyso-Gb3 area alone vs. Lyso-Gb3 injected amount or by using area response ratio of Lyso-Gb3 vs. Lyso-Gb3 injected amounts. Using Lyso-Gb3 AUC alone, a considerable deviation from a good fit with r^2 values of 0.9068 and 0.9279 was observed. When this AUC data is expressed relative to the AUC data of the m/z 280.3 daughter ion derived from the GSG internal standard from the same sample the r^2 values increase to 0.9999. This demonstrates that the GSG internal standard very effectively compensates for variability in the entire analytical process and that the assay is applicable to the samples of interest over the entire clinically relevant concentration range (Figure 3.15).

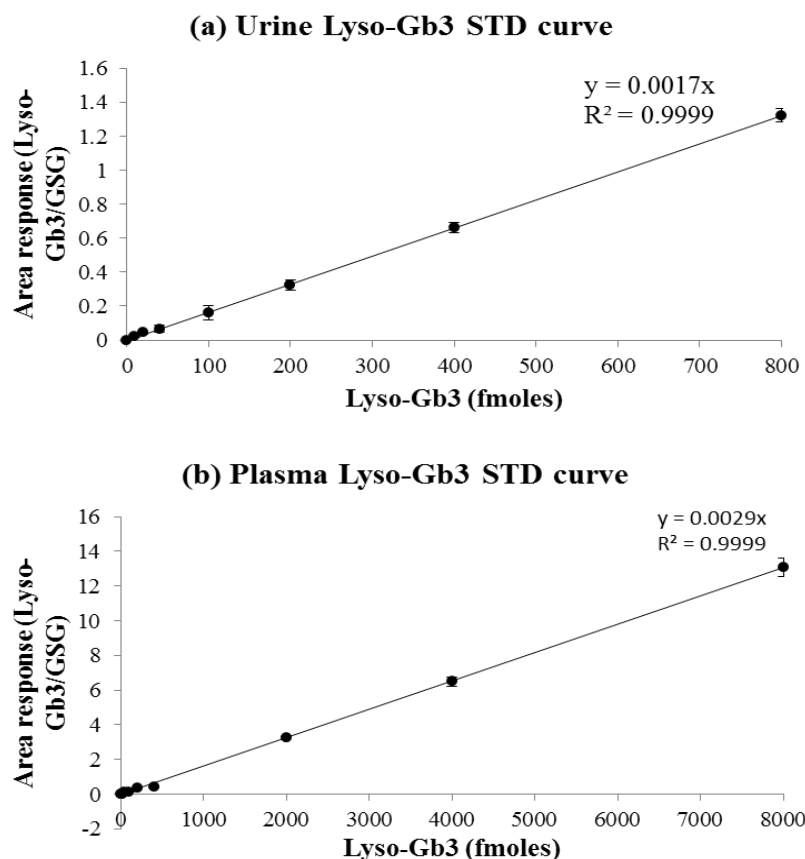


Figure 3.15 Lyso-Gb3 standard curves using GSG IS: Lyso-Gb3 extracted from urine (a) and plasma (b). The standard curves were plotted using Lyso-Gb3/GSG response of AUC. The standard curves show a linear response with GSG. A constant amount of GSG is used with different amounts of Lyso-Gb3 (covering the clinical ranges) showing a reproducible stable response for Lyso-Gb3 to GSG each time (Note: The used GSG internal standard is derived from plant).

3.3.6 Assay Performance Characteristics

Once the relevant variables of the extraction, HPLC resolution and MS analysis were optimised it was a priority to demonstrate that the assay is valid over the concentration range of Lyso-Gb3 seen in Fabry patients plasma and urine and to determine the lowest level of Lyso-Gb3 that can be detected and quantified reliably as well as the variability of the assay.

3.3.6.1 Lyso-Gb3 Quality Controls

As the intention of this work was to establish an assay for the analysis of large numbers of clinical samples over an extended period of time it was important to demonstrate that the

assay was reproducible over time. Urine and plasma quality controls (QCs) would be suitable for this purpose but they are not available commercially and the ethics in place for this project would not allow use of patient samples for this purpose. Therefore, we prepared our own QCs by adding Lyso-Gb3 to bulk samples before freezing aliquots that were assayed, identically to the clinical samples, with every batch processed.

Two 250mL samples of the same charcoal stripped (twice) and filtered (0.2µm) plasma from healthy controls had Lyso-Gb3 added to give a low concentration (2.5nM) and a high concentration (50 nM). After thorough mixing the samples were aliquoted and frozen at -80°C until used. For urine 2 identical samples from a healthy individual (500mL) was spiked with Lyso-Gb3 to give a low (0.5 nM) or high (6 nM) concentration. Again after extensive mixing aliquots were frozen at -80°C until used. These samples were processed regularly throughout the batches of samples and the results recorded in order that the consistency of the assay could be monitored.

Samples prepared in this way have significant limitations, for example, patient samples will contain variable amounts of metabolites and analogues related to Lyso-Gb3. Such related molecules could have effects on the extraction efficiency of the Lyso-Gb3/GSG or modify the extent of ionisation of the analytes in the MS analysis. However, even with these limitations the QC samples will allow an assessment of consistency, if not the accuracy, of the entire analytical process over time.

3.3.6.2 Lyso-Gb3 stability test

A stability test was used in order to study the stability, reproducibility and repeatability of the HPLC-MS system prior any analysis of biological samples. The stability test solution was prepared by mixing 25nM of Lyso-Gb3 standard with 8nM GSG internal standard (1:1, v:v) and stored at -20°C until used. The stability test solution was analysed 6 times at the

beginning of each run and the intensities, AUC and retention times recorded. The AUC response of Lyso-Gb3 to GSG internal standard and the retention time for the six run should be identical before analysing any clinical samples. This test allows following the instruments performance in each single run. Figure 3.16 shows extracted ion chromatogram of Lyso-Gb3 and GSG internal standard used in stability test.

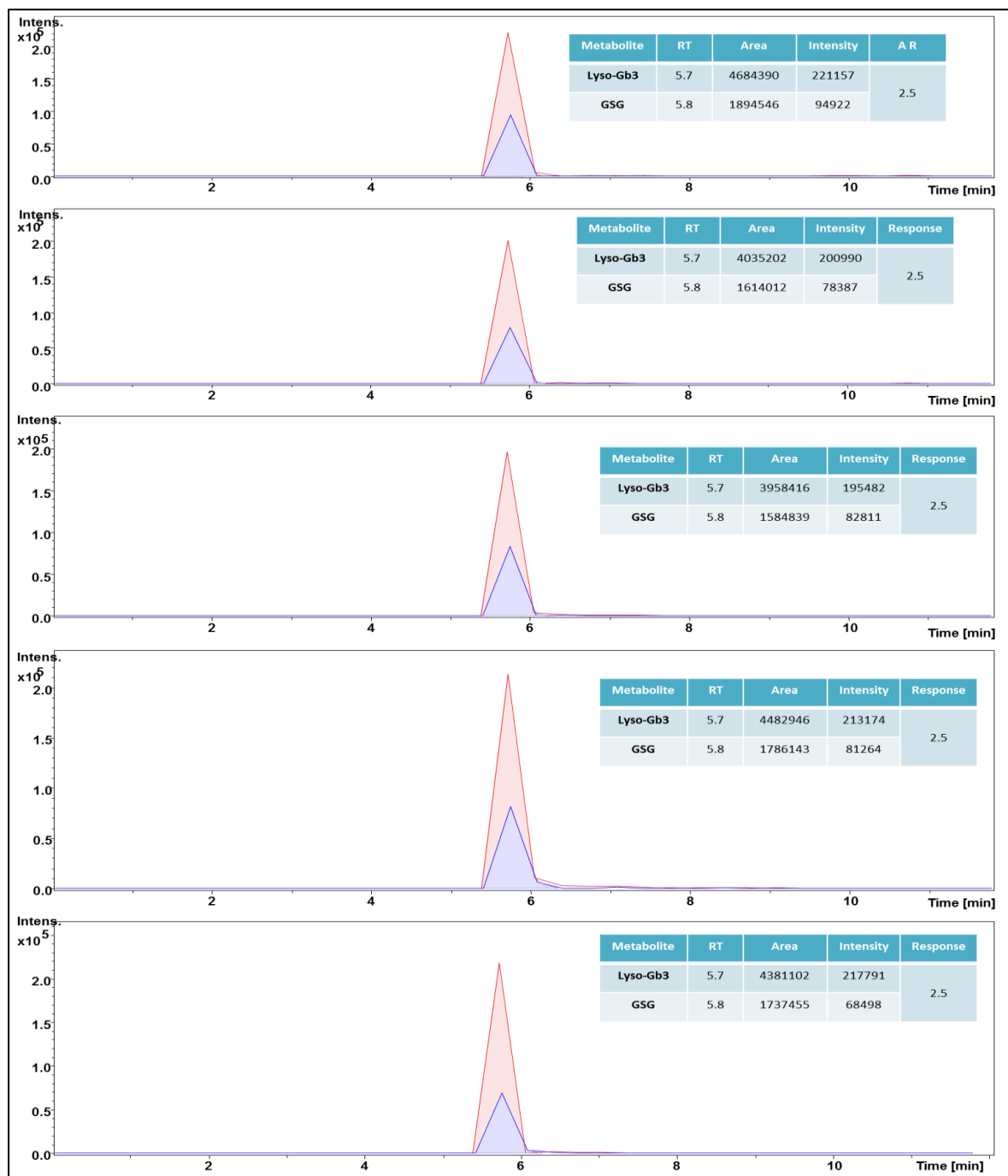


Figure 3-16 Stability test: extracted ion chromatogram of Lyso-Gb3 and GSG internal standard in stability test to study the instrument performance in each single day analysis.

3.3.6.3 Lyso-Gb3 Intra-Assay and Inter-Assay

Plasma QCs and urinary QCs were analysed in quadruplicate on the same day to calculate the intra-assay variability and this was repeated on four independent occasions on different 4 days to calculate the inter-assay variability. The following formula was used to calculate the intra-assay variability:

$$\text{Intra-assay variability} = [(Stdev\ of\ LQC\ area\ response / mean\ of\ low\ QC\ area\ response * 100) + (Stdev\ of\ HQC\ area\ response / mean\ of\ HQC\ area\ response * 100)] / 2 \dots\dots\dots (Equation\ 3.4)$$

The inter-assay variability was calculated using the following formula:

$$\text{Inter-assay variability} = [(Stdev\ of\ LQC\ area\ response\ means / mean\ of\ LQC\ mean\ area\ response * 100) + (Stdev\ of\ HQC\ area\ response\ means / means\ of\ HQC\ area\ response\ means * 100)] / 2 \dots\dots\dots (Equation\ 3.5)$$

Intra-assay variability (CV %) was calculated for plasma QCs as: 5.13%. Inter-assay variability (CV %) was calculated as: 8.79%.

Intra-assay variability (CV %) was calculated for urine QCs as: 1.93%. Inter-assay variability (CV %) was calculated as: 2.46%. The acceptable intra- and inter-assays variability is $\leq 15\%$.

3.3.6.4 Limits of Detection and Quantitation

A range of concentrations of synthetic Lyso-Gb3, were added to either healthy control urine or pooled human plasma stripped twice with charcoal samples which were extracted and analysed using the optimised methods given above. The concentration ranges used were 0, 0.125, 0.25, 0.5 and 1.0 nM for plasma and 0, 25, 50, 100, 200, 400 pM for urine. Limit of detection (LOD) and limit of quantitation of both plasma and urinary Lyso-Gb3 were determined using (Equation 3.6) and (Equation 3.7) respectively, where $f = 3.3$ and 10 for

LOD and LOQ respectively, *stdev* = standard deviation and *b*: Slope of the regression line (Figure 3.10 b and c) respectively.

$$LOD = f \times stdev/b \dots\dots\dots (Equation 3.6)$$

$$LOQ = f \times stdev/b \dots\dots\dots (Equation 3.7)$$

For plasma LOD was 0.13 nM and LOQ was 0.25 nM. However, for urine LOD was 50 pM and LOQ was 100 pM.

3.4 DISCUSSION

We have developed an HPLC-MS/MS approach for analysis of plasma and urinary Lyso-Gb3 and its analogues. Our pilot data on method optimisation has shown a clear advantage of SPE extraction, HPLC separation and MS/MS assays.

SPE was preferred over liquid-liquid extraction (Bligh and Dyer extraction), because of its simple procedure, shorter processing time and comparable recoveries. Moreover it does not require halogenated solvents (e.g. chloroform) where laboratories avoid the use of chloroform and other halogenated solvents for environmental reasons.

The MRM acquisition mode used here has the advantage that the selection of the parent ion before fragmentation removes any contaminants (other unwanted peaks) from the MS spectrum giving very clean spectra and allowing accurate and sensitive quantitation.

The use of a suitable internal standard in quantitative mass spectrometry approaches is essential. Stable isotopes versions are typically used as they are chemically identical to the parent but vary in mass by a known amount. However, as no isotopic form of Lyso-Gb3 is commercially available and although synthetic protocols have been developed they are complex and require specialist knowledge [25, 257]. Therefore GSG was selected as internal

standard since it is closely related to Lyso-Gb3 and behaves in an identical fashion in the extraction, HPLC and MS protocols utilized here.

GSG derived from plant was superior over GSG-bovine origin as an internal standard. While the later gives rise to exactly the same fragment likes Lys-Gb3 m/z 282.3 which could cause carry over and/or confusion, the plant GSG contains an extra double-bond in the sphingosine moiety giving a fragment of m/z 280.3. The use of GSG-plant origin removes any potential carry over, and used in all project studies.

Similarly, to optimize the analytical process, I have tested the use of the newly developed nano-Viper fittings and found to be superior in terms of quantitation of Lyso-Gb3 and other metabolites. The use of nano-Viper fittings connection prevents any chromatography dead space and improves the limit of detection of the tested lipids.

As this project deals with clinical samples, it was critical to ensure the instrument performance is stable. So, we have prepared our own in-house Lyso-Gb3 QCs to monitor the performance. MS performance displayed high stability with acceptable intra- and inter-assay variability.

In conclusion, this chapter illustrates the optimization conditions of this research project. GSG-plant source internal standard and instrumentation have been chosen with optimal quality control benchmarks achieved. We have established a mass spectrometry based assay for Lyso-Gb3 and other metabolites. We have validated the assays using samples from Fabry patients. This assay is cheap and employs a virtually non-invasive method to collect body fluids such as urine and plasma facilitating regular monitoring. The relevant metabolites were extracted using SPE then separated by HPLC before being linked online to MS. The detected mass alone does not definitely identify the metabolite. Therefore the ions are fragmented using MS/MS giving a characteristic fragmentation pattern of daughter ions. GSLs

concentrations were measured using Lyso-Gb3 standard calibration curve after measuring the area response ratio of the examined analyte to GSG internal standard. Having optimised the assays to detect and quantify Lyso-Gb3 and its associated analogues, this protocol was used to examine and quantify the levels of these metabolites in plasma and urine samples obtained from Fabry patients. These data are presented in detail in Chapter 4 and Chapter 5.

CHAPTER - 4
THE CLINICAL UTILITY OF PLASMA LYSO-GB3
AND ITS ANALOGUES IN THE DIAGNOSIS AND
MONITORING OF FABRY DISEASE

4.1 INTRODUCTION

Fabry disease (FD) results from mutations in the α -gal-A gene. The human genome mutation database has identified approximately 800 mutations thus far, some of which result in a complete loss of enzymatic activity while others result in diminished activity. As outlined in Chapter-1, the α -gal-A enzyme activity test shows the deficiency of enzymatic activity in Fabry patients. This deficiency can identify classical Fabry patients especially in hemizygous males, where the enzyme activity is considerably reduced or absent (<1%). Nevertheless, classical heterozygous Fabry females and both genders of late-onset variant Fabry patients can show residual enzymatic activity.

Currently, mutation analysis and the α -gal-A enzyme activity test along with clinical signs are used to establish the diagnosis of FD. A subgroup of Fabry patients lack the classical features of FD and present with single organ involvement (e.g. LVH and kidney disease) and are classified as atypical variants [79, 224, 258]. They usually present later in life than classical Fabry patients and tend to have symptoms which are not specific to FD making them a diagnostic challenge. The correct diagnosis in atypical variant Fabry patients usually takes many years if not decades. At the other end of the spectrum, increased awareness of FD has led to screening studies resulting in identification of patients with mutations of unknown significance which has led to over diagnosing FD in some patients [259].

Lyso-Gb3 has been increasingly suggested as a promising diagnostic biomarker and some evidence suggest that Lyso-Gb3 may be of value in monitoring FD [46, 162, 215]. Patel et al [260] reported that FD is associated with a high burden of cardiac morbidity and mortality. Adverse cardiac outcomes are associated with age, global disease severity and advanced cardiac disease but not the presence of cardiac genetic variants.

The work presented in this chapter aims to measure the levels of plasma Lyso-Gb3 and its analogues and to study the clinical utility of these GSLs as biomarkers for the diagnosis and monitoring of FD in both classical and cardiac variant Fabry patients.

4.2 SAMPLE COLLECTION FROM FABRY PATIENTS

Blood samples were collected from 100 Fabry patients. Plasma samples were generated after centrifugation at 3500 rpm/10mins to pellet and remove contaminating blood cells. Plasma samples were stored at -80°C until required for processing. All Fabry patients were diagnosed either by screening for the presence of *GLA* gene mutation and/or by α -gal-A enzymatic activity assay. Of the 100 Fabry patients enrolled in the study, 56 patients were from London, and 44 were from the West Midlands and Sheffield. Samples were obtained from 33 healthy volunteers and an additional 76 non-Fabry patients; the latter was obtained from 5 clinics at the Queen Elizabeth Hospital, Birmingham, UK; the latter were receiving treatment for diseases unrelated to FD. Table 4.1 shows the subgroups of the control subjects and the anthropometric data of the patients and control subjects are shown in Table 2.1.

Control Group	Subjects number
Healthy volunteers	33
Renal clinic patients	15
Adrenal clinic patients	15
Inflammatory bowel disease (IBD) patients	16
Autoimmune liver disease patients	15
Vascular disease patients	15
Total control subjects	109

Table 4.1 Control subjects groups: Table showing the groups of 109 control subjects.

4.3 RESULTS

4.3.1 Control groups:

Blood samples were collected from 76 non-Fabry patients obtained from 5 different clinics. As far as possible, these were age and gender matched. These included patients receiving treatment for renal, adrenal, inflammatory bowel disease (IBD), autoimmune liver disease and vascular disease. Plasma samples generated from this group were analysed for the levels of Lyso-Gb3 and its analogues. This analysis was performed to determine whether Lyso-Gb3 constitutes a specific biomarker for Fabry disease patients. The mean concentration of Lyso-Gb3 was 0.88 nM, with a median of 0.73 nM and range of (0.28-2.30) nM. The results of this analysis revealed that all non-Fabry patients from the five clinics had comparable levels of plasma Lyso-Gb3 that were similar to those of healthy control subjects. Similarly, the healthy control volunteers (n=33) showed mean concentration of Lyso-Gb3 of 1.3 nM, with a median of 1.3 nM and range of (0.9-2.1) nM. Hence, all 76 non-Fabry patients data was pulled together in the study to increase the number of control subjects from 33 to be 109 (Figure 4-1).

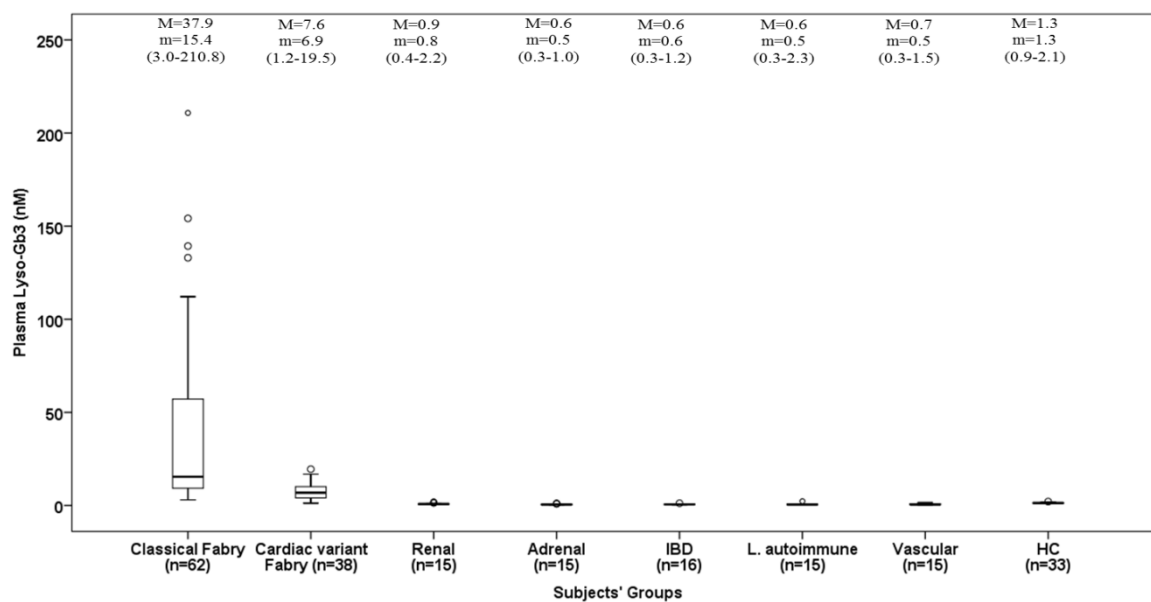


Figure 4.1 Plasma Lyso-Gb3 levels: Figure showing the levels of Lyso-Gb3 in plasma samples generated from classical Fabry patients, cardiac variant Fabry patients and non-Fabry patients recruited from the five different clinics (renal, adrenal, inflammatory bowel disease (IBD), autoimmune liver disease (L. autoimmune), and vascular disease), in addition to healthy control individuals (HC). Those non-Fabry patients enrolled in the study as controls with the healthy control individuals elevating the number of control subjects from 33 to 109 subjects.

4.3.2 Plasma Lyso-Gb3 levels in classical Fabry patients

To determine whether the levels of plasma Lyso-Gb3 could be used as a diagnostic tool to diagnose patients with classical FD, it was necessary to compare the levels of Lyso-Gb3 in Fabry patients and control subjects. A total of 62 Fabry patients were categorized as classical and the remaining 38 patients were as cardiac variants Fabry patients. The diagnosis of classical Fabry patients was initially confirmed by screening for the presence of known typical α -gal-A mutations (Table 4.2), the presence of typical FD manifestations, and the presence of low enzymatic activity of α -gal-A (<1%).

Mutation		Classical Fabry patients		
Protein Sequence Change	Nucleotide change	male	female	total subjects
A309P	c.925 G>C	0	5	5
R227X	c.679 C>T	1	4	5
P205T	c.613 C>A	0	4	4
V316E	c.947 T>A	1	2	3
I317T	c.950 T>C	0	3	3
358delE	c.1072_1074del	2	1	3
G325D	c.974 G>A	2	1	3
I117S	c.350 T>G	2	0	2
L372P	c.1115 T>C	1	1	2
C.90Y	C.269 G>A	1	1	2
Y184X	C.5525A	0	2	2
N33D	not available	0	2	2
V269A	c.806 T>C	0	1	1
N263S	c.788 A>G	1	0	1
T41fs*79	c.123delC	1	0	1
R220X	c.658 C>T	1	0	1
R301G	c.901 C>G	0	1	1
R301X	c.901 C>T	1	0	1
not available	C.466delG	0	1	1
not available	C.359_364delTAGCTA	1	0	1
G411D	c.1232 G>A	0	1	1
D93Y	c.277 G>T	1	0	1
not available	EXON2 DELETION	0	1	1
A13P	c.37 G>C	0	1	1
not available	C.802-3_802-2delCA	0	1	1
A121T	c.361 G>A	0	1	1
M42V	c.124 A>G	1	0	1
P293H	c.878 C>A	0	1	1
R301P	c.902 G>C	1	0	1
R112C	c.334 C>T	1	0	1
C174Vfs*17	c.520delT	0	1	1
not available	not available	4	3	7
Total subjects		23	39	62

Table 4.2 Mutations list for classical Fabry patients.

The levels of plasma Lyso-Gb3 in treatment-naïve classical Fabry patients [male (n = 8) and female (n = 12)] were compared to treated classical Fabry patients [males (n = 15) and females (n = 27)] and control subjects [male (n=63) and female (n = 46)].

As predicted, higher levels of Lyso-Gb3 were observed in treatment-naïve classical Fabry males compared to the other groups. The concentration of plasma Lyso-Gb3 in untreated classical Fabry males (mean \pm SEM = 117.8 \pm 18.8) was significantly higher compared to treated classical Fabry males (mean \pm SEM = 61.3 nM \pm 4.4) ($p < 0.01$), and control males (mean \pm SEM = 0.9 nM \pm 0.5) ($p < 0.0001$). Significant differences were also noted between treated Fabry males and control males ($p < 0.0001$). A comparison of untreated Fabry males and females showed nearly ten-fold higher levels of Lyso-Gb3 in Fabry males. The difference observed was highly statistically significant ($p < 0.0001$).

In the female subgroup, the concentration of plasma Lyso-Gb3 was twelve-fold higher in classical Fabry females (mean \pm SEM = 15.2 nM \pm 3.7) pre-ERT, and (mean \pm SEM = 11.4 \pm 0.9) post-ERT compared to control females (mean \pm SEM = 0.8 nM, \pm 0.07) ($p < 0.0001$). However, the variation in the levels of plasma Lyso-Gb3 in classical Fabry females' pre-ERT and post-ERT was not statistically significant ($p > 0.05$). Furthermore, the difference between plasma Lyso-Gb3 levels in the two control genders was not statistically significant ($p > 0.05$) (Figure 4-2).

These results confirm that the levels of plasma Lyso-Gb3 are significantly higher in untreated hemizygous Fabry males and appear to drop in treated hemizygous Fabry males. There was no overlap between the levels of plasma Lyso-Gb3 in untreated classical Fabry males and females. Furthermore, there was no overlap in the levels of Lyso-Gb3 in classical Fabry patients and control subjects. Plasma Lyso-Gb3 levels in classical Fabry patients were found

to be associated patient's gender (Pearson correlation p value of < 0.0001), and treatment status (Pearson correlation p value of < 0.05).

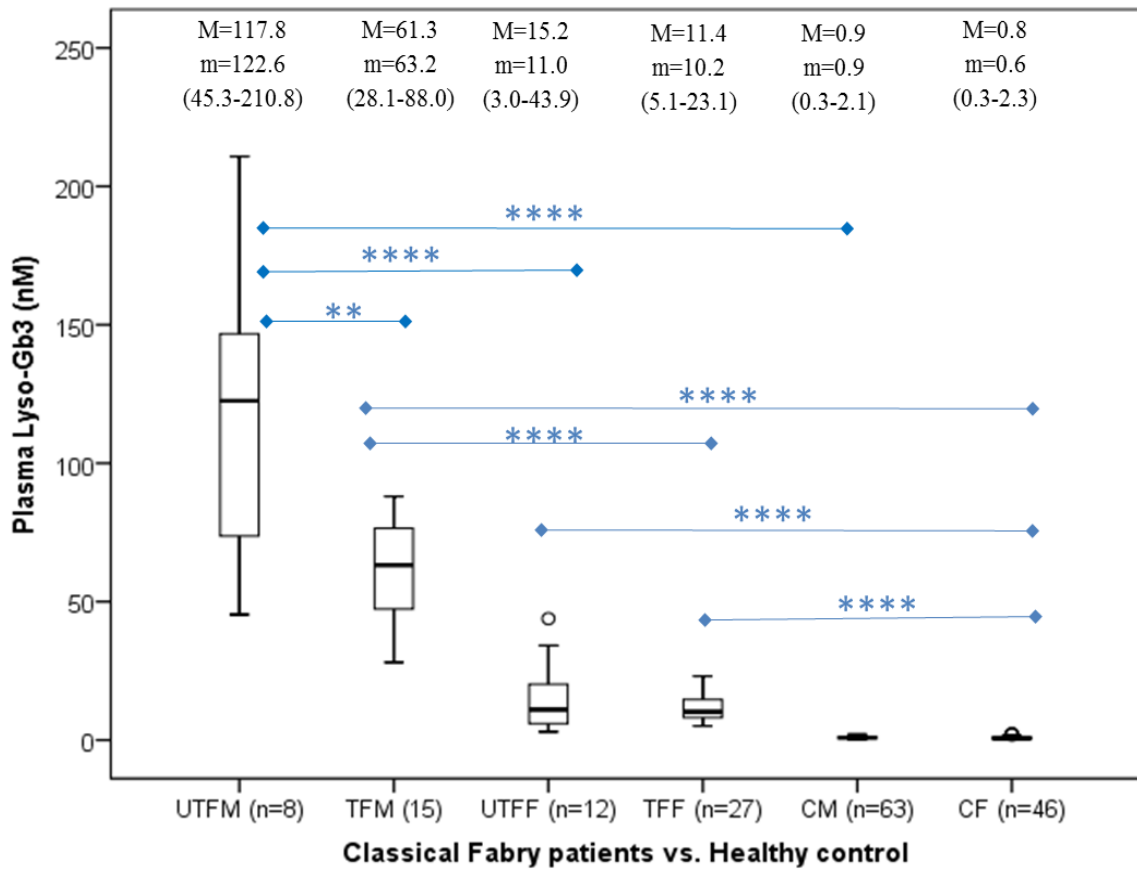


Figure 4.2 The levels of plasma Lyso-Gb3 in classical Fabry patients: Data were pooled from the patients and control subjects and presented as Whiskers-box plots. The concentrations were measured using Lyso-Gb3 calibration curve. Data were analysed using SPSS. The subjects were divided to 6 groups as follows: untreated Fabry male (UTFM), treated Fabry male (TFM), untreated Fabry female (UTFE), treated Fabry female (TFE), control male (CM) and control female (CF). For each group: n = number of subject; M = Mean; m = median; the range of minimum and maximum values in brackets; box= maximum and minimum quartiles; line inside the box = median; whiskers = maximum and minimum non-outlier values; o = outlier values. Various groups were compared against each other using Mann-Whitney U tests. The differences were considered statistically significant if the p value < 0.05 (**= $p < 0.01$ and ****= $p < 0.0001$).

In order to assess the diagnostic accuracy of plasma Lyso-Gb3 levels in diagnosing classical FD in males, Receiver Operator Characteristic (ROC) analysis performed. Plasma Lyso-Gb3 levels in 7 confirmed untreated classical Fabry males and 63 control males were assessed.

ROC curves were generated using SPSS software. A statistically significant result was generated with favourable area under the curve (1.000, p value <0.0001). Plasma Lyso-Gb3 value equal to or higher than 2.7 indicated the presence of FD with a sensitivity of 100% and specificity of 100% (Figure 4.3).

The diagnostic accuracy of Plasma Lyso-Gb3 levels in diagnosing classical FD in females was assessed using ROC analysis. Plasma Lyso-Gb3 levels in 12 confirmed untreated classical Fabry females and 46 control females were assessed. The ROC curve generated showed a statistically significant result with high area under the curve (1.000, p value <0.0001). Plasma Lyso-Gb3 levels equal to or higher than 2.6 nM indicated the presence of FD with a sensitivity of 100% and specificity of 100%. This implies that females with plasma Lyso-Gb3 levels equal to or higher than 2.6 nM would be correctly identified as having FD (Figure 4.3).

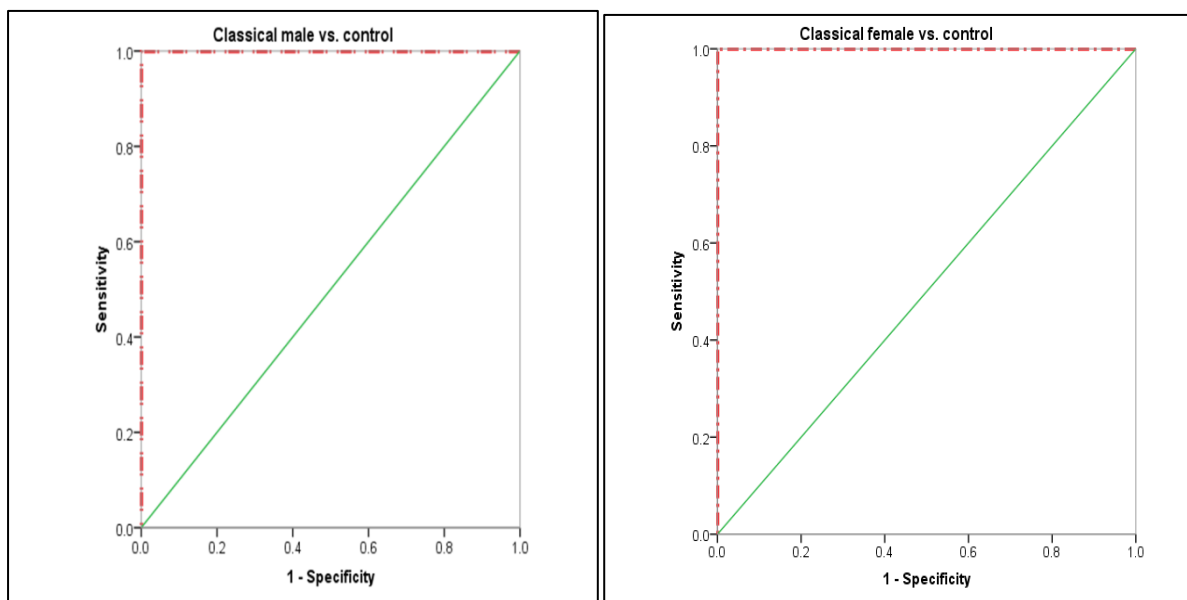


Figure 4-3 ROC curve plasma Lyso-Gb3 levels in untreated hemizygous classical Fabry males vs. control males, the reference line is in green (**left panel**), cut-off value of 2.7 nM. ROC curve plasma Lyso-Gb3 levels in untreated heterozygous classical Fabry females vs. control females, reference line is in green (**right panel**), cut-off value of 2.6 nM. Graphs were generated using SPSS software.

4.3.3 Plasma Lyso-Gb3 levels in cardiac variant Fabry patients

A total of 38 Fabry patients were diagnosed as carrying cardiac variants. The diagnosis was confirmed by mutation analysis of the α -gal-A gene. Among this group, 34 patients were shown to harbor a sequence change (N215S) in the *GLA* gene (an established mutation in cardiac FD variants). The other 4 patients had sequence changes of R118C, A301G, R301Q and P490T in the α -gal-A protein (Table 4.3).

Mutation		Cardiac variants' Fabry patients		
protein Sequence Change	nucleotide change	male	female	total subjects
N215S	c.644 A>G	20	14	34
R118C	c.352 C>T	0	1	1
A301G	c.902 G>A	0	1	1
R301Q	c.902 G>A	0	1	1
P490T	not available	0	1	1
Total subjects		20	18	38

Table 4.3: The mutations listed for cardiac variant Fabry patients.

Cardiac variant Fabry patients showed lower levels of plasma Lyso-Gb3 (1.2-19.5 nM) compared to classical Fabry patients (3.0-210.8 nM) (Figure 4.1). Plasma Lyso-Gb3 concentration in untreated cardiac variant Fabry males (n = 7, mean \pm SEM = 10.1 nM \pm 2.1) was significantly higher than in control males and in untreated cardiac variant Females [(n = 63, mean \pm SEM = 0.9 nM \pm 0.05), *p* value <0.0001], [(n =13, mean \pm SEM = 5.3 nM \pm 0.95), *p* value <0.05] respectively. However, there was no significant variation in the concentration of plasma Lyso-Gb3 between cardiac variant Fabry males pre-ERT and post-ERT [n =13, mean \pm SEM = 9.4 nM \pm 1.1), *p* value >0.05]. Treated cardiac variant Fabry males showed significantly higher levels of plasma Lyso-Gb3 than control males (*p* value <0.0001). Both untreated cardiac variant Fabry females (n = 63, mean \pm SEM = 0.9 nM \pm 0.05) and treated cardiac variant Fabry females (n = 5, mean \pm SEM = 5.7 nM \pm 1.8) showed significantly higher levels of plasma Lyso-Gb3 compared to control females (n =46, mean \pm

SEM = 0.8 nM ± 0.07) with *p* values of <0.0001 and <0.001 respectively. Similarly, the variation in Lyso-Gb3 concentration in females' pre-ERT and post-ERT did not reach the statistical significance (*p* value >0.05), (Figure 4.4).

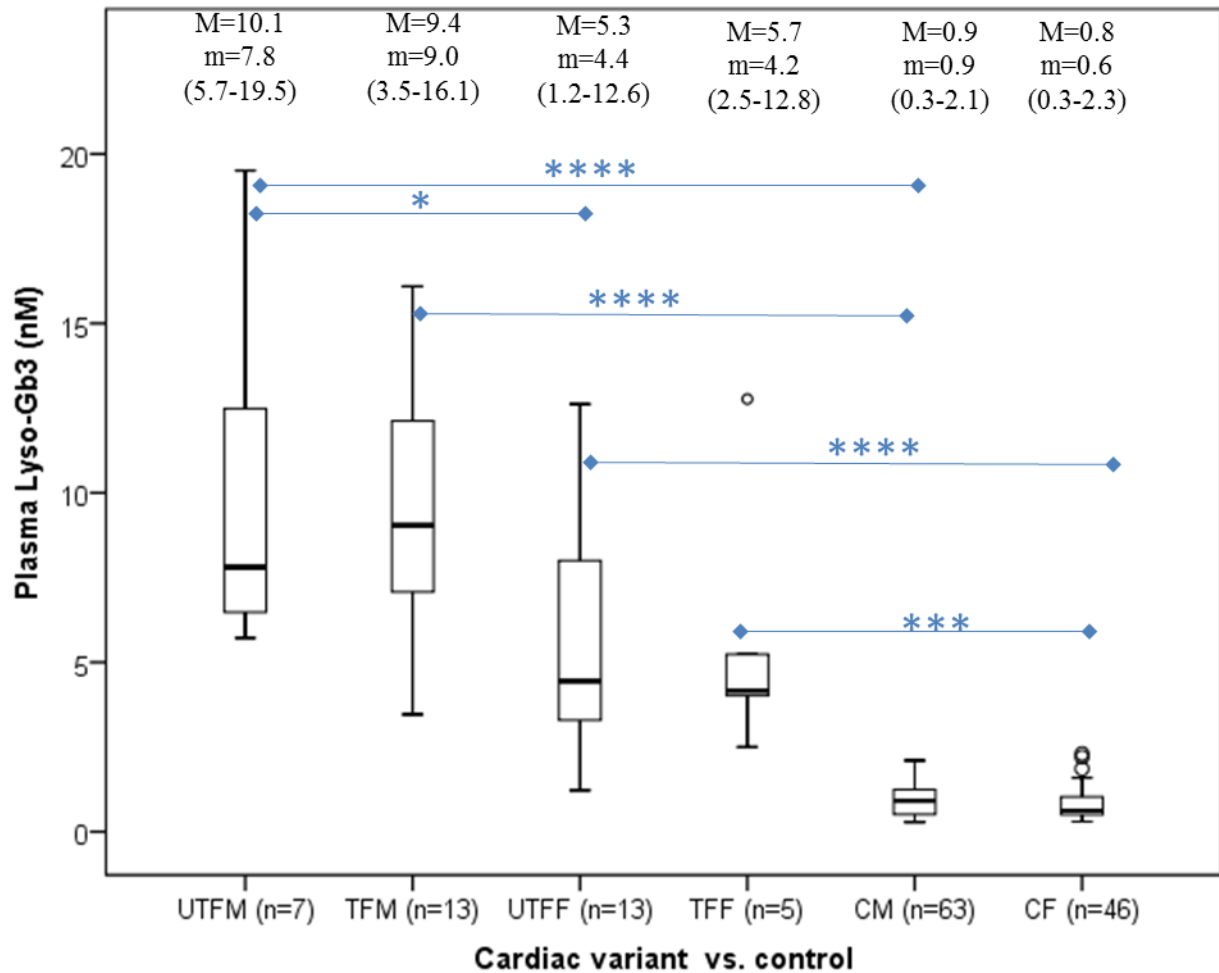


Figure 4.4: The levels of plasma Lyso-Gb3 in cardiac variant Fabry patients: Data were pooled from the patients and control subjects and presented as Whiskers-box plots. The concentrations were measured using Lyso-Gb3 calibration curve. Data were analysed using SPSS. The subjects were divided to 6 groups as follows: untreated Fabry male (UTFM), treated Fabry male (TFM), untreated Fabry female (UTFE), treated Fabry female (TFE), control male (CM) and control female (CF). For each group: n = number of subject; M = Mean; m = median; the range of minimum and maximum values in brackets; box= maximum and minimum quartiles; line inside the box = median; whiskers = maximum and minimum non-outlier values; o = outlier values. Various groups were compared against each other using Mann-Whitney U tests. The differences were considered statistically significant if the *p* value < 0.05 (**=*p*<0.01, ***=*p*<0.001 and ****=*p*<0.0001).

The diagnostic accuracy of plasma Lyso-Gb3 in diagnosing cardiac variant form of FD in males assessed. Plasma Lyso-Gb3 levels in 7 males with cardiac variant FD and 63 control males were assessed. Similar to classical Fabry males, the ROC curve analysis showed excellent diagnostic accuracy. Plasma Lyso-Gb3 levels equal to or higher than 2.7 nM carried a diagnostic sensitivity and specificity of 100% (AUC=1, p value <0.0001). This implies that all individuals with plasma Lyso-Gb3 levels >2.7 nM are expected to be positive for cardiac variant FD (Figure 4.5).

In order to assess the diagnostic accuracy of plasma Lyso-Gb3 levels in diagnosing cardiac variant FD in females, ROC analysis performed. Plasma Lyso-Gb3 levels in 13 confirmed untreated cardiac variant Fabry females and 46 control females were assessed. ROC analysis showed a statistically significant result with high area under the curve (0.965, p value <0.001). Plasma Lyso-Gb3 value equal to or higher than 2.7 nM indicated the presence of FD with a sensitivity of nearly 77% and specificity of 100%. Moreover, the cut-off value corresponding to 100% sensitivity was 2.4 nM. However, specificity at this point was only 80.4%. This implies that, depending on whether the test is aimed to be used as a screening test or a confirmatory test, an optimum cut-off value with the highest sensitivity or specificity might be selected (Figure 4.5).

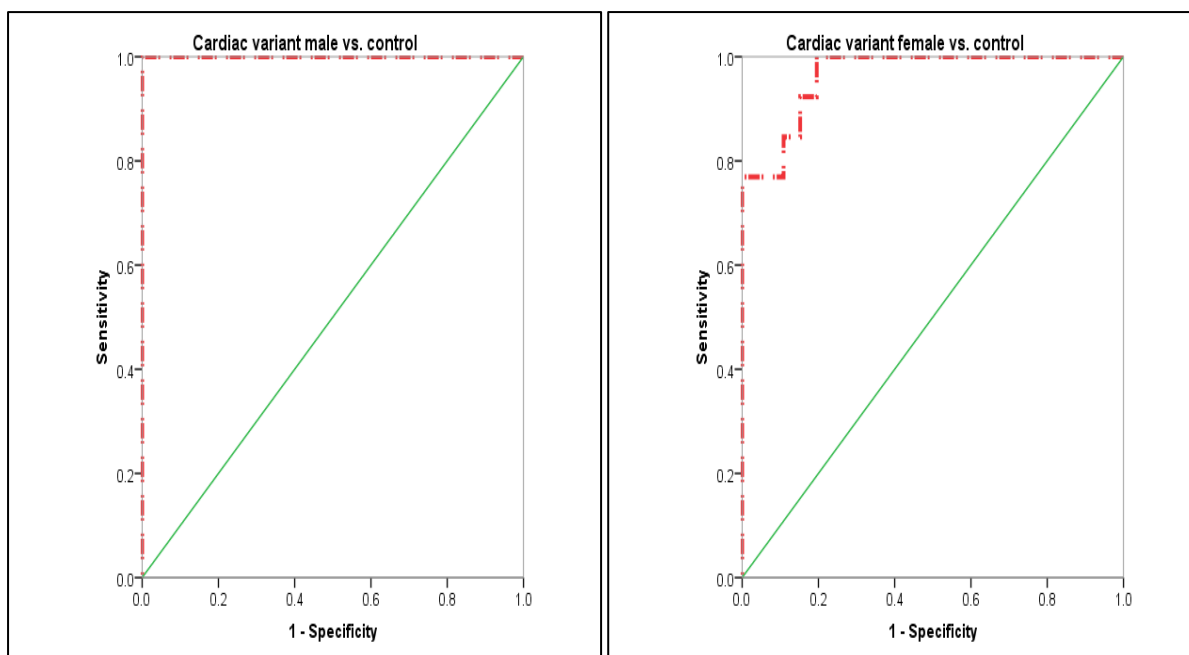


Figure 4.5 ROC curve plasma Lyso-Gb3 levels in untreated hemizygous cardiac variant Fabry males vs. control males (**left panel**), cut-off value of 2.7 nM. ROC curve plasma Lyso-Gb3 levels in untreated heterozygous cardiac variant Fabry females vs. control females (**right panel**), cut-off value of 2.4 nM. Graphs were generated using SPSS software.

4.3.4 Levels of plasma Lyso-Gb3 analogues in classical Fabry patients

The relative concentration of various Lyso-Gb3 analogues was analysed in our cohort of classical Fabry patients. This data is presented in the form of Whiskers box plots (Figure 4.6). This comparison included both untreated and treated male and female Fabry patients and control subjects. As shown in Figure 4.6, the relative concentration of each Lyso-Gb3 analogues in classical Fabry patients varied considerably. While levels of Lyso-Gb3 (-2 Da) analogue in males, were found at the highest concentration (0.1-50.7 nM), levels of Lyso-Gb3 (+18 Da) analogue were somewhat lower (1.6-17.5 nM), while levels of Lyso-Gb3 (-28 Da) varied between (0.4-5.5 nM). The levels of Lyso-Gb3 (+34 Da) varied between (0.9-13.8 nM), while the concentration of Lyso-Gb3 (+16 Da) was extremely low (0.1-2.2 nM). The levels of Lyso-Gb3 (+50 Da) were virtually undetectable. In all cases, the levels of each

analogue were undetectable in control male and female subjects. Again, differences were observed in the levels of all Lyso-Gb3 analogues between male and female classical Fabry patients, with female Fabry patients having concentrations that were lower than classical Fabry males but marginally elevated compared to control subjects. In response to ERT treatment, the concentration of each Lyso-Gb3 analogue decreased in male Fabry patients (Figure 4.6).

The relative distribution of plasma Lyso-Gb3 and its analogues in the untreated classical Fabry patients is shown in Figure 4.7. In both male and female Fabry patients, the levels of plasma Lyso-Gb3 constituted the highest percentage of total Lyso-Gb3 (males 76% vs females 79%) while small trace amounts of Lyso-Gb3 analogues were detected. In both male and female cases, the levels of Lyso-Gb3 (-2 Da), Lyso-Gb3 (+18 Da), Lyso-Gb3 (+34 Da) and Lyso-Gb3 (-28 Da), which arise through modification of the sphingosine moiety, were broadly similar in both male and female cases, comprising 12% and 11% for Lyso-Gb3 (-2 Da), 5% for Lyso-Gb3 (+18 Da), 4% and 2% for Lyso-Gb3 (+34 Da), 2% for Lyso-Gb3 (-28 Da), and 1% for Lyso-Gb3 (+16 Da), in males and females respectively.

Figure 4.8 shows a ROC analysis of plasma Lyso-Gb3 and its 6 analogues. The results show that plasma Lyso-Gb3 and three analogues including Lyso-Gb3 (-28 Da), Lyso-Gb3 (+16 Da) and Lyso-Gb3 (+18 Da), show high sensitivity and specificity as a diagnostic predictor for FD. While Lyso-Gb3 has been proven to have a potential diagnostic utility, caution should be taken with regards to the use of the aforementioned analogues given their extremely low abundance and variability in detection between Fabry patients, even though they are absent in samples taken from healthy control subjects.

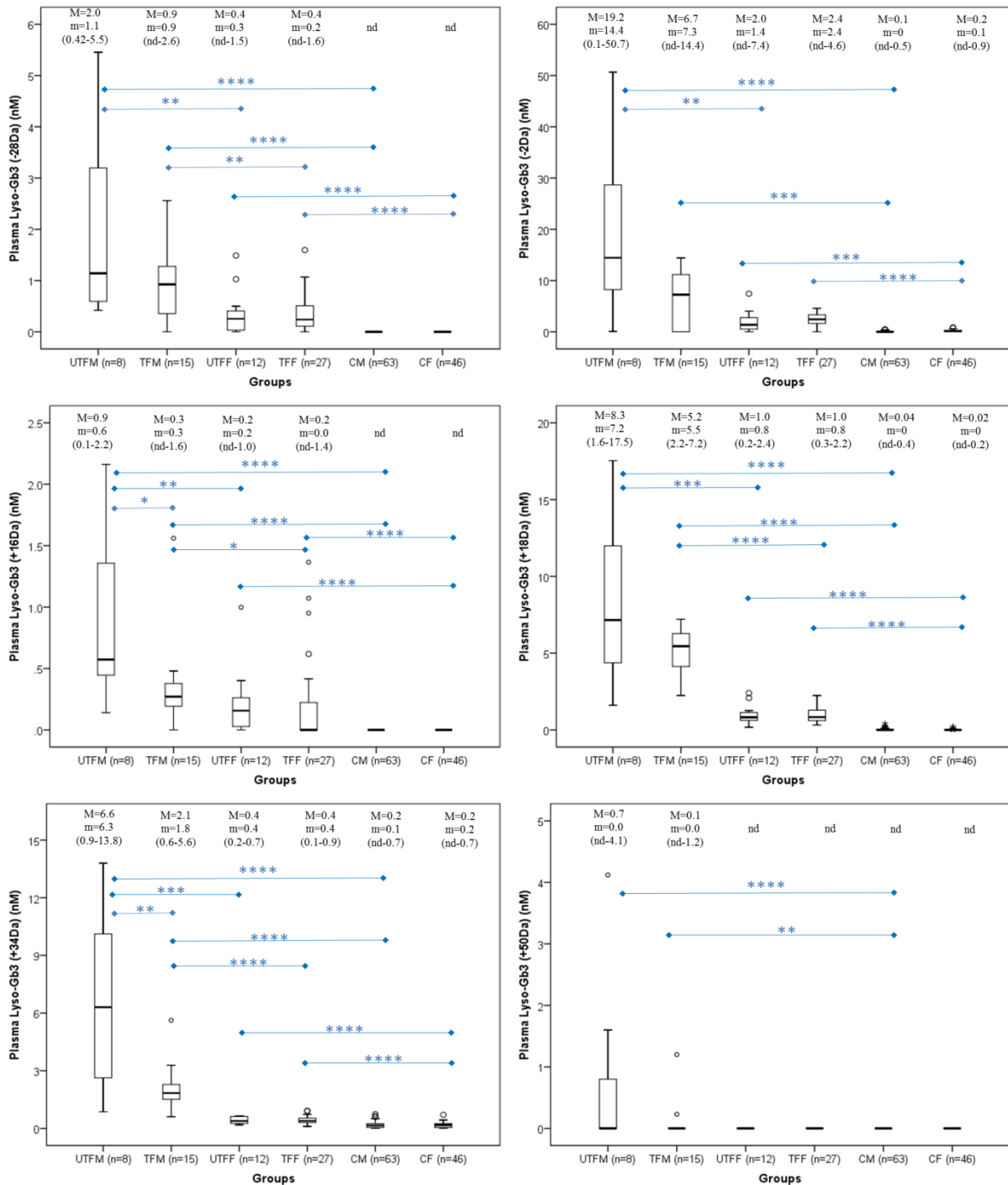


Figure 4.6 The levels of plasma Lyso-Gb3 analogues in classical Fabry patients: Data were pooled from the patients and control subjects and presented as Whiskers-box plots. The concentrations were measured using Lyso-Gb3 calibration curve. Data were analysed using SPSS. The subjects were divided to 6 groups as follows: untreated Fabry male (UTFM), treated Fabry male (TFM), untreated Fabry female (UTFF), treated Fabry female (TFF), control male (CM) and control female (CF). For each group: n = number of subject; M = Mean; m = median; the range of minimum and maximum values in brackets; box= maximum and minimum quartiles; line inside the box = median; whiskers = maximum and minimum non-outlier values; o = outlier values. Various groups were compared against each other using Mann-Whitney U tests. The differences were considered statistically significant if the p value < 0.05 (*= $p < 0.01$, **= $p < 0.001$ and ****= $p < 0.0001$).

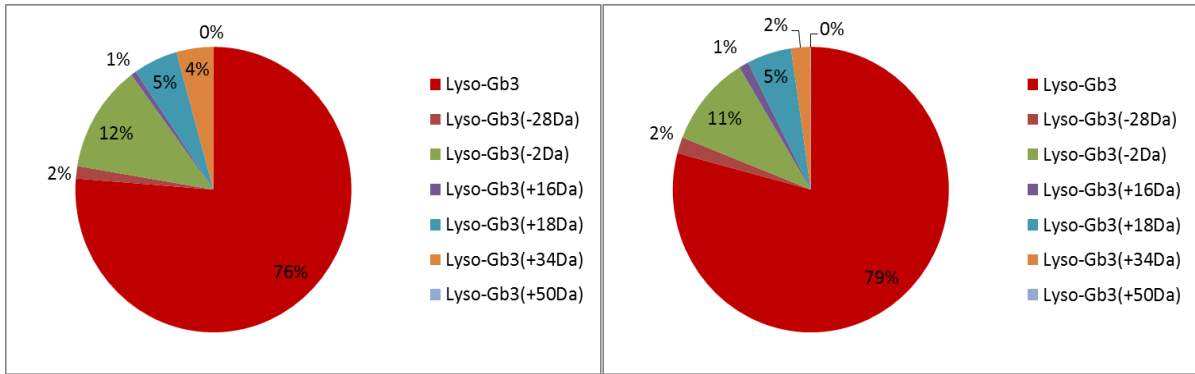


Figure 4.7: The relative distribution of plasma Lyso-Gb3 and its analogues in untreated classical Fabry males (n=8, **left panel**) and untreated classical females (n=12, **right panel**). All classical Fabry males and females showed high percentage of Lyso-Gb3 in their plasma while trace amounts were converted to Lyso-Gb3 analogues attributed to modifications in the sphingosine moiety.

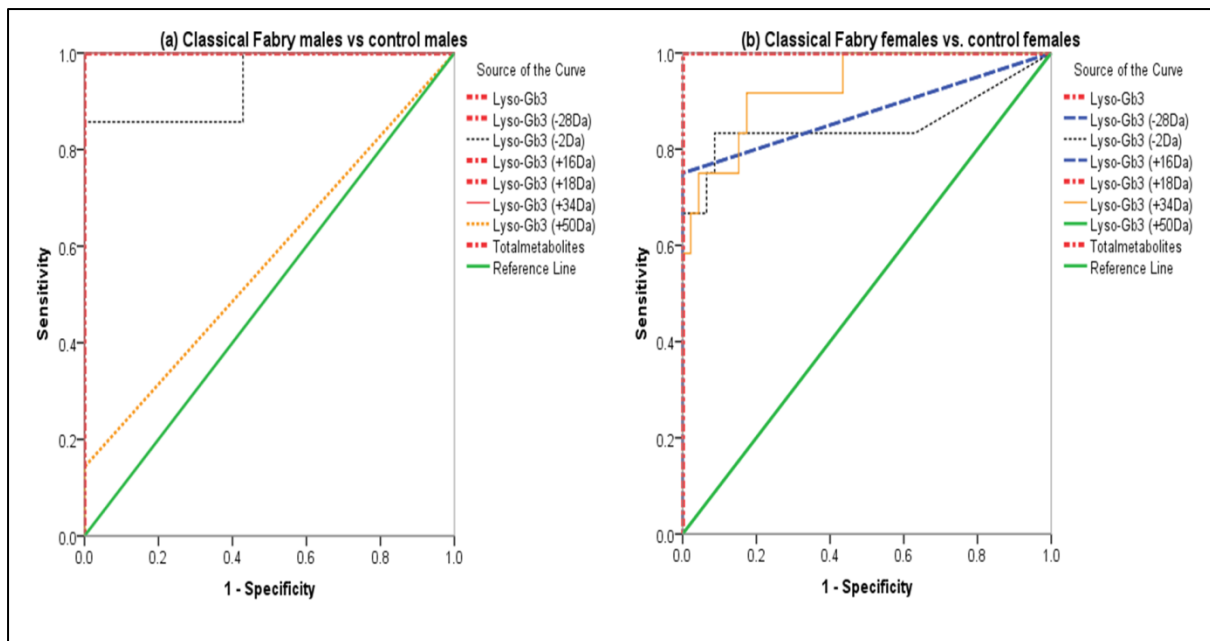


Figure 4.8: ROC analysis for diagnostic accuracy of plasma Lyso-Gb3 analogues to predict Fabry disease in classical Fabry patients: (a) untreated hemizygous classical Fabry males vs. control males. (b) Untreated heterozygous classical Fabry females vs. control females. Graphs were generated using SPSS software.

4.3.5 Levels of plasma Lyso-Gb3 analogues in cardiac variant Fabry patients:

Building on the analysis outlined above, I set out to determine the levels of various Lyso-Gb3 analogues in cardiac variants Fabry patients. Again, this comparison included both untreated and treated male and female Fabry patients carrying the cardiac variant mutations and normal control subjects. As shown in Figure 4.9, the relative concentration of each Lyso-Gb3 analogue varied considerably and, compared to classical Fabry patients were an order of magnitude lower. As with classical Fabry patients, the levels of Lyso-Gb3 (-2 Da) were found at the highest concentration (0-4 nM), while levels of Lyso-Gb3 (+18 Da) were somewhat lower (0-2 nM), and levels of Lyso-Gb3 (-28 Da) very low (0-1 nM). The levels of Lyso-Gb3 (+34 Da) varied between (0-0.5 nM) while the concentration of Lyso-Gb3 (+16 Da) was extremely small (0-0.9 nM). The levels of Lyso-Gb3 (+50 Da) were undetectable. In all cases, the levels of each analogue were undetectable in control male and female subjects. Unlike classical Fabry patients, there was less of a distinction between male and female Fabry patients carrying the cardiac variant on the levels of all Lyso-Gb3 analogues. In response to ERT treatment, a reduction in the concentration of specific Lyso-Gb3 analogues was only observed in male patients, with reductions in the concentrations of Lyso-Gb3 (-28 Da), Lyso-Gb3 (+16 Da) and, to a lesser extent in Lyso-Gb3 (-2 Da). The concentrations of Lyso-Gb3 (+18 Da) were moderately affected, while the concentrations of Lyso-Gb3 (+34 Da) and Lyso-Gb3 (+50 Da) were virtually undetectable.

The relative distribution of plasma Lyso-Gb3 and its various analogues was examined in the untreated cardiac variant Fabry patients. This data is presented in the form of a pie chart (Figure 4.10). In keeping with findings from classical Fabry patients, the levels of plasma Lyso-Gb3 constituted the highest percentage of total Lyso-Gb3 in both male and female patients (males 77% vs. females 72%). Again, small trace amounts of Lyso-Gb3 analogues

were detected in both male and female cases at broadly similar levels. The levels of Lyso-Gb3 (-2 Da), Lyso-Gb3 (+18 Da), Lyso-Gb3 (+34 Da) and Lyso-Gb3 (-28 Da), which arise through modification of the sphingosine moiety, were broadly similar in both male and female cases, comprising 7% and 9% for Lyso-Gb3 (-2 Da); 9% and 6% for Lyso-Gb3 (+18 Da); 5% and 3% for Lyso-Gb3 (+34 Da); 4% and 3% for Lyso-Gb3 (-28 Da); and 3% and 2% for Lyso-Gb3 (+16 Da) in males and females respectively.

Figure 4.11 shows a ROC analysis of plasma Lyso-Gb3 and its 6 analogues in cardiac variant Fabry patients. The results show that plasma Lyso-Gb3 and the analogue Lyso-Gb3 (+18 Da) show high sensitivity and specificity as a diagnostic predictor for FD. The results proven that Lyso-Gb3 has a potential diagnostic utility. Despite some healthy control subjects didn't show any detectable levels of the analogue Lyso-Gb3 (+18 Da), caution should be taken with regards to the use of the aforementioned analogues due to the extremely low abundance and variability in detection between Fabry patients.

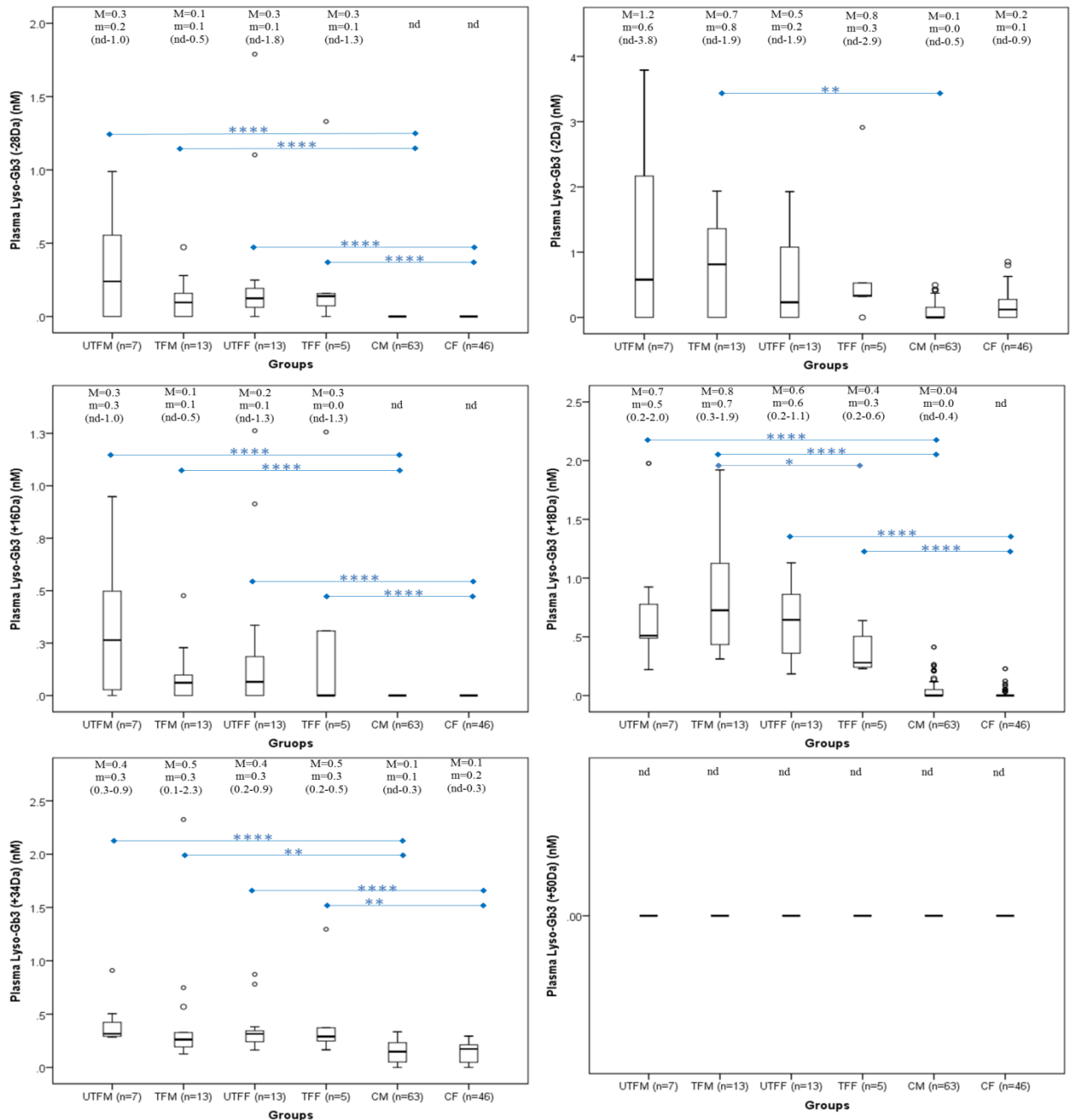


Figure 4.9 Plasma Lyso-Gb3 analogues levels in cardiac variant Fabry patients: Data were pooled from the patients and control subjects and presented as Whiskers-box plots. The concentrations were measured using Lyso-Gb3 calibration curve. Data were analysed using SPSS. The subjects were divided to 6 groups as follows: untreated Fabry male (UTFM), treated Fabry male (TFM), untreated Fabry female (UTFF), treated Fabry female (TFF), control male (CM) and control female (CF). For each group: n = number of subject; M = Mean; m = median; the range of minimum and maximum values in brackets; box= maximum and minimum quartiles; line inside the box = median; whiskers = maximum and minimum non-outlier values; o = outlier values. Various groups were compared against each other using Mann-Whitney U tests. The differences were considered statistically significant if the *p* value < 0.05 (**=*p*<0.01, ***=*p*<0.001 and ****=*p*<0.0001).

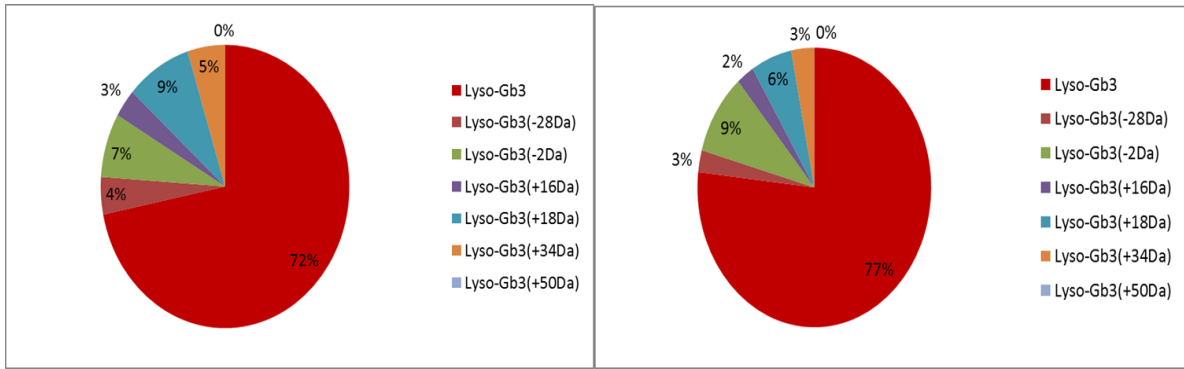


Figure 4.10 The relative distribution of plasma Lyso-Gb3 and its analogues in cardiac variant Fabry patients: untreated cardiac variant Fabry males (n=7, left panel) and females (n=13, right panel). All cardiac variant Fabry males and females showed a high percent of Lyso-Gb3 in their plasma while small trace amounts of Lyso-Gb3 analogues were present.

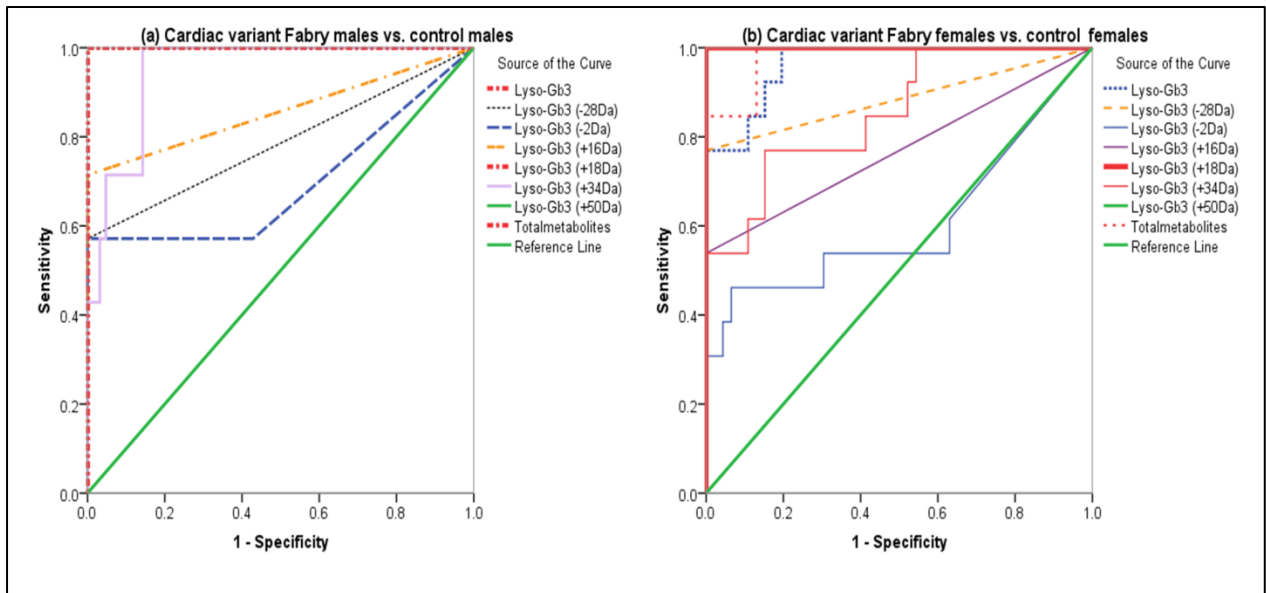


Figure 4.11: ROC analysis for diagnostic accuracy of plasma Lyso-Gb3 analogues to predict Fabry disease in cardiac variant Fabry patients: (a) untreated hemizygous cardiac variant Fabry males vs. control males. (b) Untreated heterozygous cardiac variant Fabry females vs. control females. Graphs were generated using SPSS software.

4.3.6 Plasma Lyso-Gb3 levels in classical Fabry patients following ERT

We next set out to examine the effects of ERT treatment on the levels of plasma Lyso-Gb3 in classical Fabry patients. Blood samples were collected from three patients and the levels of Lyso-Gb3 analysed from plasma as described previously. In the three cases blood sample were available pre and post treatment. In the classical Fabry male patient (Figure 4-12 a), elevated levels of plasma Lyso-Gb3 were found pre-ERT (154 nM). However, in response to the treatment by ERT, the levels of Lyso-Gb3 were reduced by almost two-fold one month after treatment (83 nM). 10 months after treatment the levels of Lyso-Gb3 had reduced again to (62 nM). By 18 and 22 months, post-ERT the levels of Lyso-Gb3 increased slightly, but were still almost two fold lower compared to the levels observed pre-ERT. While the pre-ERT levels of Lyso-Gb3 were 77.1 nM in the other Fabry male patient (Figure 4-12 b). However, the levels of Lyso-Gb3 were reduced 5 months post-ERT, and continued to drop at 11, 26 months in response to continual treatment. Here, the concentrations of Lyso-Gb3 were almost 2.6 fold lower 26 months post-ERT compared to starting point (pre-ERT). For the classical Fabry female patient (Figure 4-12 c), the levels of Lyso-Gb3 were elevated pre-ERT (43.9 nM), and decreased by almost two fold (20.1 nM) at the first time point post-ERT (after 2 months from the initiation of ERT). At the second time point (after 7 months from the initiation of ERT), the levels of Lyso-Gb3 had increased slightly (25.0 nM), yet were still almost two – fold lower than levels observed pre-ERT.

Building upon the above findings, the levels of Plasma Lyso-Gb3 in Fabry patients following ERT treatment was extended to include a larger cohort of patients. As shown in Figure 4.13, the levels of plasma Lyso-Gb3 were measured in an additional 8 classical Fabry patients following treatment by ERT. Patient samples 1,2 and 3 were previously described in Figure 4-12). In broad agreement with the previous findings, the overall levels of Lyso-Gb3 were reduced in classical Fabry patients following treatment with ERT (Figure 4.13).

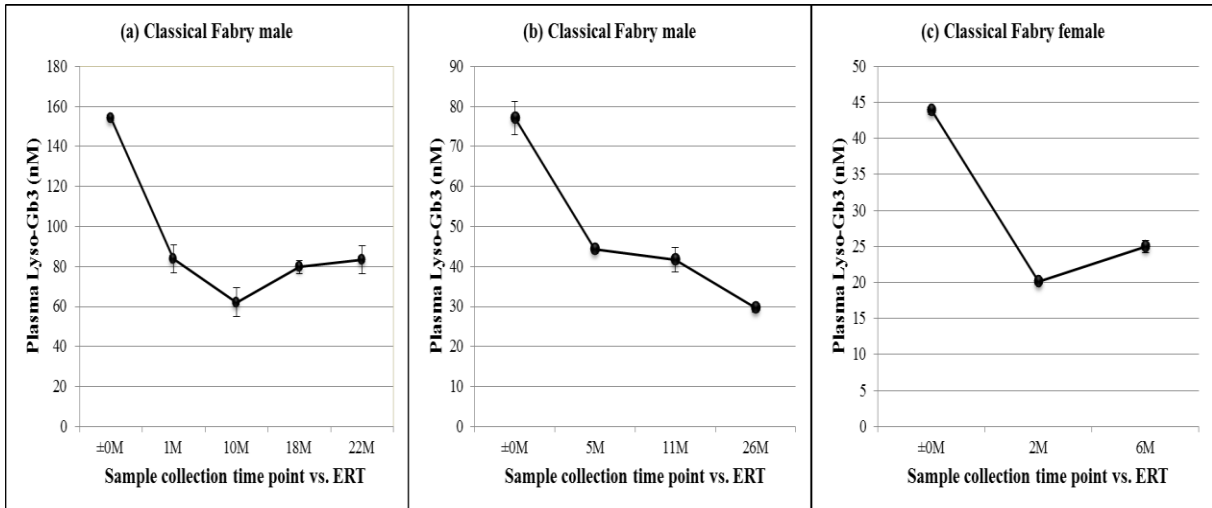


Figure 4.12 Following up ERT management in classical Fabry patients: Figure showing the levels of plasma Lyso-Gb3 levels in 3 classical Fabry patients’ pre- and post-ERT. The plasma samples were collected from the 3 patients at different time-points.

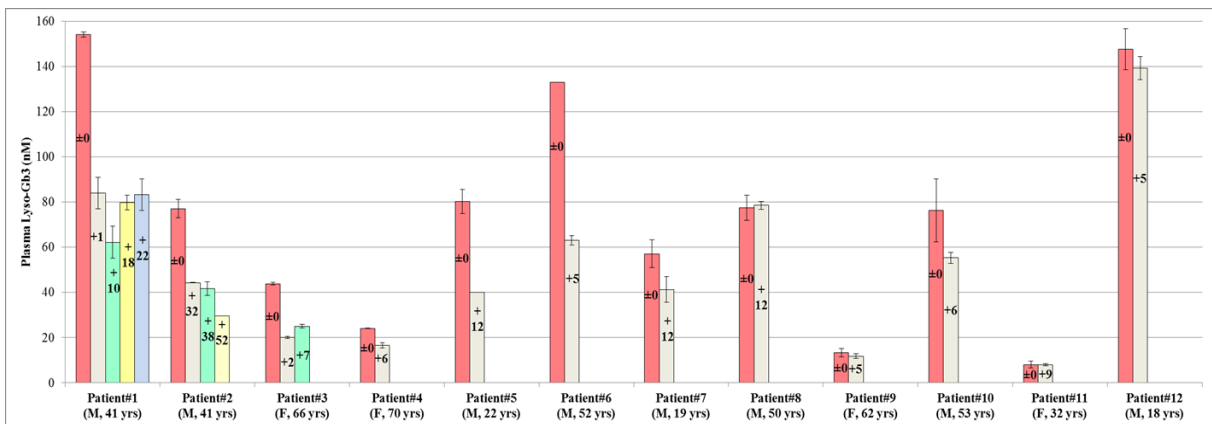


Figure 4.13 Plasma Lyso-Gb3 levels through ERT monitoring in classical Fabry patients: A figure shows the variations in plasma Lyso-Gb3 levels in 12 classical Fabry patients’ were undergone to ERT. The time points of sample collection were varied from a patient to another and here are expressed by numbers (months) inside the column.

4.3.7 Plasma Lyso-Gb3 levels in cardiac variant Fabry patients following ERT

Continuing with the theme of the previous section, the levels of Plasma Lyso-Gb3 were also measured in Fabry patients carrying cardiac variants pre and post-ERT treatment. As shown in Figure 4.14, the levels of plasma Lyso-Gb3 were lower than those observed in classical Fabry patients, yet in a number of cases; the levels of Lyso-Gb3 were reduced in patients following treatment with ERT.

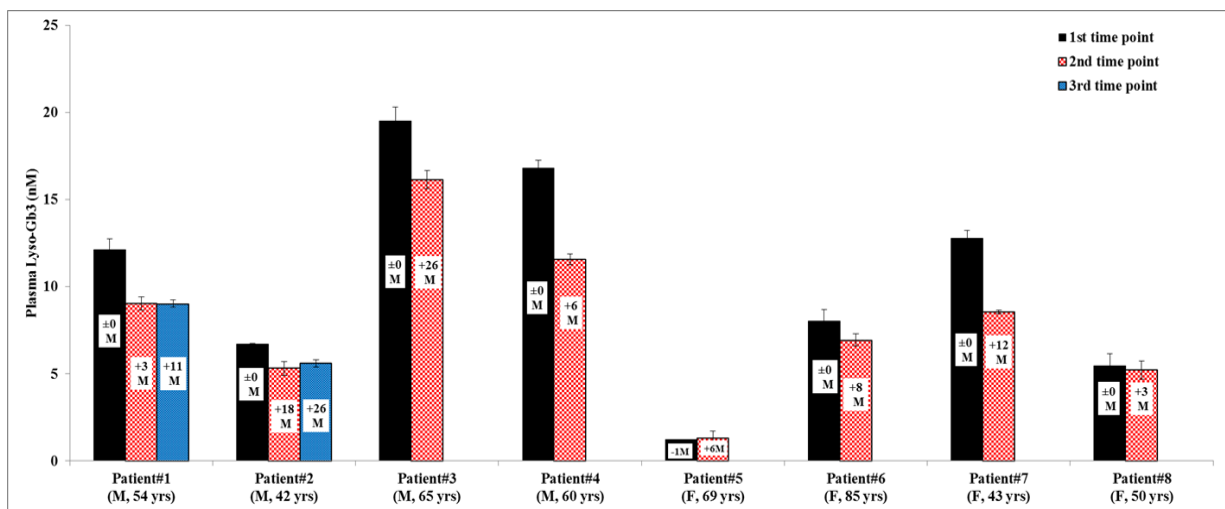


Figure 4.14 Plasma Lyso-Gb3 levels through ERT monitoring in cardiac variant Fabry patients: Plasma Lyso-Gb3 levels in cardiac variant Fabry patients' pre and post-ERT.

In order to follow FD progression without ERT treatment, the levels of Lyso-Gb3 were available at 2 different time-points for only 3 cardiac variant Fabry patients. The results show slight increase in the levels of Lyso-Gb3 with time (Figure 3-15).

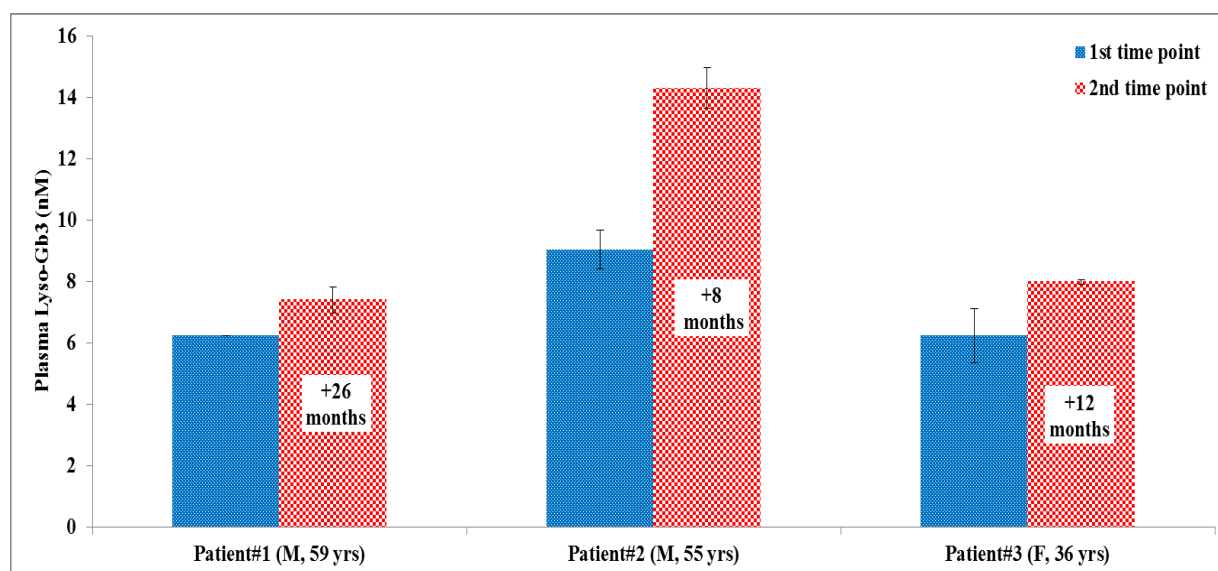


Figure 4.15 Plasma Lyso-Gb3 levels vs. time in cardiac variant Fabry patients without any ERT management: Plasma Lyso-Gb3 concentrations in 3 cardiac variant Fabry patients with the nonsense mutation N215S. Plasma samples were collected from the patients at 2 different time-points where the patients were not undergone for any type of ERT at both the 2 time-points.

4.3.8 Correlation between the levels of plasma Lyso-Gb3 and its various analogues in Fabry patients:

We next set out to explore whether the concentrations of specific Lyso-Gb3 analogues correlated with the levels of Lyso-Gb3 in plasma samples from Fabry patients. As shown in Figure 4.16, a strong correlation was observed between the levels of Lyso-Gb3 and its analogues: Lyso-Gb3 (+18 Da) and Lyso-Gb3 (+34 Da); and to a lesser extent with the other analogues in plasma samples from Fabry patients. In all cases, the correlations between the levels of plasma Lyso-Gb3 and its analogues were statistically significant with Pearson Chi-Square p value < 0.0001.

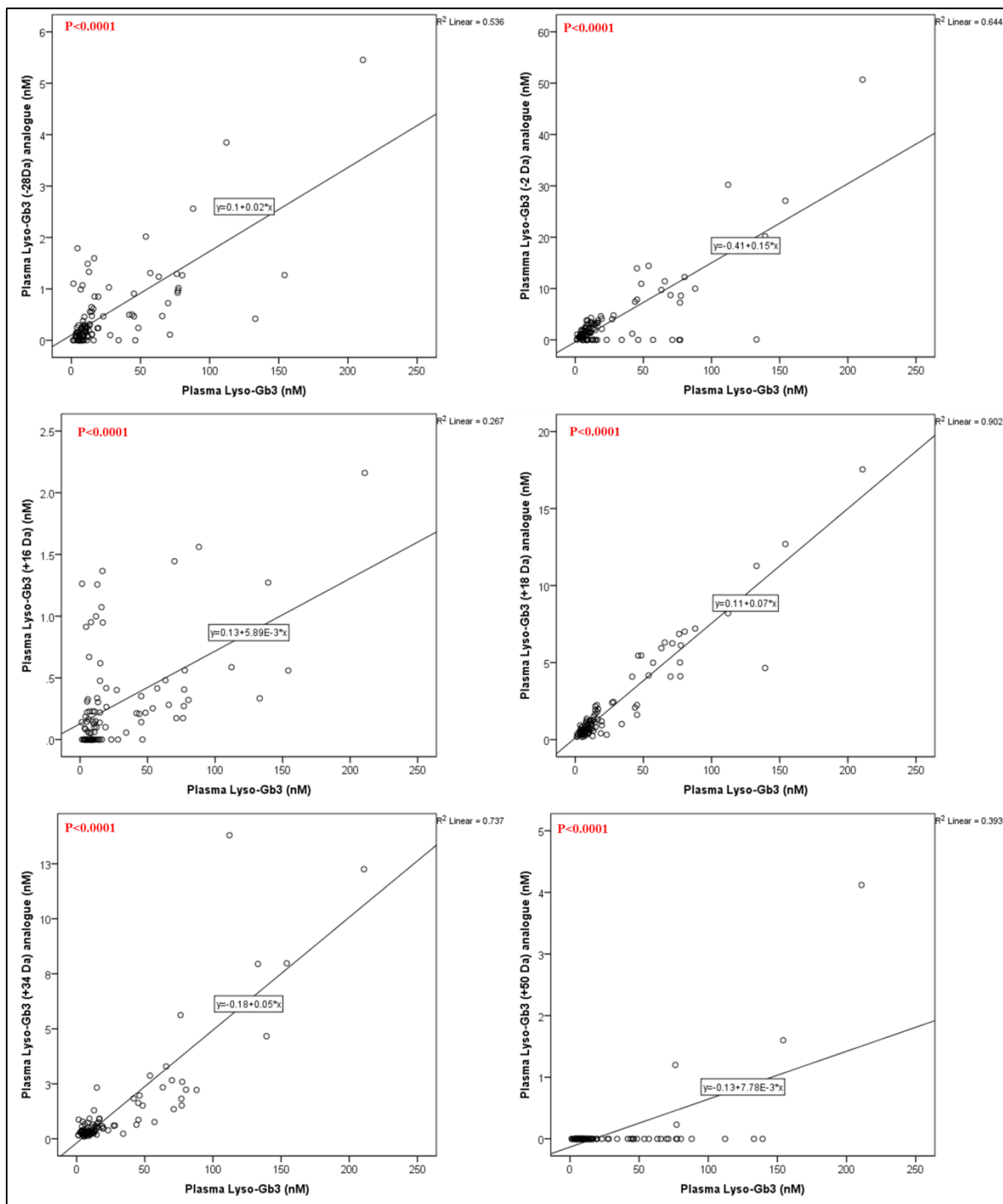


Figure 4.16 Correlation between plasma Lyso-Gb3 and its analogues: The graphs show a strong correlation between the levels of plasma Lyso-Gb3 and its analogues in Fabry patients. The correlation was statistically significant with Pearson Chi-Square p value < 0.0001 in all cases. Graphs were generated using SPSS software.

4.3.9 Correlation between the levels of plasma Lyso-Gb3 and age of Fabry patients:

The correlation between the levels of plasma Lyso-Gb3 and the age of Fabry patients did not reach the statistical significance (Figure 4.17).

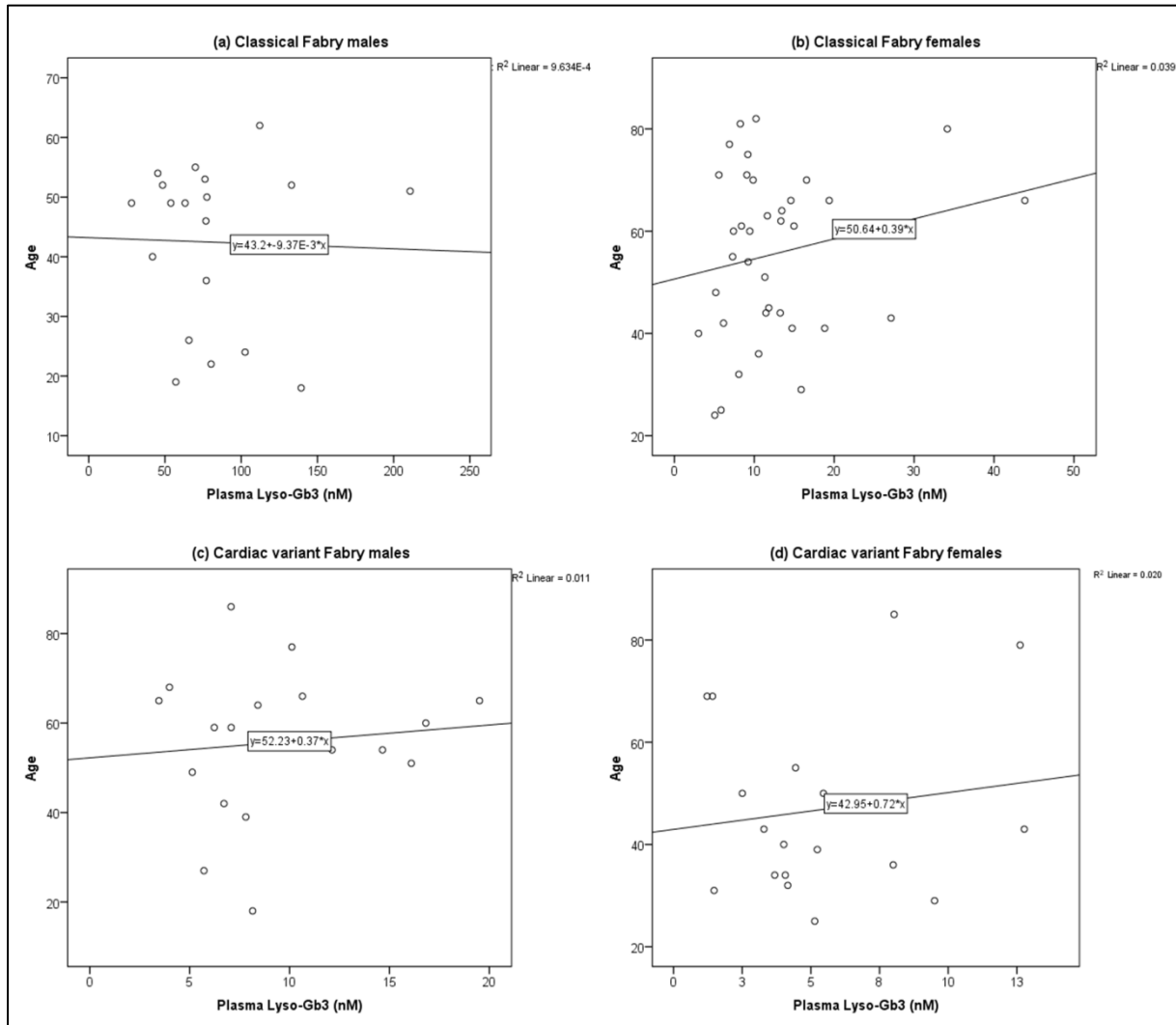


Figure 4.17 Correlation between plasma Lyso-Gb3 levels and the age: The graphs show the correlation between the levels of plasma Lyso-Gb3 and the age of Fabry patients. The correlation was statistically not significant in all groups. Graphs were generated using SPSS software.

4.4 DISCUSSION

There is a lack of available biomarkers that can be used to diagnose the various forms of FD, or to establish disease severity and monitor disease progression. As stated previously, two GSLs have been identified which constitute promising biomarkers that may aid the diagnosis and monitoring of the disease in Fabry patients. These are Gb3 and Lyso-Gb3; the latter is very similar to Gb3 but lacks the fatty acid chain.

Several recent publications advocate the use of Lyso-Gb3 as a diagnostic biomarker for FD [46, 166, 222, 224]. The concentrations of plasma Lyso-Gb3 have shown utility in diagnosing the condition and, at least in one study, shown to correlate with disease severity [215]. Findings presented in this Chapter further substantiate these claims and support the utility of measuring plasma Lyso-Gb3 levels in the diagnosis of classical and cardiac variant forms of the disease.

Results presented in this chapter set out to explore the utility of Lyso-Gb3 as a potential biomarker in the diagnosis and monitoring of FD. Findings presented in the first part of the chapter demonstrate that Lyso-Gb3 may serve as a useful biomarker in the detection of classical Fabry males, while it is still useful in diagnosing classical Fabry females despite their residual enzyme activity.

There was no overlap between any classical Fabry patients and control males. Also, there was no overlap between cardiac variant Fabry males and control males. However, it is important to highlight that the levels of Lyso-Gb3 in some cardiac variant females overlapped or were very close to the levels in control females and hence caution is advised interpreting plasma Lyso-Gb3 levels in this subgroup. Based on the results of the remaining subgroups of Fabry patients (classical males, classical females and cardiac variant males) Lyso-Gb3 has a potential value to be used as a diagnostic tool for FD.

In clinical practice this implies that in males, positive plasma Lyso-Gb3 is likely to be diagnostic and a negative Lyso-Gb3 should reliably exclude FD as a diagnosis. In females positive plasma Lyso-Gb3 levels again should be considered diagnostic but negative results cannot exclude FD with certainty. As discussed previously, Fabry females continue to pose a diagnostic dilemma in FD. It is likely that use of plasma Lyso-Gb3 in many of these situations, especially when the levels are high, help to establish the diagnosis.

Another area where we suggest plasma Lyso-Gb3 is likely to be of benefit is in patients with mutations of unknown significance. In male patients with mutations of unknown significance a positive or negative results should help in confirming or excluding the diagnosis of FD. However in female patients a positive result can reliably confirm the diagnosis of FD but a negative result cannot exclude it. To expand the argument further most of female patients including cardiac variants had levels above control females. Hence in situations where the suspicion of FD is low, a negative result of plasma Lyso-Gb3 will add some confidence along with other clinical information in attempting to exclude FD.

In terms of monitoring of FD, plasma Lyso-Gb3 may be useful as a biomarker in classical Fabry males, where plasma Lyso-Gb3 levels were found to drop in response to ERT therapy. Subsequent to the initial drop, increases in the levels of Lyso-Gb3 were observed in only two cases from a total of twelve classical Fabry patients examined during their follow-up. This phenomenon has previously been suggested to be due to development of an immune response to ERT [46, 159-162]. It is not clear whether this increase in Lyso-Gb3 levels after the initial decline, correlates with a worsening of symptoms and disease progression.

In essence, these finding suggest that Lyso-Gb3 may serve as a useful biomarker in monitoring Fabry patients' response to ERT. Moreover, in agreement with a study by van Breemen and colleagues [165], we have shown that the levels of plasma Lyso-Grb3 are

reduced in classical Fabry males receiving ERT and may, therefore, have utility in monitoring a patients' response to ERT.

While a correlation was observed between the levels of plasma Lyso-Gb3 and the presence of selected Lyso-Gb3 analogues, the overall concentrations of these analogues were deemed too low to be of use as selective biomarkers in Fabry Patients.

The results show that plasma Lyso-Gb3 and its analogues: Lyso-Gb3 (-28 Da) Lyso-Gb3 (+16 Da) and Lyso-Gb3 (+18 Da) show high sensitivity and specificity as diagnostic predictors for classical FD in males. However, plasma Lyso-Gb3 and only the analogue Lyso-Gb3 (+18 Da) show high sensitivity and specificity as a diagnostic predictor for classical FD in females and for cardiac variant form of FD in both genders.

While Lyso-Gb3 has been proven to have a potential diagnostic utility, caution should be taken with regards to the use of the aforementioned analogues given their extremely low abundance and variability in detection between Fabry patients, even though they are absent in samples taken from healthy control subjects.

CHAPTER - 5
THE CLINICAL UTILITY OF URINARY LYSO-GB3
AND ITS ANALOGUES IN THE DIAGNOSIS OF
FABRY DISEASE

5.1 INTRODUCTION

The deficiency of α -gal-A activity in Fabry patients results in the intracellular accumulation of Gb3 and Lyso-Gb3 in various organs. It has been suggested that the genetic and epigenetic modifications contribute to residual enzyme activity.

While less serious clinical manifestations of FD include pain in the hands and feet, more serious complications include diseases of the kidney, heart and brain. Fabry nephropathy is the involvement of the kidneys in Fabry patients. Over time, the progressive accumulation of Gb3 and Lyso-Gb3 and their related isoforms and analogues in cells that form the distal tubule and the loop of Henle, disrupts cell function and leads to renal pathologies. These manifest as proteinuria and reduced eGFR and can lead to chronic kidney disease and progression to renal decline [261-263].

The longitudinal databases on FD collected by The Fabry Registry (Genzyme) [49, 264, 265] and The Fabry Outcome Survey (FOS; Shire) [27], have shown a long delay in the diagnosis of FD after the onset of initial symptoms [261]. It has been shown that before renal dialysis and renal transplants became widely available, the life span of male Fabry patients was markedly reduced with an average age at death of 42 years [266-268]. However, the availability of renal dialysis and renal transplants worldwide in the past decades have extended the lifespan of Fabry patients [266].

As outlined in previous chapters, it is now well established that the diagnostic procedures (mutation analysis and α -gal-A enzyme activity test), while useful in the initial diagnosis of FD, have no utility for disease monitoring. This fact underscores the importance of identifying other methods that not only aid diagnosis, but can be used to monitor Fabry patients receiving treatment.

Plasma Lyso-Gb3 has progressively gained more importance as biomarker in Fabry in recent years but as explained in previous chapters it has its limitation as a diagnostic and monitoring biomarker especially in female variant patients. Having another biomarker to compliment plasma Lyso-Gb3 may provide some more diagnostic confidence in groups like cardiac variants and patients with genetic variant of unknown significance.

In this chapter we aimed to measure the levels of urinary Lyso-Gb3 and its analogues and to determine the clinical utility of these GSLs as biomarkers for the diagnosis of FD.

5.2 SAMPLE COLLECTION FROM FABRY PATIENTS

Urine samples were collected from 42 Fabry patients. Samples were collected in universal urine tubes and stored at -80°C without prior purification or centrifugation until further processing. A total of 42 patients were sourced from the West Midlands or Sheffield. All the patients provided matching plasma samples at the same time of collection. All patients were diagnosed as FD sufferers either by mutation screening of the *GLA* gene or by the α -gal-A enzymatic activity test. Samples were obtained from 23 healthy volunteers and an additional 25 non-Fabry patients; the latter, who were receiving treatment for diseases unrelated to FD, were obtained from renal and adrenal clinics at the Queen Elizabeth Hospital, Birmingham, UK. Table 5-1 shows demographic data of the patients and control subjects.

		Fabry patients				Control subjects	
		classical		cardiac variant		male	female
		male	female	male	Female		
Number		13	7	11	11	22	26
Age (y)	mean	39.8±15.1	58.7±16.1	49.6±12.9	51.6±19.9	46.7±14.9	59.4±18.3
	median	45.5±4.8	62.0±6.1	54.0±3.9	46.5±6.3	57.2±5.6	56.8±7.4
	range	19-55	32-80	18-65	29-85	20-82	29-80

Table 5.1 Anthropometric data of Fabry patients and control subjects: Table showing the total number, age and gender of all subgroups of Fabry patients and control subjects.

5.3 RESULTS

5.3.1 Control groups:

Our control population consisted of 23 healthy volunteers and 25 non-Fabry patients. Urine samples were obtained from 25 non-Fabry patients attending two different clinics at the Queen Elizabeth hospital, Birmingham, UK. As far as possible, these were age and gender matched. These included patients receiving treatment for renal (n=15) or adrenal (n=10) diseases. Urine samples generated from this group were analysed for the levels of Lyso-Gb3 and its analogues by LC-MS/MS, as outlined in section 2.4, 2.5 and 2.6 of Chapter 2. This analysis was performed to determine whether urinary Lyso-Gb3 might constitute a specific biomarker for FD and whether it is prevalent in other disease conditions. The results of this analysis revealed that all non-Fabry patients from both clinics had comparable levels of urinary Lyso-Gb3 that were broadly similar to those of healthy control subjects. The levels of Lyso-Gb3 were virtually undetectable in both non-Fabry patients (from renal and adrenal clinics) and healthy volunteers (n= 23), where the levels of Lyso-Gb3 typically below the limit of detection in urine samples. All 25 non-Fabry patients were enrolled in the study as controls alongside healthy volunteers, increasing the number of control subjects from 23 to be 48 (Figure 5-1). Urinary Lyso-Gb3 was detected in classical Fabry patients (mean ± SEM = 105.8 pmol/mmol creatinine ± 20.8). Moreover, Lyso-Gb3 was also detected in most of

Fabry patients carrying cardiac variant mutations of the α -gal-A gene, although in this case, substantially lower levels (approximately 6 fold) of Lyso-Gb3 were present (mean \pm SEM = 16.9 pmol/mmol creatinine \pm 4.9).

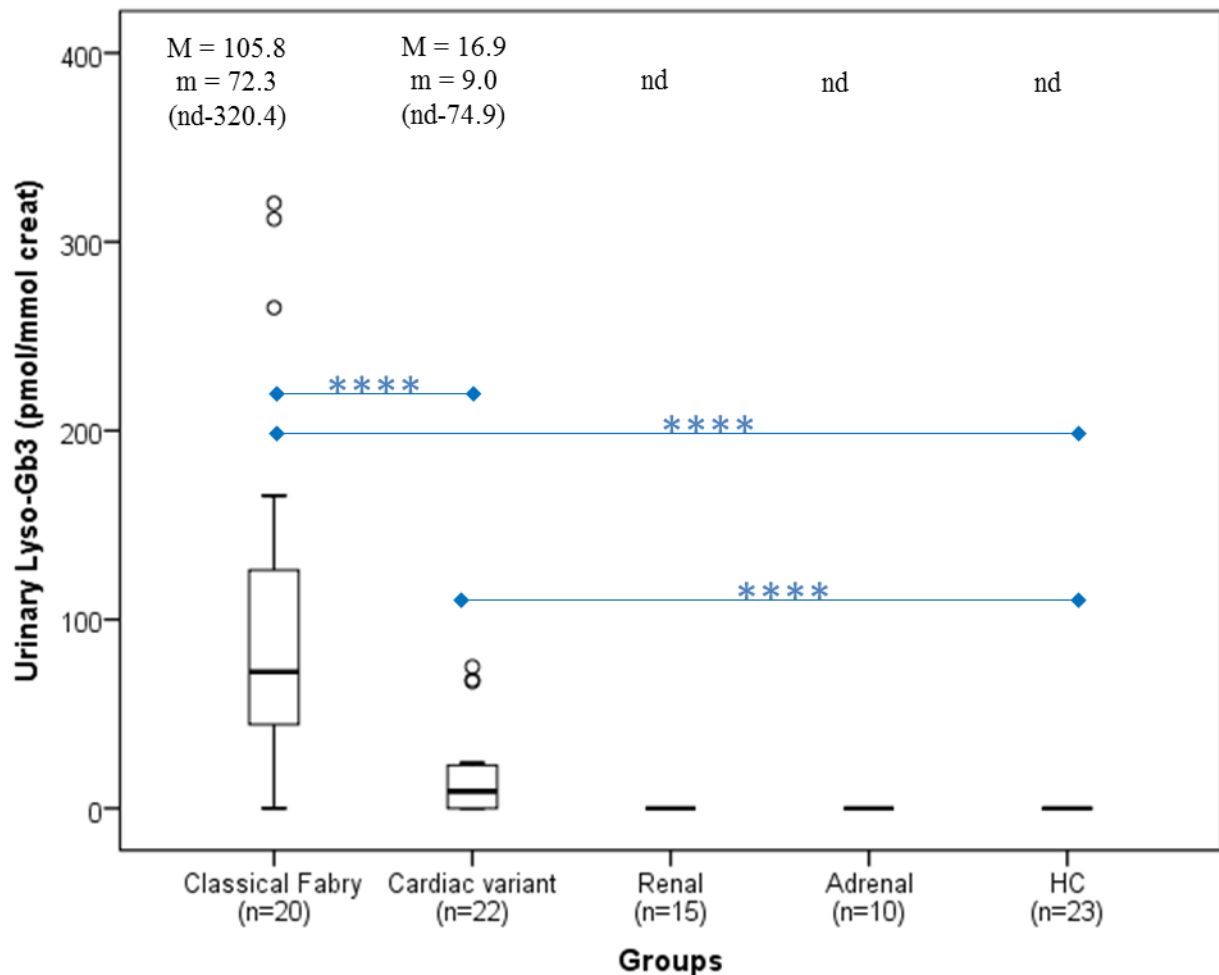


Figure 5-1 Urinary Lyso-Gb3 levels in Fabry patients and control subjects: The figure shows the levels of Lyso-Gb3 in urine samples generated from classical Fabry patients, cardiac variant Fabry patients and non-Fabry patients from the two different clinics (renal diseases clinic and adrenal diseases clinic), in addition to healthy control individuals (HC). The results revealed that there are no differences in the levels of Lyso-Gb3 between healthy controls and non-Fabry patients from renal and adrenal groups. All non-Fabry groups showed undetectable Lyso-Gb3 levels exactly like healthy control volunteers where their urinary Lyso-Gb3 was undetectable. Consequently, those non-Fabry patients were pulled together to form large control. For each group: n = number of subject; M = Mean; m = median; the range of minimum and maximum values in brackets; box= maximum and minimum quartiles; line inside the box = median; whiskers = maximum and minimum non-outlier values; various groups were compared against each other using Mann-Whitney U tests. The differences were considered statistically significant if the p value < 0.05 (****= $p < 0.0001$).

5.3.2 Urinary Lyso-Gb3 levels in classical Fabry patients

To determine whether the levels of urinary Lyso-Gb3 could be used as a diagnostic tool to diagnose patients with classical FD, it was necessary to compare the levels of urinary Lyso-Gb3 in Fabry patients to those of control subjects. The levels of urinary Lyso-Gb3 in hemizygous male (n = 12) and heterozygous female (n = 7) classical Fabry patients were compared to each other and to control male (n = 22) and control female (n = 26) subjects.

The levels of urinary Lyso-Gb3 were significantly higher in classical Fabry males (mean \pm SEM = 135.0 ± 30.8) compared to classical Fabry females (mean \pm SEM = 64.3 ± 18.1 , p value < 0.05), with a noticeable overlap in urinary Lyso-Gb3 levels between genders. Nonetheless, the concentrations of urinary Lyso-Gb3 in male and female classical Fabry patients were significantly higher than those of male and female control subjects, where the levels of urinary Lyso-Gb3 were undetectable (p value < 0.0001).

There was no overlap between the levels of urine Lyso-Gb3 in classical Fabry males and control male subjects. Nevertheless, of a total of seven heterozygous classical Fabry females, only one patient did not show any detectable level of urinary Lyso-Gb3.

These results confirm that the levels of urinary Lyso-Gb3 were significantly higher in hemizygous classical Fabry males and, to a lesser extent in heterozygous classical Fabry females, and point to the potential utility of urinary Lyso-Gb3 in the diagnosis of classical FD in both male and female subjects however caution is advised interpreting a negative result in classical females.

In order to assess the diagnostic accuracy of urinary Lyso-Gb3 levels in diagnosing classical FD in males, ROC analysis performed. Urinary Lyso-Gb3 levels in 4 confirmed untreated classical Fabry males and 22 control males were assessed. A statistically significant result was generated with favourable AUC (1.000, p value < 0.01). Urinary Lyso-Gb3 levels equal

to or higher than 9.4 pmol/mmol creatinine indicated the presence of FD with a sensitivity of 100% and specificity of 100% Figure (5-3).

On the other hand, ROC analysis performed to assess the diagnostic accuracy of urinary Lyso-Gb3 levels in diagnosing classical FD in females. Urinary Lyso-Gb3 levels in 4 confirmed untreated classical Fabry females and 26 control females were assessed. ROC curve showed a statistically significant result with AUC (0.875, p value <0.05). Urinary Lyso-Gb3 value equal to or higher than 12.8 pmol/mmol creatinine indicated the presence of FD with a sensitivity of 75% and specificity of 100% (Figure 5-3).

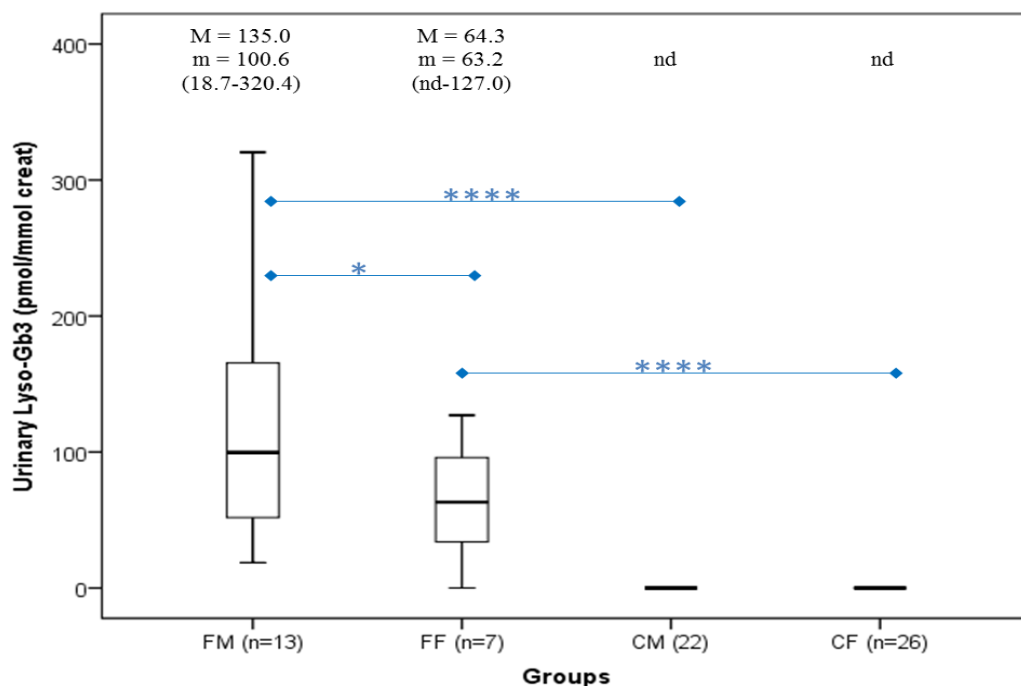


Figure 5-2 Urinary Lyso-Gb3 levels in classical Fabry patients: Data were pooled from the patients and control subjects and presented as box plots. The concentrations were measured using Lyso-Gb3 calibration curve. Data were analysed using SPSS. The subjects were divided to 4 groups as follows: Fabry male (FM), Fabry female (FF), control male (CM) and control female (CF). For each group: n = number of subject; M = Mean; m = median; the range of minimum and maximum values in brackets; box= maximum and minimum quartiles; line inside the box = median; whiskers = maximum and minimum non-outlier values; various groups were compared against each other using Mann-Whitney U tests. The differences were considered statistically significant if the p value < 0.05 (* = p <0.01 and **** = p <0001).

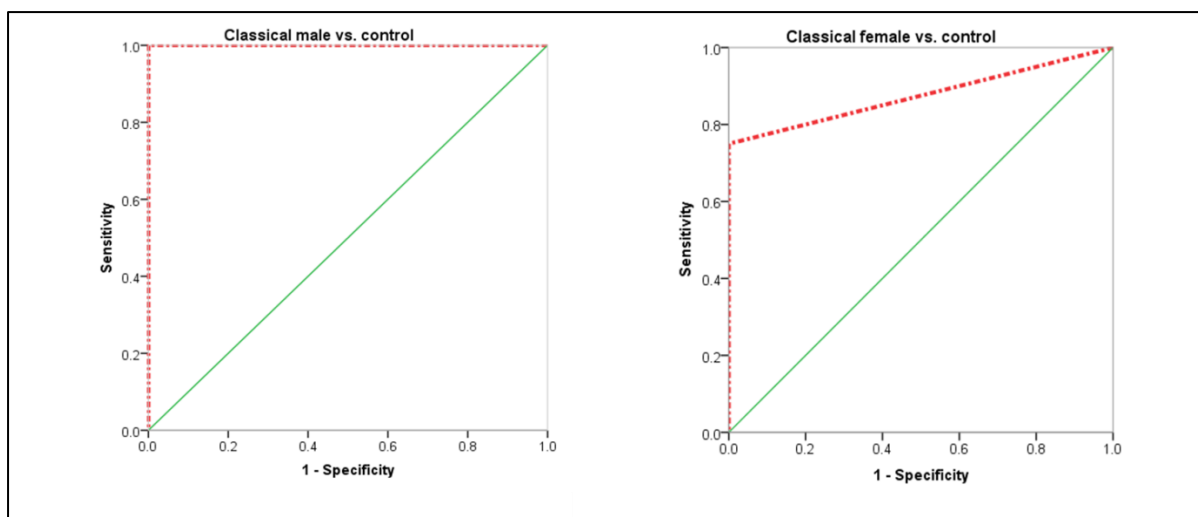


Figure 5.3 ROC analysis for diagnostic accuracy of urinary Lyso-Gb3 to predict Fabry disease in classical Fabry patients: Urinary Lyso-Gb3 levels in hemizygous classical Fabry males vs. control males (**left panel**), reference line is in green. ROC curve urinary Lyso-Gb3 levels in untreated heterozygous classical Fabry females vs. control females (**right panel**), reference line is in green. Graphs were generated using SPSS software.

5.3.3 Urinary Lyso-Gb3 levels in cardiac variant Fabry patients

A total of 22 Fabry patients for whom we have urinary samples have a diagnosis of cardiac variant. The diagnosis was confirmed by mutation analysis of the *GLA* gene. Among this group, 19 patients were shown to harbor a sequence change (N215S) in the α -gal-A gene (an established mutation in cardiac FD variants). The other 3 patients had sequence changes of R118C, A301G and R301Q in the *GLA* protein. Urinary Lyso-Gb3 levels in cardiac variant Fabry patients [(mean \pm SEM = 16.9 pmol/mmol creatinine \pm 4.9) with range of (0.0-74.9 pmol/mmol creatinine)] were significantly lower than the levels in classical Fabry patients [(mean \pm SEM = 105.8 pmol/mmol creatinine \pm 20.8) with range of (0.0-320.4 pmol/mmol creatinine), p value < 0001] (Figure 5.1). The average urinary Lyso-Gb3 concentration in cardiac variant Fabry males (n = 11, mean \pm SEM = 14.5 pmol/mmol creatinine \pm 5.7) was significantly higher than in control males [(n = 22, mean \pm SEM = 0.0 pmol/mmol creatinine \pm 0.0), p value < 0.0001]. Moreover, cardiac variant Fabry Females showed significantly

higher levels of urinary Lyso-Gb3 [(n = 11, mean ± SEM = 21.0 pmol/mmol creatinine ± 9.9)] compared to control females [(n = 26, mean ± SEM = 0.0 pmol/mmol creatinine ± 0.0), *p* value < 0.0001]. Nevertheless, there was no significant variation in the average concentration of urinary Lyso-Gb3 between the two genders of cardiac variant Fabry patients (*p* value > 0.05) (Figure 5-4).

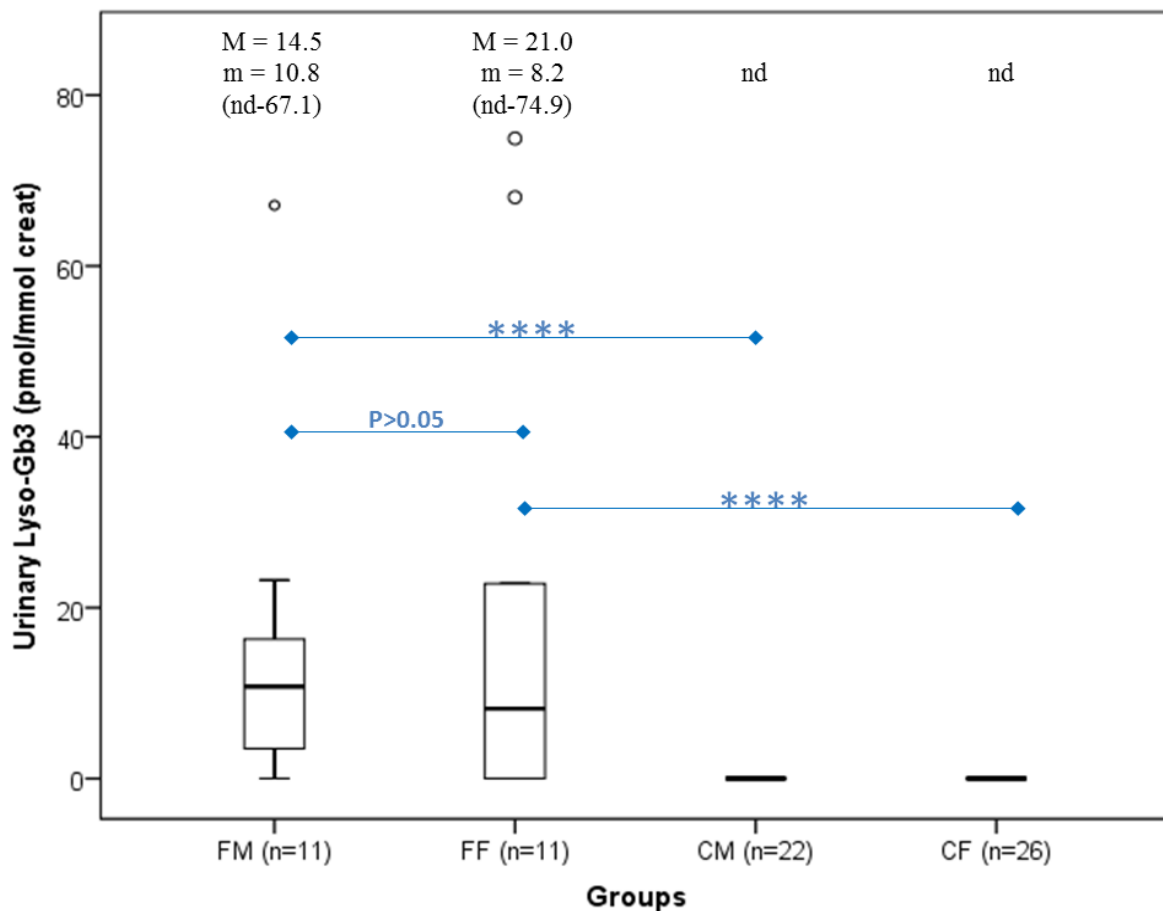


Figure 5.4: Urinary Lyso-Gb3 levels in cardiac variant Fabry patients. Data were pooled from the patients and control subjects and presented as box plots. The concentrations were measured using Lyso-Gb3 calibration curve. Data were analysed using SPSS. The subjects were divided to 4 groups as follows: Fabry male (FM), Fabry female (FF), control male (CM) and control female (CF). For each group: n = number of subject; M = Mean; m = median; the range of minimum and maximum values in brackets; box= maximum and minimum quartiles; line inside the box = median; whiskers = maximum and minimum non-outlier values; o = outlier values. Various groups were compared against each other using Mann-Whitney U tests. The differences were considered statistically significant if the *p* value < 0.05 (****=*p*<0001).

ROC analysis performed to assess the diagnostic accuracy of urinary Lyso-Gb3 levels in diagnosing cardiac variant FD in males. Urinary Lyso-Gb3 levels in 6 confirmed untreated cardiac variant Fabry males and 22 control males were assessed. ROC curve showed a statistically significant result with AUC (0.833, p value <0.05). Urinary Lyso-Gb3 value equal to or higher than 1.4 pmol/mmol creatinine indicated the presence of FD with a sensitivity of 67% and specificity of 100% (Figure 5.5).

The diagnostic accuracy of urinary Lyso-Gb3 levels in diagnosing cardiac variant FD in females was tested. Urinary Lyso-Gb3 levels in 7 confirmed untreated cardiac variant Fabry females and 26 control females were assessed. ROC curve showed a statistically significant result with AUC (0.857, p value <0.01). Urinary Lyso-Gb3 levels equal to or higher than 2.6 pmol/mmol creatinine indicated the presence of FD with a sensitivity of 71% and specificity of 100% (Figure 5.5).

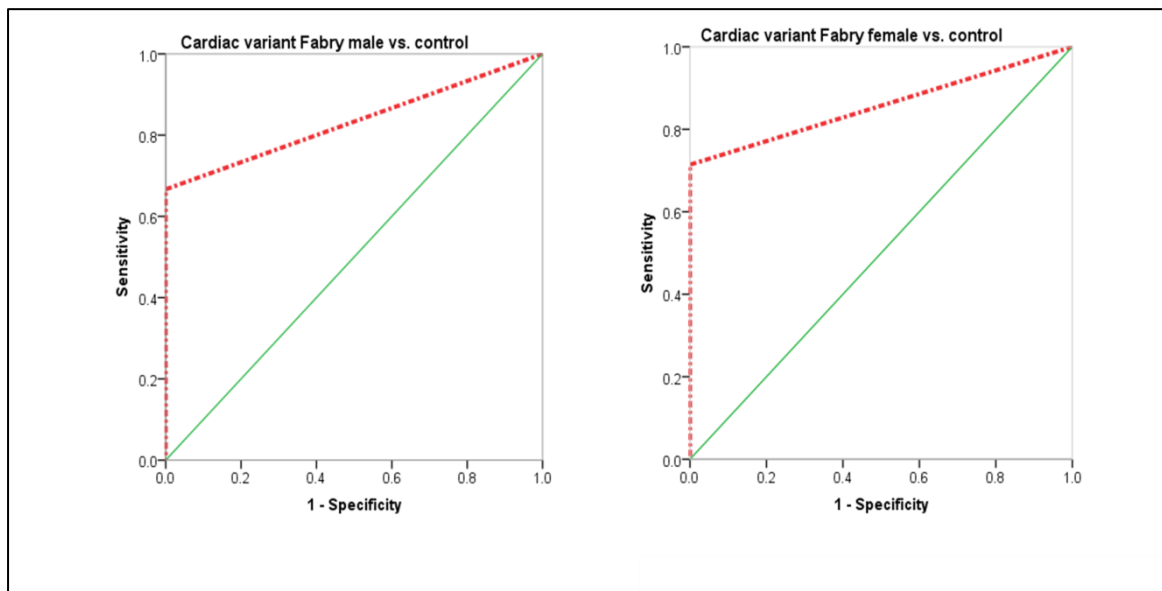


Figure 5.5: ROC analysis for diagnostic accuracy of urinary Lyso-Gb3 to predict Fabry disease in cardiac variant Fabry patients: urinary Lyso-Gb3 levels in hemizygous cardiac variant Fabry males vs. control males (**left panel**). ROC curve urinary Lyso-Gb3 levels in heterozygous cardiac variant Fabry females vs. control females (**right panel**). Graphs were generated using SPSS software.

5.3.4 The levels of urinary Lyso-Gb3 analogues in classical Fabry patients

The relative concentration of various urinary Lyso-Gb3 analogues was analysed in our cohort of classical Fabry patients. This data is presented in the form of box plots (Figure 5.6). This comparison included both male and female classical Fabry patients and normal control subjects. As shown in Figure 4.6, the relative concentrations of Lyso-Gb3 analogues varied considerably in classical Fabry males. While the levels of Lyso-Gb3 (+16 Da) analogue were found at the highest concentration (77.2-1607.7 pmol/mmol creatinine), levels of Lyso-Gb3 (+34 Da) analogue were somewhat lower (12.2-698.6 pmol/mmol creatinine), while levels of Lyso-Gb3 (-2 Da) analogue varied between (33.8-231.8 pmol/mmol creatinine). The concentrations of Lyso-Gb3 (+50 Da) ranged between (0-62.1 Da), while the levels of Lyso-Gb3 (-12 Da), Lyso-Gb3 (-28 Da) and Lyso-Gb3 (+14 Da) analogues varied between (0-41 pmol/mmol creatinine).

In all cases, the levels of each analogue were undetectable in control male and female subjects. Again, differences were observed in the levels of all Lyso-Gb3 analogues between male and female classical Fabry patients, with female Fabry patients having concentrations that were marginally elevated compared to control subjects (Figure 5.6).

The relative distribution of urinary Lyso-Gb3 and its various analogues was analysed in our cohort of classical Fabry patients. This data is presented in the form of a pie chart (Figure 5.7). In both male and female Fabry patients, the levels of urinary Lyso-Gb3 constituted only small percentage of Lyso-Gb3 (males 12% vs females 21%) while the large remaining amounts were distributed between Lyso-Gb3 analogues which arise through modifications of the sphingosine moiety.

The levels of Lyso-Gb3 (+16 Da) constituted the highest percentage of the total measured metabolites (males 44% vs females 36%). While the levels of Lyso-Gb3 (+34 Da) were

approximately two fold lower (males 25% vs females 19%), the analogue Lyso-Gb3 (-2 Da) was somewhat lower with relative percentages of 13% for males and 15% for females.

However, the levels of Lyso-Gb3 (+50 Da), Lyso-Gb3 (-28 Da), Lyso-Gb3 (+14 Da) and Lyso-Gb3 (-12 Da) were broadly similar in both male and female cases, comprising 1% and 7% for Lyso-Gb3 (+50 Da), 2% for Lyso-Gb3 (-28 Da), 2% and 0% for Lyso-Gb3 (+14 Da), 1% and 0% for Lyso-Gb3 (-12 Da) in males and females respectively.

Figure 5.8 shows ROC analysis of urinary Lyso-Gb3 and its analogues in classical Fabry patients. The results showed that urinary Lyso-Gb3 and the analogues Lyso-Gb3 (-28 Da, -2 Da, +14 Da, +16Da and +34 Da), in addition to the total pool of Lyso-Gb3, all showed high sensitivity and specificity. As such, they may constitute a useful diagnostic predictor for FD in males. Unlike the case for Fabry males, only the total pool of urinary Lyso-Gb3 and analogues showed high sensitivity and specificity in Fabry females. In this case, analysis of the total pool of Lyso-Gb3 can be used as a diagnostic predictor for FD in females. While control subjects did not show detectable levels of urinary Lyso-Gb3 analogues, caution should be taken with regards to their use individually for diagnostic purposes due to their low abundance and variability between Fabry patients.

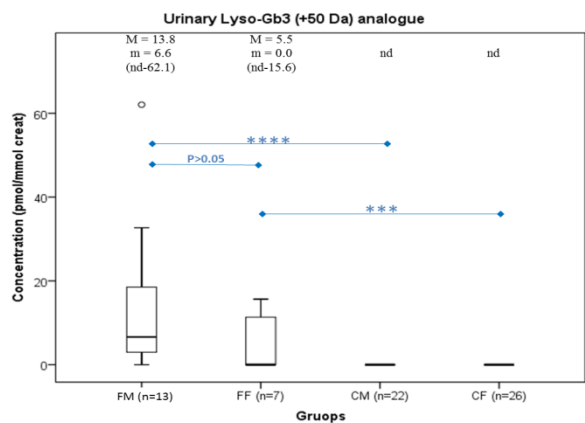
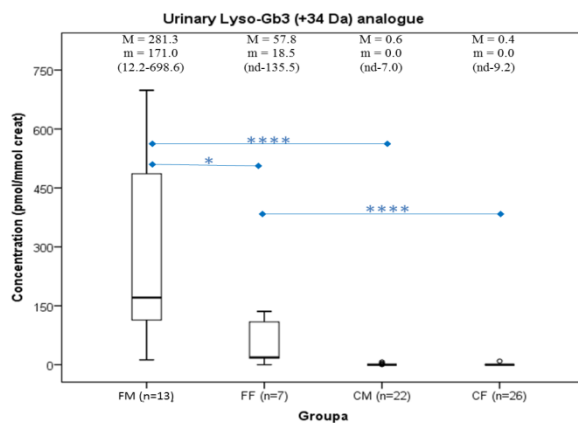
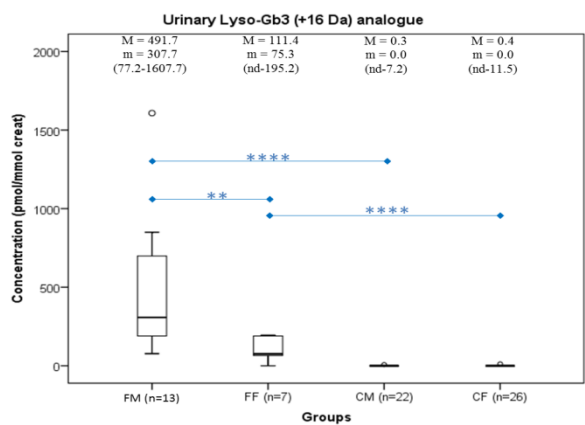
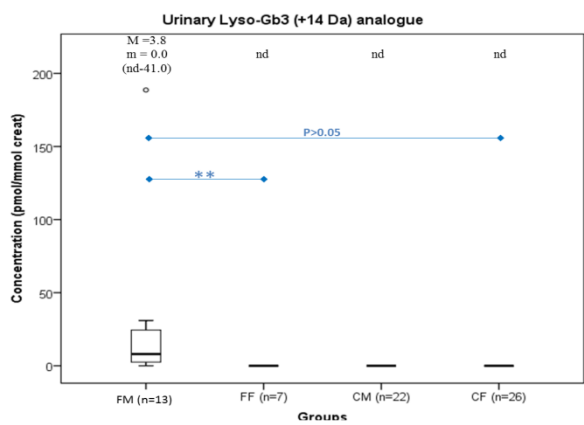
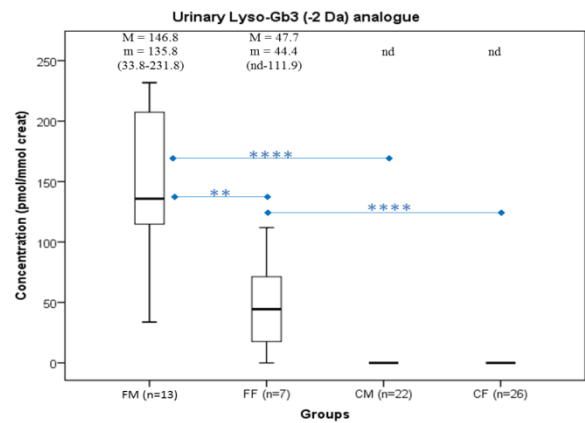
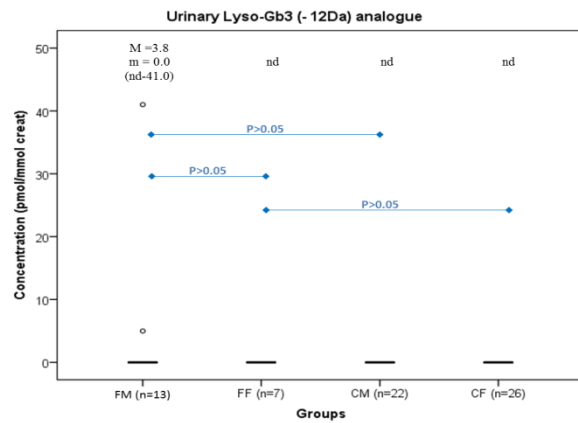
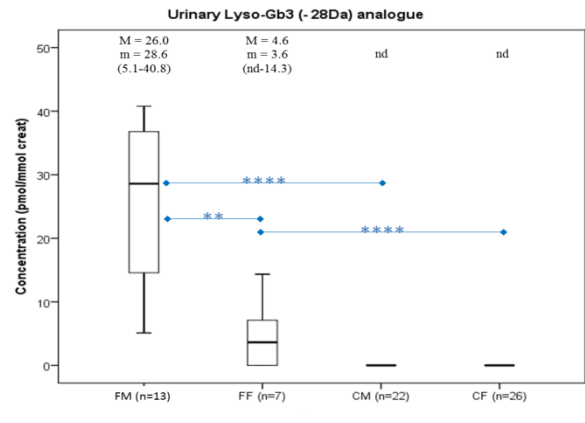
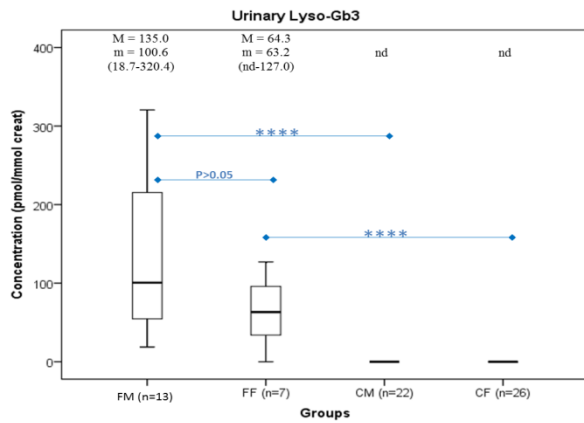


Figure 5.6: The levels of urinary Lyso-Gb3 analogues in classical Fabry patients. Data were pooled from the patients and control subjects and presented as box plots. The concentrations were measured using Lyso-Gb3 calibration curve. Data were analysed using SPSS. The subjects were divided to 4 groups as follows: Fabry male (FM), Fabry female (FF), control male (CM) and control female (CF). For each group: n = number of subject; M = Mean; m = median; the range of minimum and maximum values in brackets; box= maximum and minimum quartiles; line inside the box = median; whiskers = maximum and minimum non-outlier values; o = outlier values. Various groups were compared against each other using Mann-Whitney U tests. The differences were considered statistically significant if the *p* value < 0.05 (*=*p*<0.05, **=*p*<0.01, ***=*p*<0.001 and ****=*p*<0.0001).

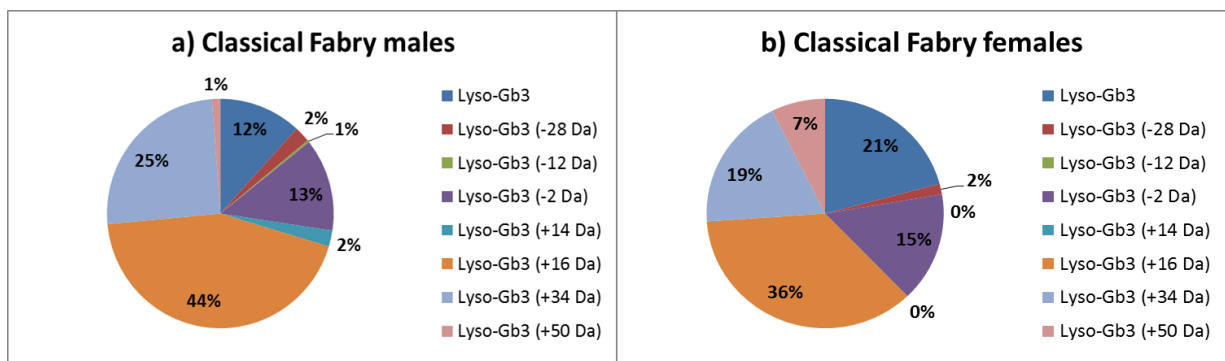


Figure 5.7: The relative distribution of urinary Lyso-Gb3 and its analogues in classical Fabry patients. Classical Fabry males (n=13, a) and in classical Fabry females (n=7, b). All classical Fabry males and females showed low percentage of Lyso-Gb3 in their urine compared to the total measured metabolites. Nonetheless, larger amounts were converted to Lyso-Gb3 analogues attributed to modifications in the sphingosine moiety.

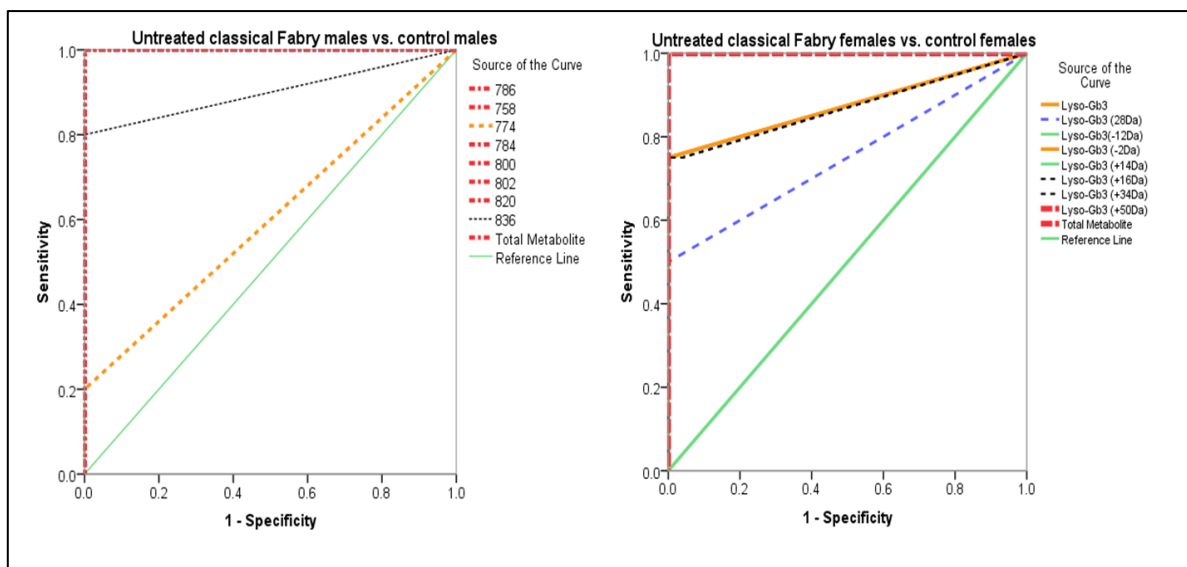


Figure 5.8: ROC analysis for diagnostic accuracy of urinary Lyso-Gb3 analogues to predict Fabry disease in classical Fabry patients: untreated hemizygous classical Fabry males vs. control males (left panel). Untreated heterozygous classical Fabry females vs. control females (right panel). Graphs were generated using SPSS software.

In order to test the utility of using total urinary Lyso-Gb3 and its analogue as a biomarker for the diagnosis and monitoring FD, the total metabolites has been used and the statistical variations between the various groups have been measured (Figure 5-9).

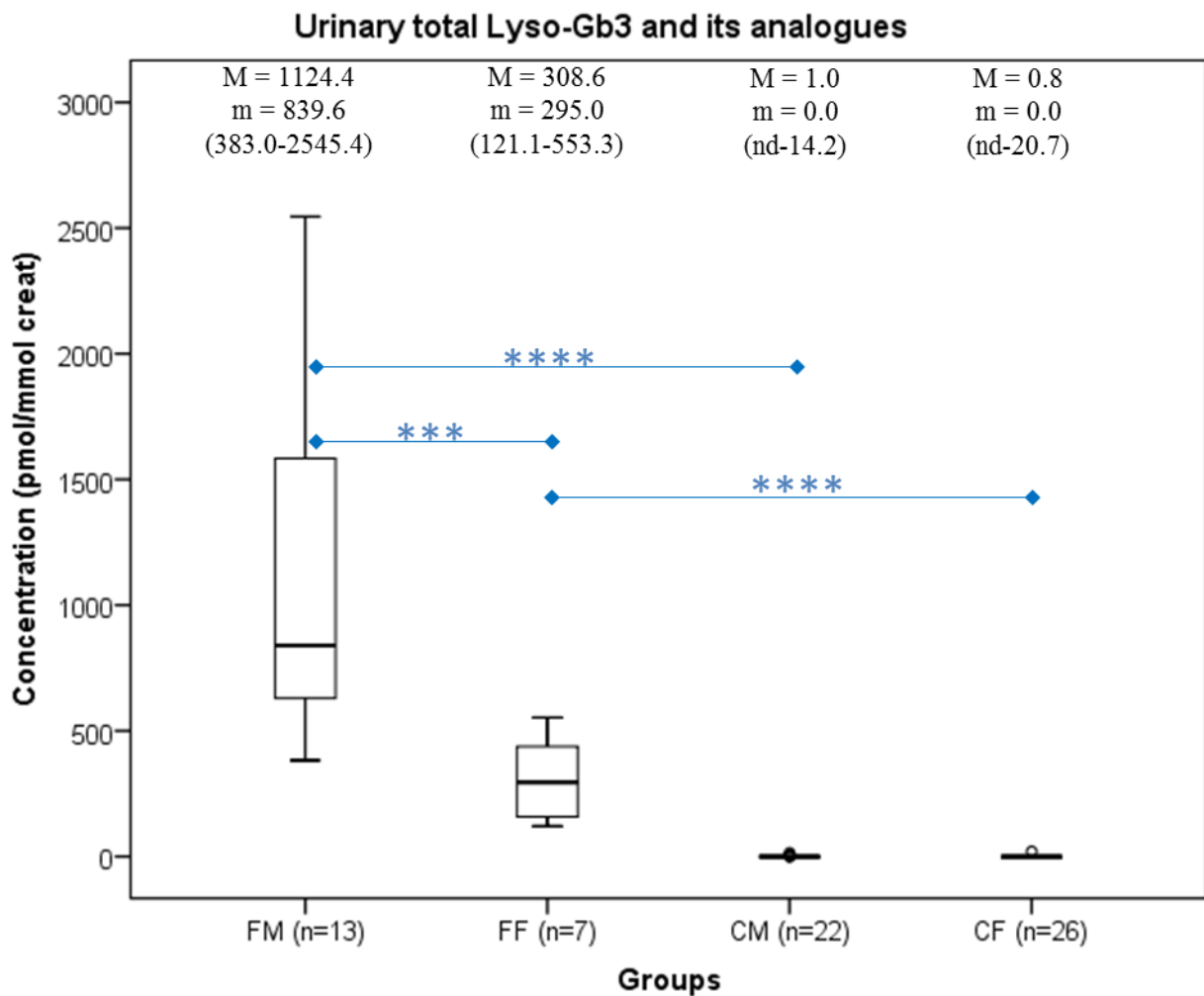


Figure 5.9: The levels of urinary total Lyso-Gb3 and its analogues in classical Fabry patients. Data were pooled from the patients and control subjects and presented as box plots. The concentrations were measured using Lyso-Gb3 calibration curve. Data were analysed using SPSS. The subjects were divided to 4 groups as follows: Fabry male (FM), Fabry female (FF), control male (CM) and control female (CF). For each group: n = number of subject; M = Mean; m = median; the range of minimum and maximum values in brackets; box= maximum and minimum quartiles; line inside the box = median; whiskers = maximum and minimum non-outlier values; o = outlier values. Various groups were compared against each other using Mann-Whitney U tests. The differences were considered statistically significant if the p value < 0.05 (***) = $p < 0.001$ and (****) = $p < 0.0001$.

5.3.5 Levels of urinary Lyso-Gb3 analogues in cardiac variant Fabry patients

Building on the analysis outlined above, I set out to determine the levels of various urinary Lyso-Gb3 analogues in cardiac variants Fabry patients. Again, this comparison included both male and female Fabry patients carrying the cardiac variant mutations and normal control subjects. As shown in Figure 5.10, the average concentration of each Lyso-Gb3 analogue varied considerably and, compared to classical Fabry patients were an order of magnitude lower. The levels of Lyso-Gb3 (+34 Da) were found at the highest concentration (0.0-355.5 pmol/mmol creatinine) for males and (7.0-181.1 pmol/mmol creatinine) for females, while levels of Lyso-Gb3 (+16 Da) were somewhat lower (20.6-246.2 pmol/mmol creatinine) for males and (0.0-148.3 pmol/mmol creatinine) for females. The levels of Lyso-Gb3 (-28 Da) were very low and ranged between (0.0-38.0 pmol/mmol creatinine). However, the analogues Lyso-Gb3 (+50 Da), Lyso-Gb3 (+14 Da) and Lyso-Gb3 (-12 Da) were virtually undetectable. In all cases, the levels of each analogue were undetectable in control male and female subjects. Unlike classical Fabry patients, there was less of a distinction between male and female Fabry patients carrying the cardiac variant on the levels of all Lyso-Gb3 analogues. Collectively, the observed levels of urinary Lyso-Gb3 and its analogues in both genders of cardiac variant Fabry patients were very low.

The relative distribution of urinary Lyso-Gb3 and its various analogues was examined in our cohort of Fabry patients diagnosed with the cardiac variants. This data is presented in the form of a pie chart (Figure 5.11). In keeping with findings from classical Fabry patients, the levels of urinary Lyso-Gb3 constituted small percentage from the total observed metabolites in both male and female patients (males 6% vs. females 7%). However, the large percentage of Lyso-Gb3 was converted to its analogues. These analogues were detected in both male and female cases at broadly similar levels, comprising 31% and 19% for Lyso-Gb3 (+34 Da); 40% and 19% for Lyso-Gb3 (+16 Da), 7% and 43% for Lyso-Gb3 (+50 Da), 11% and 6% for

Lyso-Gb3 (-2 Da), 4% for Lyso-Gb3 (+14 Da), 1% for Lyso-Gb3 (-28 Da), 0% and 1% for Lyso-Gb3 (-12 Da) in males and females respectively.

Figure 5.12 shows ROC analysis of urinary Lyso-Gb3 and its analogues in cardiac variant Fabry patients. The results show that urinary Lyso-Gb3 analogues (-2 Da, +16 Da, +34 Da and the total pool of Lyso-Gb3 analogues) showed high sensitivity and specificity as a diagnostic predictor for cardiac variant Fabry males. However, in the case of Fabry females, only the total pool of urinary Lyso-Gb3 analogues showed high sensitivity and specificity, suggesting its utility as a diagnostic predictor for cardiac variant Fabry females. While control subjects did not show detectable levels of urinary Lyso-Gb3 analogues, caution should be taken with regards to their use individually for diagnostic purposes due to their low abundance and variability between Fabry patients.

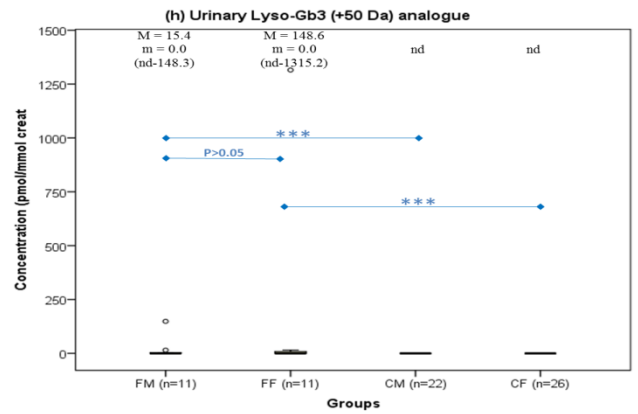
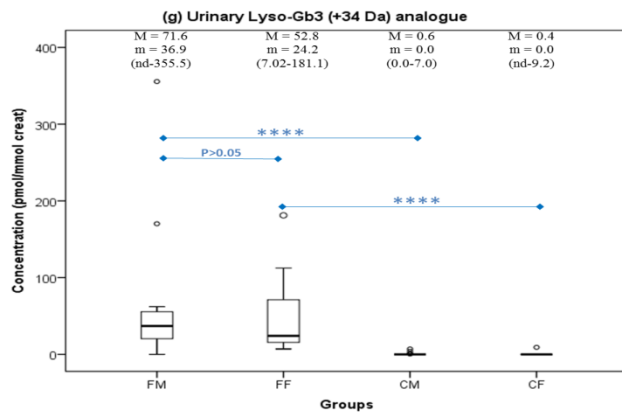
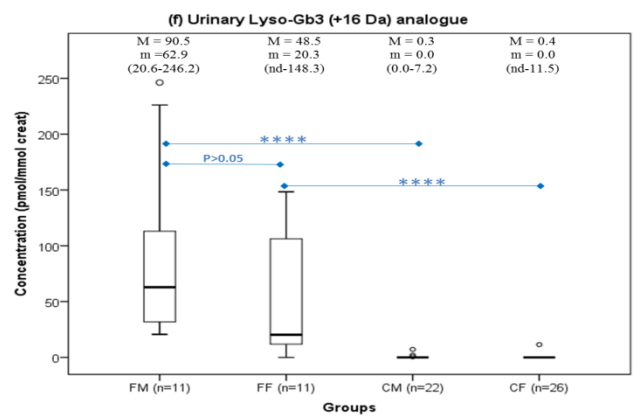
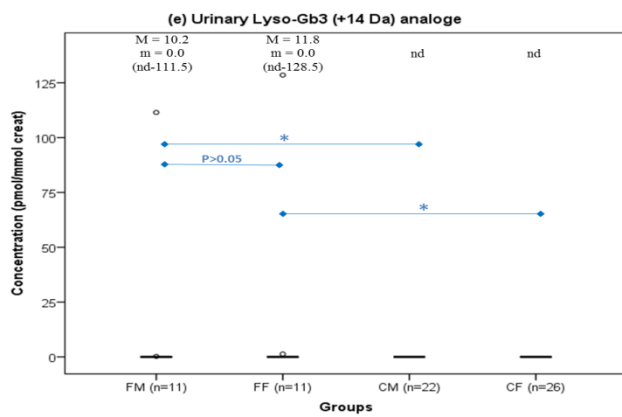
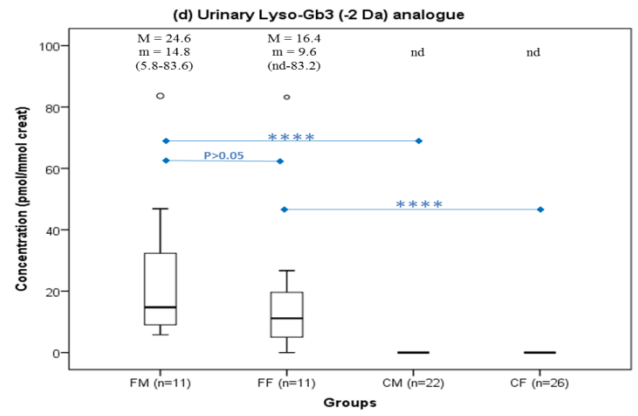
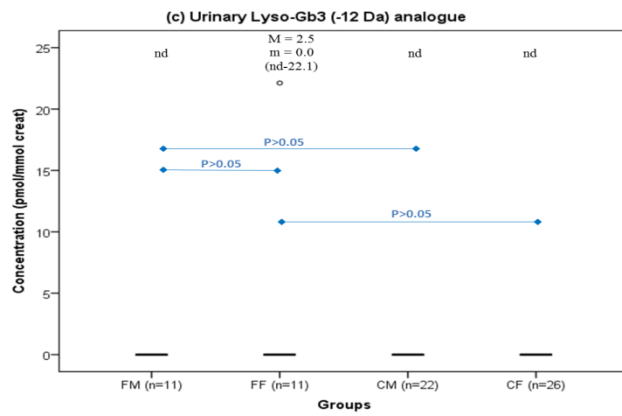
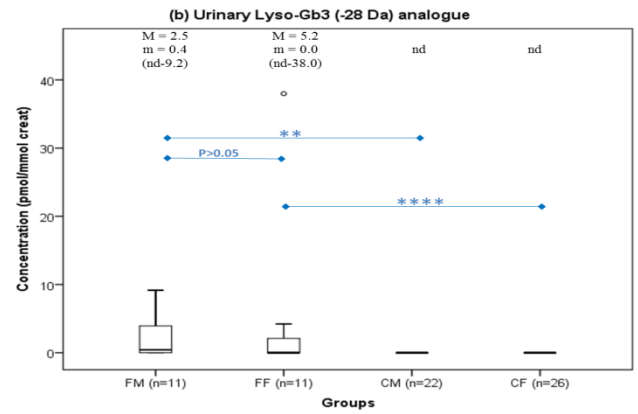
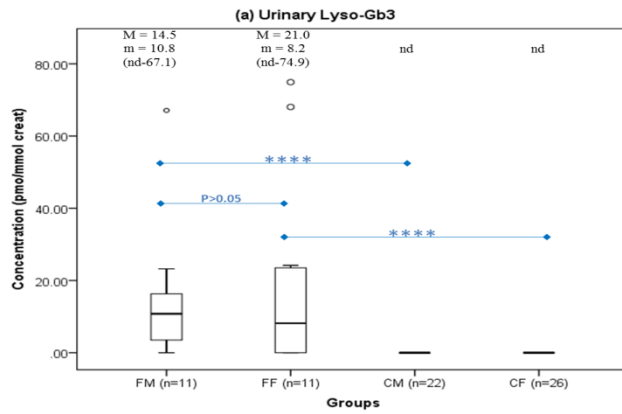


Figure 5.10: Urinary Lyso-Gb3 analogues levels in cardiac variant Fabry patients: Data were pooled from the patients and control subjects and presented as box plots. The concentrations were measured using Lyso-Gb3 calibration curve. Data were analysed using SPSS. The subjects were divided to 4 groups as follows: Fabry male (FM), Fabry female (FF), control male (CM) and control female (CF). For each group: n = number of subject; M = Mean; m = median; the range of minimum and maximum values in brackets; box= maximum and minimum quartiles; line inside the box = median; whiskers = maximum and minimum non-outlier values; o = outlier values. Various groups were compared against each other using Mann-Whitney U tests. The differences were considered statistically significant if the *p* value < 0.05 (**=*p*<0.01, ***=*p*<0.001 and ****=*p*<0.0001).

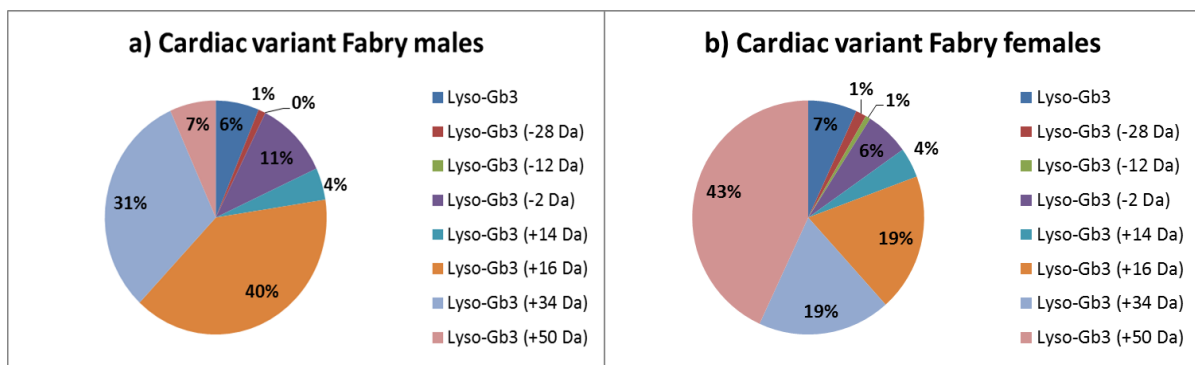


Figure 5.11: The relative distribution of urinary Lyso-Gb3 and its analogues in cardiac variant Fabry patients. (a) Cardiac variant Fabry males (n=11). **(b)** Cardiac variant Fabry females (n=11). Both cardiac variant Fabry males and females showed a low percentage of Lyso-Gb3 in their plasma compared to the total measured metabolites. Nevertheless, larger amounts of Lyso-Gb3 analogues were observed.

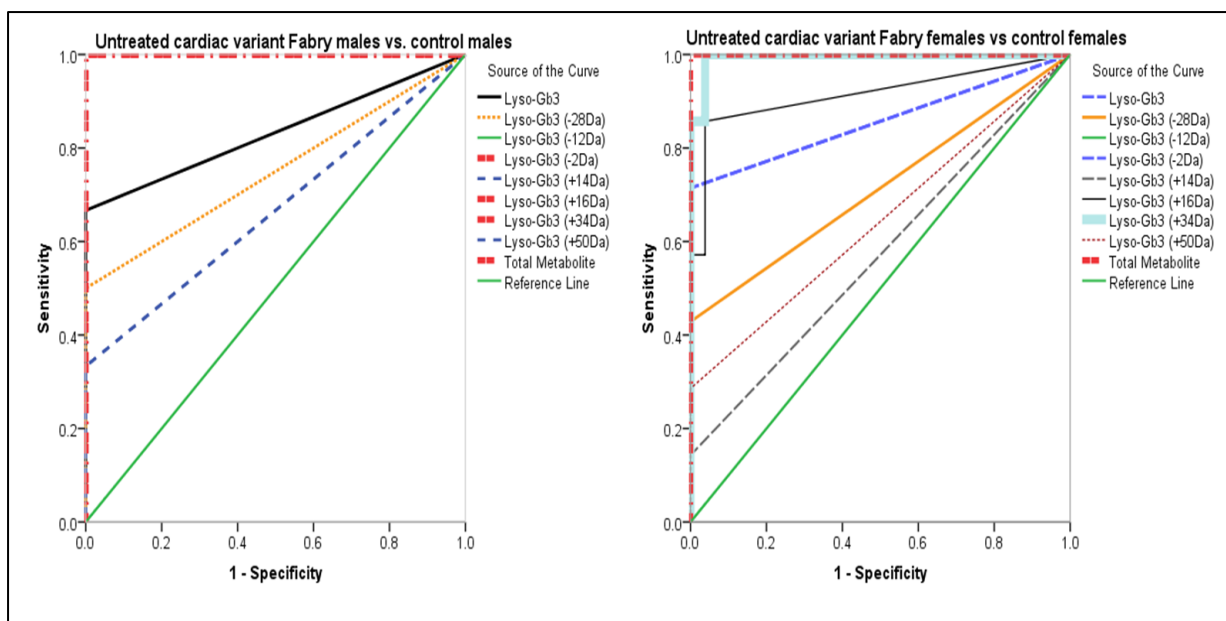


Figure 5.12: ROC analysis for diagnostic accuracy of urinary Lyso-Gb3 analogues to predict Fabry disease in cardiac variant Fabry patients: untreated hemizygous cardiac Fabry males vs. control males (left panel); untreated heterozygous cardiac Fabry females vs. control females (right panel). Graphs were generated using SPSS software.

In order to test the utility of using total urinary Lyso-Gb3 and its analogue as a biomarker for the diagnosis and monitoring FD, the total metabolites has been used and the statistical variations between the various groups have been measured (Figure 5-13).

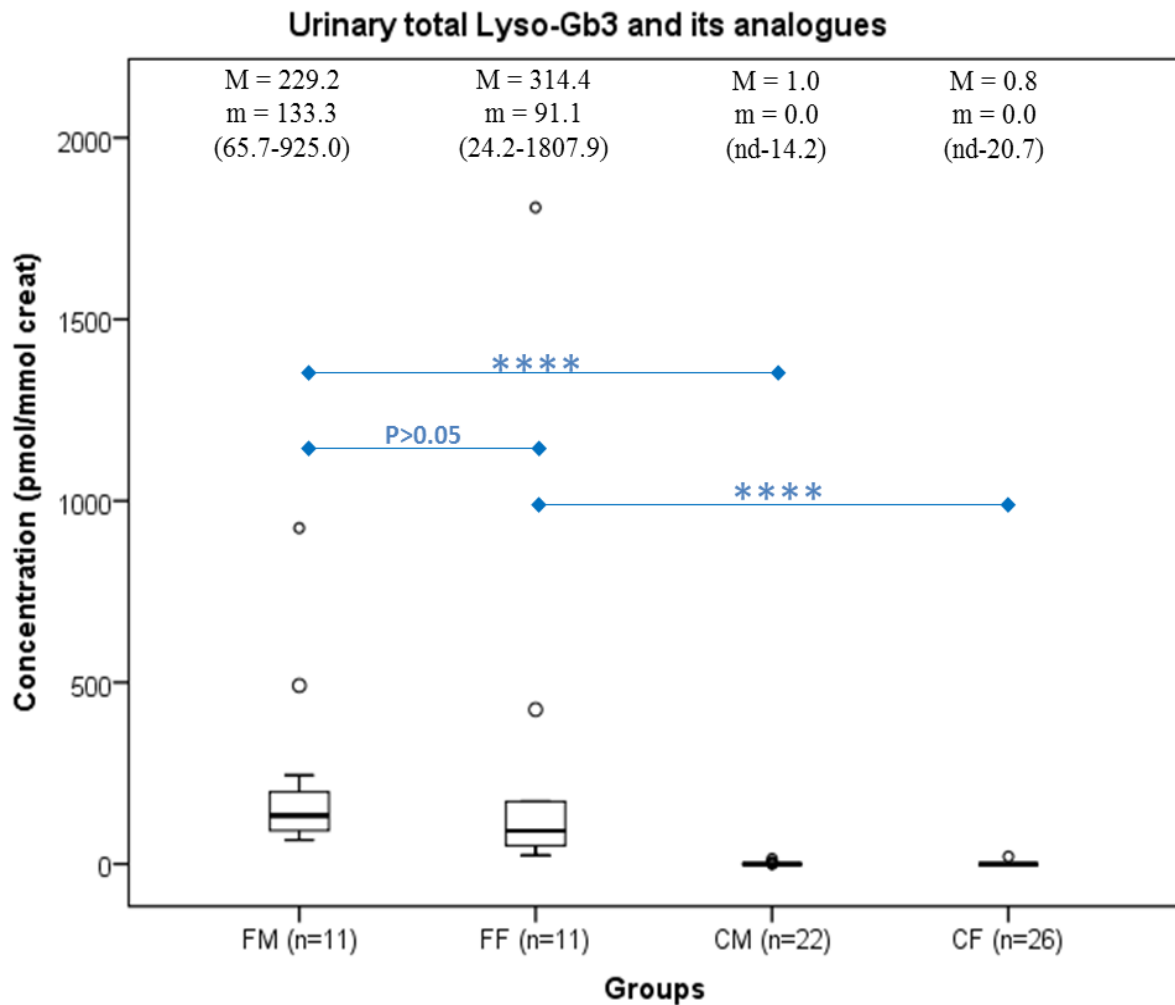


Figure 5.13 The levels of total urinary Lyso-Gb3 and its analogues in cardiac variant Fabry patients. Data were pooled from the patients and control subjects and presented as box plots. The concentrations were measured using Lyso-Gb3 calibration curve. Data were analysed using SPSS. The subjects were divided to 4 groups as follows: Fabry male (FM), Fabry female (FF), control male (CM) and control female (CF). For each group: n = number of subject; M = Mean; m = median; the range of minimum and maximum values in brackets; box= maximum and minimum quartiles; line inside the box = median; whiskers = maximum and minimum non-outlier values; o = outlier values. Various groups were compared against each other using Mann-Whitney U tests. The differences were considered statistically significant if the *p* value < 0.05 (**** = *p*<0.0001).

5.3.6 Correlation between the levels of urinary Lyso-Gb3 and its various analogues in Fabry patients

We next set out to explore whether the urinary levels of Lyso-Gb3 analogues correlated with Lyso-Gb3 levels in Fabry patients. There is no correlation observed between most of the levels of urinary Lyso-Gb3 analogues and the levels of Lyso-Gb3. This is due to the low abundance of these metabolites in urine samples. In all cases, the correlations didn't reach the statistical significance except with two analogues: Lyso-Gb3 (-28 Da) and Lyso-Gb3 (-2 Da) with Pearson correlation p values <0.01 and <0.0001 respectively (Figure 5.14).

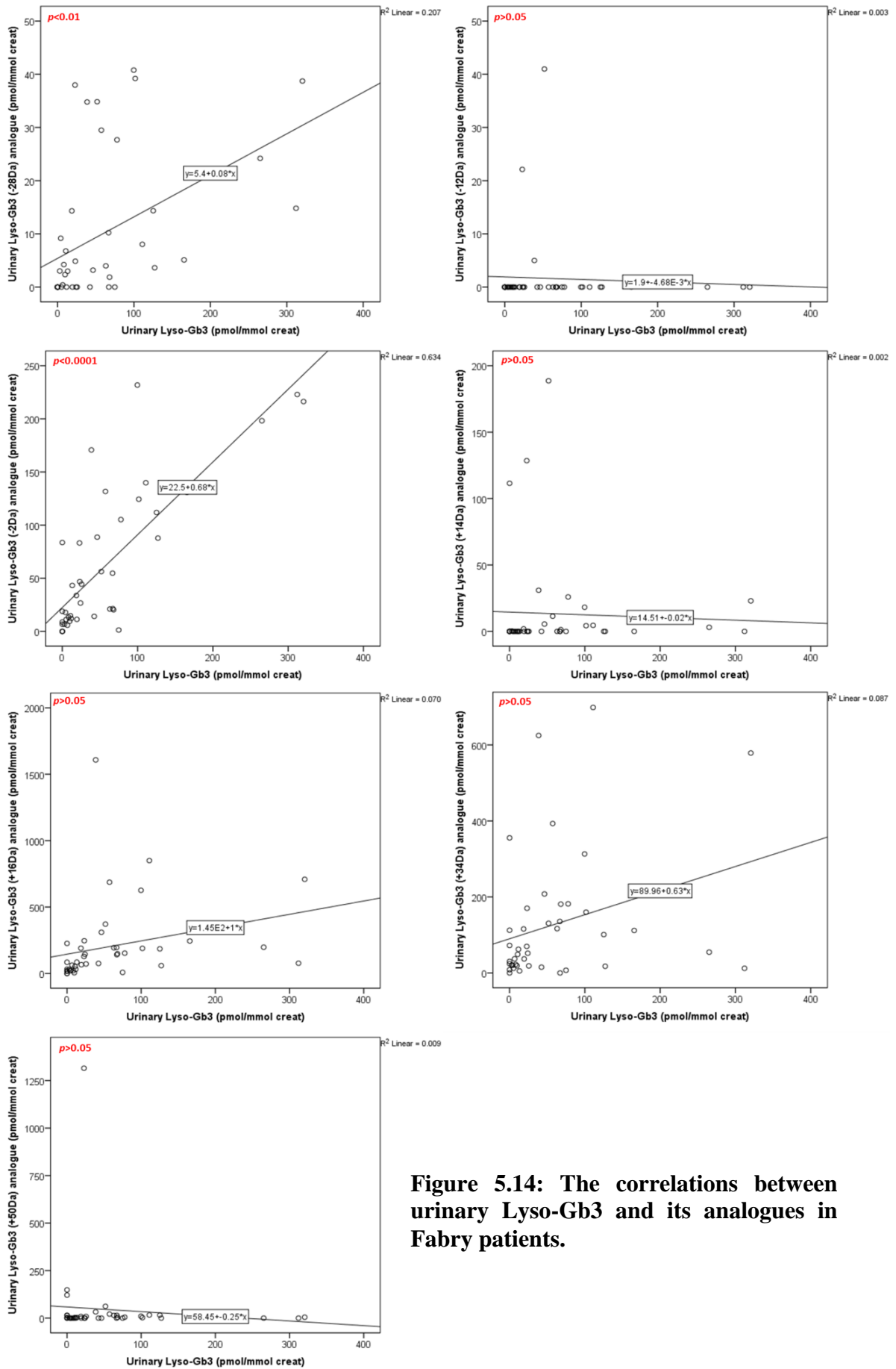


Figure 5.14: The correlations between urinary Lyso-Gb3 and its analogues in Fabry patients.

5.3.7 Correlation between the levels of urinary Lyso-Gb3 and plasma Lyso-Gb3 in Fabry patients

We next set out to explore whether the concentrations of urinary Lyso-Gb3 correlated with the levels of Lyso-Gb3 in plasma samples from the same individuals Fabry patients and at the same collection time point. There were 38 Fabry patients have dual biological samples (plasma and urine) at the same date and time point. Due to a large amount of the urinary Lyso-Gb3 being converted to various analogues, we applied two ways of comparison. Firstly, we compared the levels of Lyso-Gb3 in plasma and urine samples (Figure 5.15). Secondly, we compared the sum of the measured metabolites (Lyso-Gb3 plus its analogues) in both biological samples (Figure 5.16). Both analyses showed a significant correlation between the levels in plasma and urine. As expected the correlation between the total metabolites in plasma and urine was stronger (Pearson correlation p values <0.0001) than the correlation using Lyso-Gb3 levels only (Pearson correlation p values <0.01).

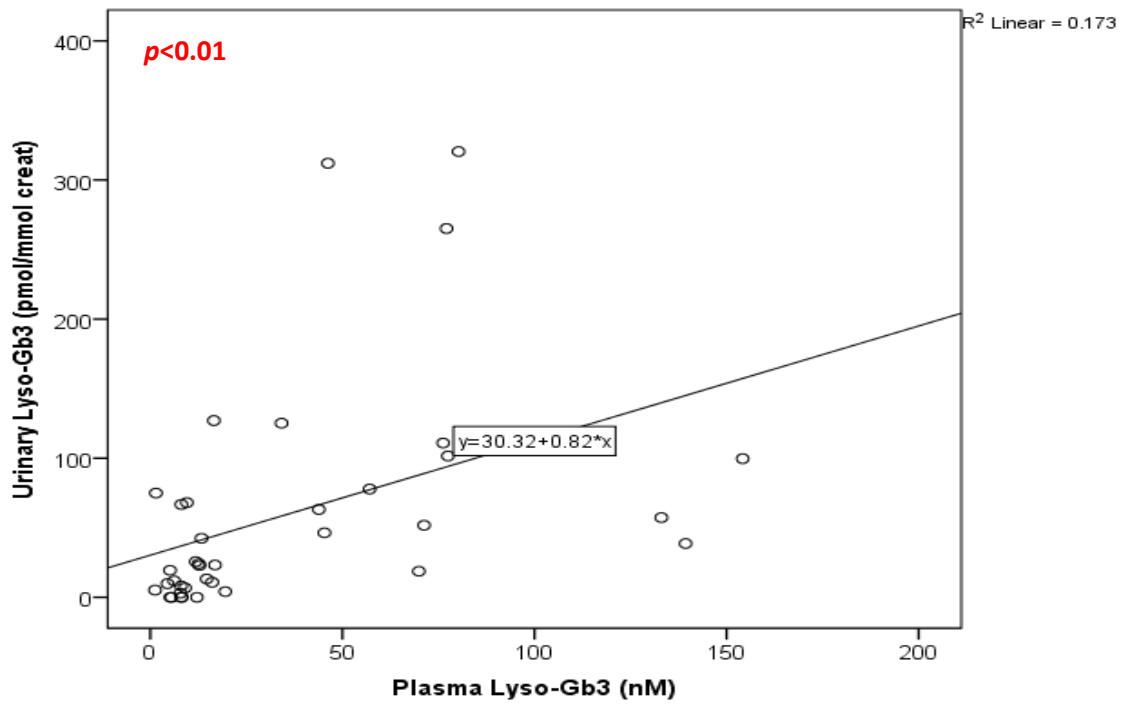


Figure 5.15 Correlation between Lyso-Gb3 levels in plasma and urine samples in Fabry patients.

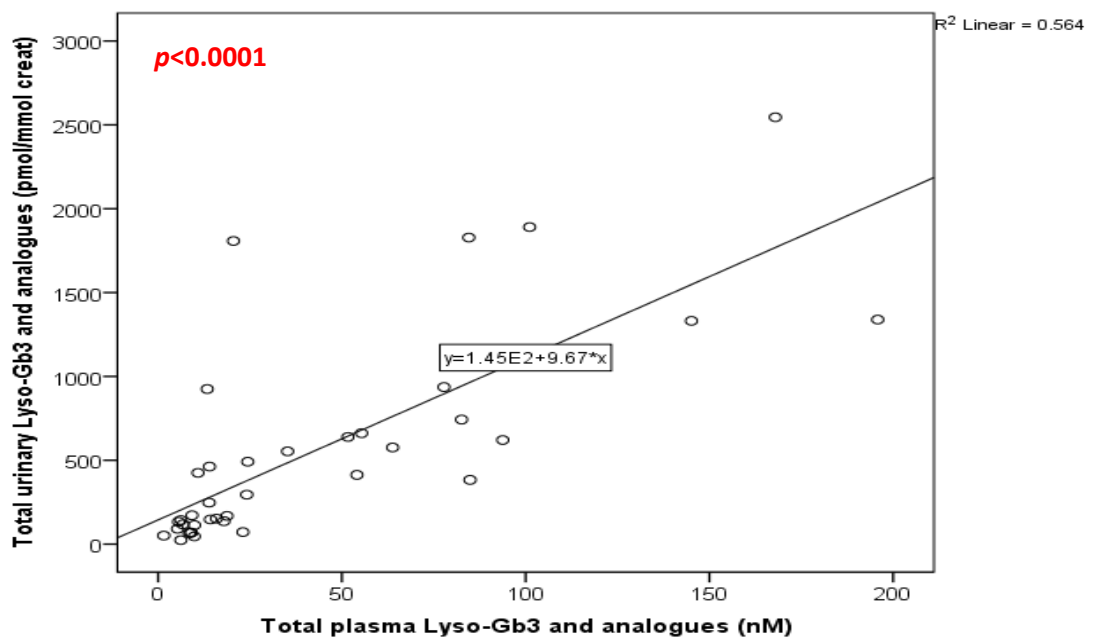


Figure 5.16 Correlation between the levels of total Lyso-Gb3 and its analogues in plasma and urine samples in Fabry patients.

5.4 DISCUSSION

Lyso-Gb3 has been detected in both the plasma [46] and urine [202] of Fabry patients. It had shown that the analysis of Lyso-Gb3 in urine samples from Fabry patients is possible despite the low abundance of Lyso-Gb3 and its various analogues in urine compared to their abundance in plasma [46, 202].

Using metabolomics approaches, a Canadian group has developed assays to measure the levels of Lyso-Gb3 and its various analogues in urine samples from Fabry patients [85, 202, 225]. In a study performed by Auray-Blais and colleagues [85] LC-MS/MS was employed to measure the levels of urinary Lyso-Gb3 and analogues in Fabry patients. This analysis constituted a major technical advancement in the field as it proved to be highly specific, very sensitive and produced highly reproducible results. This study identified seven novel Lyso-Gb3 analogues (Lyso-Gb3 (-28 Da); Lyso-Gb3 (-12 Da); Lyso-Gb3 (-2 Da); Lyso-Gb3 (+14 Da); Lyso-Gb3 (+16 Da); Lyso-Gb3 (+34 Da), and Lyso-Gb3 (+50 Da). Fragmentation studies using MS/MS confirmed that these analogues were structurally similar to Lyso-Gb3 but had modifications to the sphingosine moiety.

Using the LC-MS/MS protocol developed as a part of the current study, we attempted to quantify the levels of Lyso-Gb3 and its related analogues in urine samples taken from our cohort of classical and cardiac variant Fabry patients, comparing these to control individuals. Using this technique, we proved that it was possible to quantify the amounts of Lyso-Gb3 in urine samples from classical Fabry patients. Moreover, this protocol was sufficiently accurate and sensitive to differentiate between classical Fabry patients, cardiac variant Fabry patients and control subjects. Findings generated as part of this study are in broad agreement with those reported by Auray-Blais and colleagues [85, 202, 225] who have used similar approaches to quantify Lyso-Gb3 and its analogues as a disease-specific biomarkers in FD.

Significant differences in the levels of urinary Lyso-Gb3 were observed between classical and cardiac variant Fabry patients. Despite the later showed levels approximately six fold lower than the levels in classical Fabry patients, the amounts of Lyso-Gb3 in urine samples from cardiac variant Fabry patients were sufficiently high to differentiate this cohort of Fabry patients from control individuals.

Our results showed a significant correlation between the levels of urinary Lyso-Gb3 in Fabry patients and factors like sex and different phenotypes of Fabry patients. This is in agreement with Auray-Blais and colleagues [85, 201].

Unlike the situation in plasma, where Lyso-Gb3 constituted the major component of the total Lyso-Gb3 pool (Lyso-Gb3 and all analogues), the situation was reversed in urine samples. In plasma, where the concentrations of Lyso-Gb3 constituted about 80% of the total Lyso-Gb3 pool in both classical Fabry male and female patients, this was approximately less than 20% in urine samples. In urine samples, the average concentration of the various Lyso-Gb3 analogues constituted about 80% of the total Lyso-Gb3 pool compared to an average of approximately 20% in plasma samples. In some Fabry patients, urinary Lyso-Gb3 analogues showed a relative distribution reach to 98% from the total Lyso-Gb3 pool. This finding suggests that unlike plasma where measuring Lyso-Gb3 is sufficient, measurements of the total pool of Lyso-Gb3 and its analogues in urine samples, constitutes a better analytical tool for diagnosis.

The results of Lyso-Gb3 and its analogues revealed the presence of distinct species in the urine of both male and female classical Fabry patients, to varying extents, with Lyso-Gb3 (+16 Da), Lyso-Gb3 (+34 Da), Lyso-Gb3 (-2 Da) and Lyso-Gb3 (+50 Da), constituting the most abundant analogues. Again, in all cases, the amounts of various analogues were always higher in male Fabry patients compared to females. In all instances, these comparisons were

found to be highly statistically significant. Our findings are also in broad agreement with a recent study by Lavoie and colleagues [225] who identified the existence of seven novel urinary Lyso-Gb3 analogues Lyso-Gb3 (-28 Da), Lyso-Gb3 (-12 Da), Lyso-Gb3 (-2 Da), Lyso-Gb3 (+14 Da), Lyso-Gb3 (+16 Da), Lyso-Gb3 (+34 Da) and Lyso-Gb3 (+50 Da) in samples collected from classical Fabry patients.

Similar trends were observed in cardiac variant Fabry patients, where distinct analogues were detected in both male and female patients, with Lyso-Gb3 (+16 Da), Lyso-Gb3 (+34 Da), Lyso-Gb3 (-2 Da), Lyso-Gb3 (+14 Da) and Lyso-Gb3 (+50 Da), being the most abundant analogues. In all cases, the levels of specific analogues were higher in samples from male patients compared to females with the exception of Lyso-Gb3 (+50 Da), where broadly similar concentrations were detected. In all cases, these comparisons were found to be highly statistically significant.

All 6 analogues of Lyso-Gb3 which have been detected in plasma from Fabry patients (Chapter 4) were detected in the urine samples of Fabry patients except the analogue Lyso-Gb3 (+18 Da) which was detected in plasma only. Furthermore, the two analogues Lyso-Gb3 (-12 Da) and Lyso-Gb3 (+14 Da) were detected only in urine but not in plasma.

Given the low abundance of Lyso-Gb3 and its associated analogues in urine compared to plasma, coupled with variability in specific analogue detection between patients, we decided to repeat this analysis by quantifying the total pool of Lyso-Gb3 and its analogues in both classical Fabry patients and cardiac variant Fabry patients. In both cases, the combined total of Lyso-Gb3 plus analogues was significantly higher in Fabry patients compared to control subjects. Collectively, these analyses reveal that measurement of the total pool of Lyso-Gb3 and analogues in urine samples is sufficiently specific and sensitive to make an initial

diagnosis of FD in most patients given the complete absence of Lyso-Gb3 in the urine of control subjects.

The levels of total Lyso-Gb3 and its analogues in classical males were higher than levels in controls with no overlap suggesting that this can be used as a promising biomarker alongside plasma Lyso-Gb3 in diagnosis of FD. In classical females, most patients had Lyso-Gb3 well above the control subject but some classical females had undetectable levels like controls. In this case a positive result will add to diagnostic confidence of FD but a negative result does not rule out FD.

In cardiac variant male and female patients, most showed total Lyso-Gb3 levels above control subjects but similar to classical females some cardiac variant patients showed undetectable levels. Hence the use of urinary Lyso-Gb3 has limitations as a diagnostic biomarker in these subgroups.

In conclusion, findings presented in this chapter support the use of urinary Lyso-Gb3 and its analogues as promising biomarkers for the diagnosis of both classical and cardiac variant Fabry patients. The important issue with using urinary Lyso-Gb3 is the need to quantify all the analogues and not just Lyso-Gb3 alone due to the predominance of analogues in urine sample. We did not attempt to establish the clinical significance of any particular analogue due to limited urinary sample size. A further study powered to explore the clinical significance and variability of these analogues across sex and phenotypes of FD may cast a different light on the role of these analogues in diagnosis and monitoring.

CHAPTER - 6

**DEVELOPMENT OF A NOVEL METHOD TO ASSAY
URINARY GB3 AND ASSESSMENT ITS CLINICAL
UTILITY**

Notice: Part of the work presented in this chapter has been published in the Journal of The American Society for Mass Spectrometry [1].

6.1 INTRODUCTION

Fabry disease (FD) is a rare multi-systemic X-linked lysosomal storage disorder. This inborn error of glycosphingolipid metabolism results from the absence or deficiency of α -gal-A, (OMIM 301500) which is responsible for hydrolytic cleavage between the galactose residues in Gb3. The un-metabolised GSLs accumulate in various organs, resulting in a range of pathologies in the kidneys, heart, brain, eye, peripheral nervous system and skin. Male patients show symptoms at an early age while females display symptoms when older. The precise incidence of FD is not known: the reported figures vary widely.

The rarity of the disease and wide spectrum of symptoms displayed by Fabry patients make diagnosis based on clinical manifestation problematic. FD is currently diagnosed by measuring the activity of α -gal-A and/or by mutation analysis of the α -gal-A gene where enzyme analysis may not be informative in females [27, 28, 30, 35, 41, 47, 48]. Enzyme replacement therapy (ERT), using recombinant α -gal-A has become the standard treatment for symptomatic Fabry patients since 2001 [44, 45, 210]. However, the clinical benefit of this expensive treatment is limited, depending on the clinical stage at which therapy is initiated. Gb3 levels in plasma or urine can be used to monitor the efficacy of ERT and as a diagnostic biomarker although with limited sensitivity [26, 89, 160, 201, 207, 269, 270]. Gb3 levels in biological samples decrease considerably within two weeks of starting ERT but later increase in some patients probably due to development of α -gal-A antibodies and do not show a good correlation with effects on clinical symptoms or organ function [89, 160].

Gb3 and its isoforms/analogues have been detected at elevated levels in plasma and urine of Fabry patients using mass spectrometric approaches [26, 201, 207, 212, 213, 271]. Several analytical methods have been reported for urinary Gb3 extraction and analysis. Auray-Blais and co-workers originally used filter paper discs saturated with urine which were dried and Gb3 extracted with methanol and analysed by LC-MS/MS [201]. Later, Kruger and

colleagues [207] combined liquid extraction/protein precipitation and solid phase extraction for urinary Gb3 extraction before LC-MS/MS analysis. Despite being fairly complex and time consuming, these methods both generated invaluable data on urinary Gb3 levels in FD. Recently, an advanced multiplex method has been established by Auray-Blais team where they were successful in measuring the levels of different Gb3 isoforms and analogues using liquid-liquid extraction and LC-MS/MS [26, 212].

In this study we report the development of a rapid, robust assay for Gb3 based on a novel liquid-liquid extraction followed by MALDI-TOF MS and validate it using urine from patients with classical FD and healthy control subjects.

6.2 MATERIALS AND METHODS

6.2.1 Patient samples:

Fabry patients with diagnoses confirmed by mutation analysis and enzyme activity test were enrolled in the study after giving written informed consent (ethical approval reference: 09/H1010/75). Urine samples were collected from 19 classical Fabry patients, 21 cardiac variant Fabry patients and 47 age and gender matched control subjects (patient details are shown in Table 6-1). Urine samples were stored at -80°C until further processing without any centrifugation or filtration. Classical Fabry patients are defined as having typical Fabry disease manifestations and known classical mutations with the deficiency of α -gal-A enzyme activity (<1%). Nonetheless, cardiac variant Fabry patients were identified by having known cardiac variant mutation of *GLA* gene.

6.2.2 Chemicals

HPLC grade water and acetonitrile (ACN) were purchased from (VWR international- UK). Acetone was purchased from (Fisher Scientific-UK). HPLC grade methanol (MeOH, $\geq 99.9\%$), HPLC grade chloroform (CHCl_3 , $\geq 99.9\%$), 5-chloro-2-mercaptobenzothiazole (5C2M), 2-mercaptobenzothiazole, 6-aza-2-thiothymine, 2-(4-hydroxyphenylazo) benzoic acid, 2,5-dihydroxy benzoic acid (DHB), super-DHB, sinapic acid, 2,4,6-trihydroxy acetophenone monohydrate, 2,5-dihydroxy acetophenone, α -cyano-4-hydroxycinnamic acid, picolinic acid and 9-aminoacridine hemihydrate were purchased from (Sigma Aldrich, UK). Porcine Gb3 standard and N-Heptadecanoyl ceramide trihexoside (C17:0) internal standard were purchased from Matreya (Pleasant Gap, PA).

6.2.3 Total Gb3 standard

Total Gb3 (source: porcine RBC) was used for method development and standard curve purposes. Total Gb3 stock solution (200ng/ μl) was prepared by dissolving 10mg of Gb3 standard in 50ml of MeOH then stored at -20°C .

6.2.4 Gb3 internal standard

N-Heptadecanoyl ceramide trihexoside internal standard (0.5 mg) was dissolved in 1ml of MeOH: CHCl_3 (2:1) and then 49ml of MeOH added to generate 50 ml of internal standard at 50ng/ μL . This solution was stored at -20°C in glass until used as 2ng/ μL .

6.2.5 Extraction of urinary Gb3

100 μL each of urine, MeOH containing 200ng of Gb3 internal standard and CHCl_3 were mixed and incubated in a sonicating water bath for 5 min at room temperature. The mixture was then centrifuged at 13000rpm for 5min and 1 μL of the lower (CHCl_3) layer spotted directly onto a stainless steel 384-position MALDI target in triplicate. MALDI-TOF-MS:

Once dry, the samples were overlaid with 1 μ L of 5C2M matrix (saturated solution in 50%MeOH). MS and MS/MS (using laser induced dissociation in LIFT mode) were acquired on a Bruker Ultraflex extreme TOF instrument equipped with a 1 kHz laser in positive ion mode. The MS spectra used for quantitation were the sum of 20,000 laser shots acquired in a random walk pattern. The peak areas used for Gb3 quantitation were extracted from the spectra using ClinproTools software (Bruker).

6.2.6 Gb3 internal standard and assay calibration

N-Heptadecanoyl ceramide trihexoside was used as the internal standard throughout this study. A standard curve was produced by extracting a series of Gb3 dilutions ranging from (0 to 40) ng/ μ L made in depleted urine from a healthy individual containing 2 ng/ μ L internal standard. The depleted urine was prepared by passing through a C18 cartridge and effective removal of Gb3 confirmed by parallel depletion of urine from a classical Fabry patient analyzed by MALDI-MS (Figure 6-1). The 4 most abundant Gb3 species have been used to calculate urinary total Gb3 level. The sodiated molecular ions of Gb3 species are at mass-to-charge ratios (m/z) of: 1130.7 (C22:0), 1146.7 (C22:0-OH), 1158.7 (C24:0) and 1174.7(C24:0-OH). The main peak of the Gb3 internal standard is at m/z 1060.7 (C17:0) with a secondary peak at m/z 1076.7 (C17:0-OH). The total area of both internal standard peaks and all 4 Gb3 species were used to determine the response ratio (Equation 1) and this used in conjunction with a calibration curve to determine urinary Gb3 concentrations.

Response ratio = total area of the 4 most abundant Gb3 species / area of Gb3 internal standard (Equation-1)

Limit of detection (LOD) and limit of quantitation of total Gb3 in urine were determined using (Equation 2) and (Equation 3) respectively, where $F = 3.3$ and 10 for LOD and LOQ respectively, $stdev$ = standard deviation and b : Slope of the regression line (Figure 6-2).

$$LOD = F \times stdev/b \dots\dots\dots (Equation-2)$$

$$LOQ = F \times stdev/b \dots\dots\dots (Equation-3)$$

	Fabry patients				Control subjects	
	males		females		male	female
	classical	cardiac variant	classical	cardiac variant		
Number	12	11	7	10	22	25
Mean age (y)	39.8±15.1	49.6±12.9	58.7±16.1	51.2±21.2	50.1±20.3	67.8±13.9
Range age (y)	18-55	19-65	32-80	29-85	20-82	51-85

Table 6-1 Anthropometric data of Fabry patients and healthy controls: The table shows some details of all subjects participating in this part of the study including number and age of treated and untreated hemizygous Fabry males, heterozygous Fabry females and health control subjects.

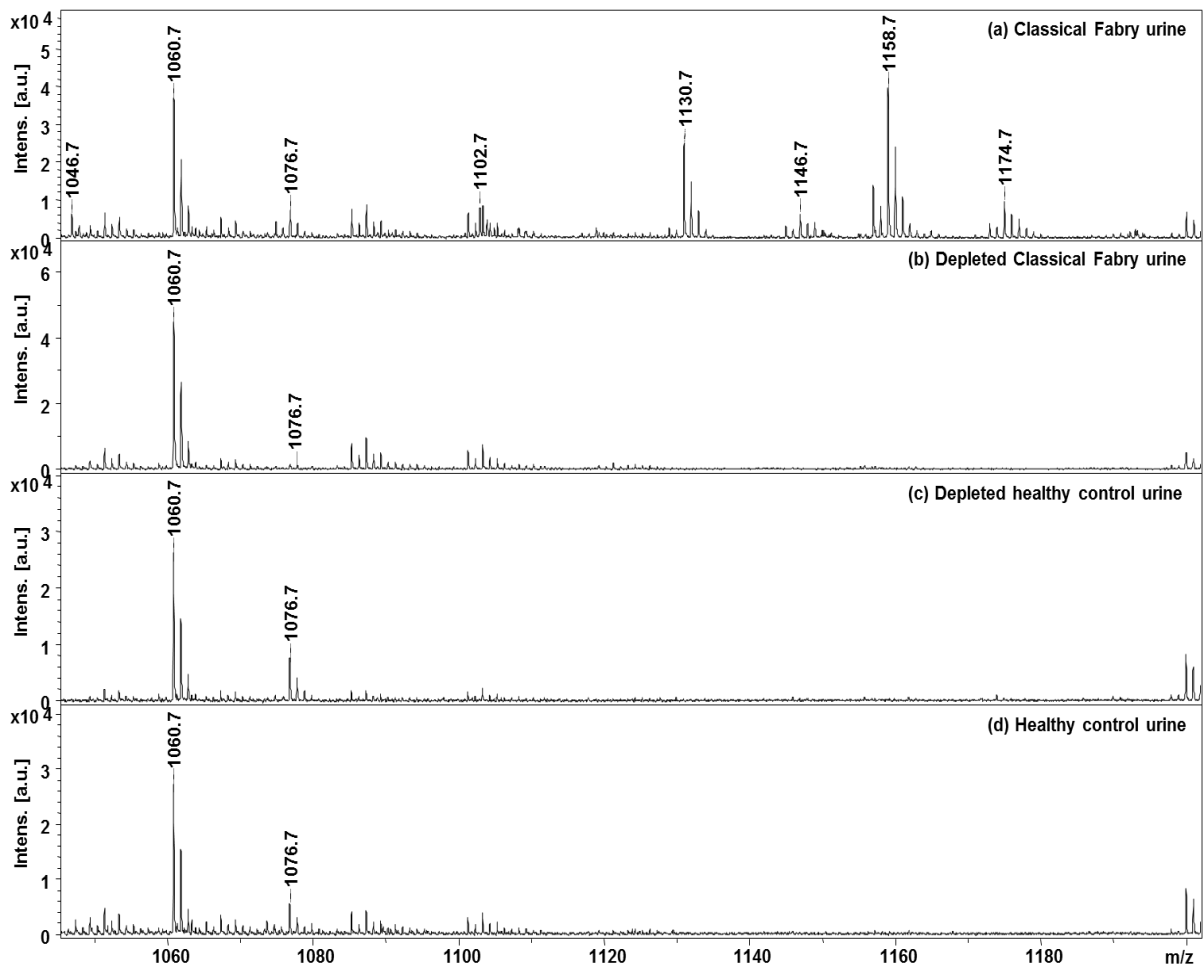


Figure 6.1 Validation of Gb3 depletion: MS spectra of urine samples pre- and post-depletion were studied. (a) Urine from classical Fabry patient pre-depletion. (b) Depleted urine from classical Fabry patient post-depletion. (c) Urine from a healthy control post-depletion. (d) Urine from healthy control pre-depletion. Gb3 internal standard is present in all spectra because it has been added post-depletion [1].

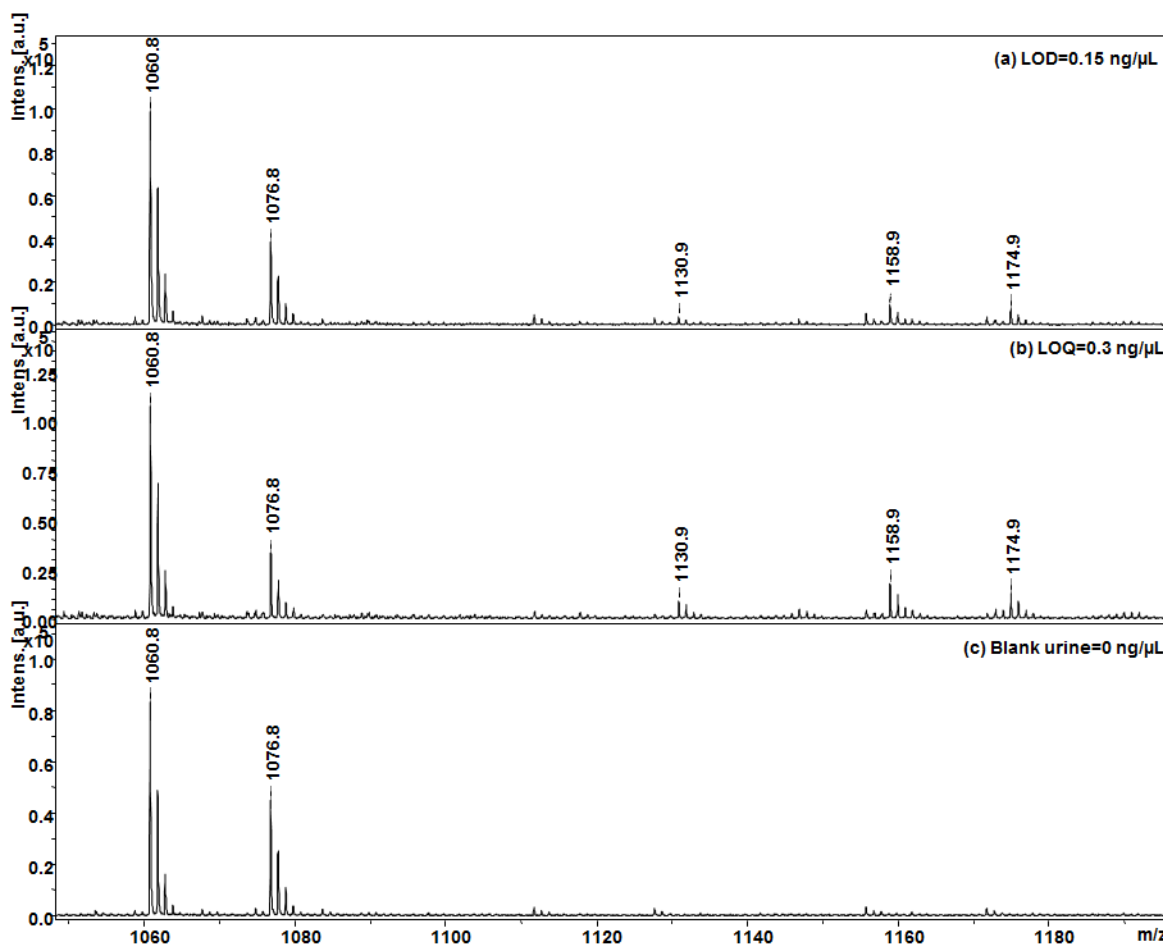


Figure 6.2 Calculation of LOD and LOQ of urinary total Gb3: the figure shows MS spectra were acquired from MALDI-TOF-MS (a) MS spectra of urinary Gb3 at the LOD, (b) MS spectra of urinary Gb3 at the LOQ, (c) MS spectra of depleted urine. LOD & LOQ were determined as follows: $LOD = \frac{F \times stdev}{b}$ & $LOQ = \frac{F \times stdev}{b}$. Where $F = 3.3$ and 10 for LOD and LOQ respectively, $stdev$ = standard deviation and b : Slope of the regression line, the figure adapted from [1].

6.3 RESULTS

6.3.1 Optimisation of Gb3 extraction from urine

Depleted urine was spiked with $2.5\mu\text{L}$ of porcine total Gb3 standard [$200\text{ng}/\mu\text{L}$] generating a final concentration of $5\text{ng}/\mu\text{L}$ and the performance of various extraction procedures involving different volumes of MeOH, acetone and CHCl_3 were assessed by MALDI-TOF-MS. We found using 1:1:1 (v:v:v) of urine, MeOH and CHCl_3 to be the optimal condition for Gb3

extraction into the hydrophobic layer (CHCl_3 layer) based on peak intensity and signal-to-noise ratio in the mass spectra (Figure 6-3) with no Gb3 detectable in the aqueous layer (Figure 6-4).

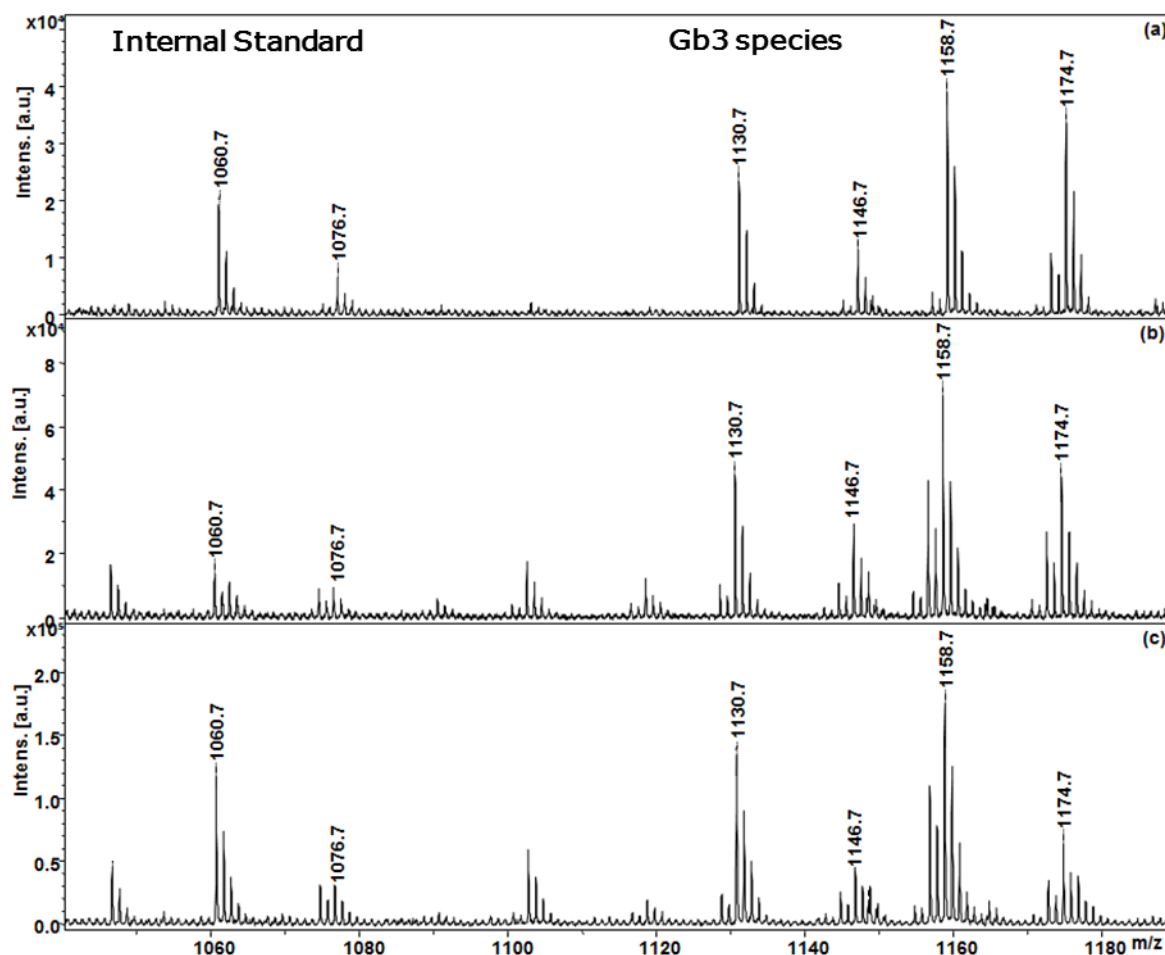


Figure 6.3 Validation of liquid-liquid extraction using different solvents: we have compared 3 different chemical solvents in order to select the optimal solvent for Gb3 extraction from urine samples. The extraction has been achieved using these 3 solvents then analysed using MALDI-TOF-MS. The peak intensities were compared and the solvent which gives higher intensities was selected for Urinary Gb3 extraction. These solvents were: (a) acetone: MeOH:H₂O, (45:45:10) (b) MeOH:CHCl₃, (2:1) (c) MeOH:CHCl₃ (1:1), the figure adapted from [1].

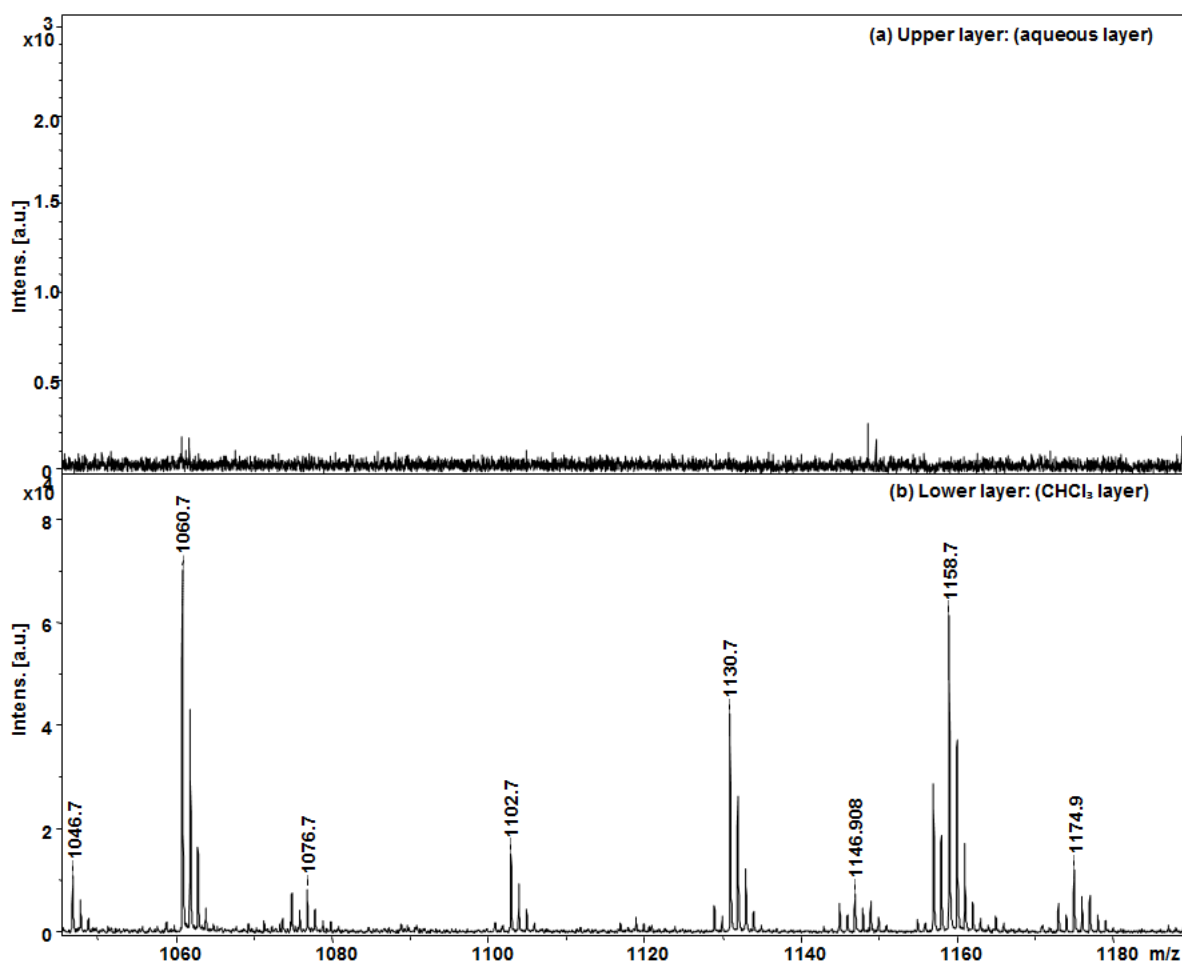


Figure 6.4 Validation of liquid-liquid extraction: MALDI-TOF-MS spectra of Gb3 species and Gb3 internal standard in: (a) the aqueous layer (upper layer) and (b) Chloroform layer (lower layer). Gb3 species and Gb3 internal standard were spiked in normal urine from a healthy control subject, the figure adapted from [1].

6.3.2 Optimization of MALDI-TOF-MS

We acquired and compared the MALDI spectra for depleted urine spiked with 5ng/ μ L Gb3 using 12 different matrix compounds (spectra shown in Figure 6-5). The matrices were used as saturated solutions both in 50%MeOH and 50%ACN. Spectra obtained with 5C2M in 50%MeOH provided the best signal intensity and signal-to-noise ratio. Example MS spectra showing Gb3 peaks in the urine of a Fabry patient, porcine Gb3 standard and a healthy control subject are shown in Figure 6-6.

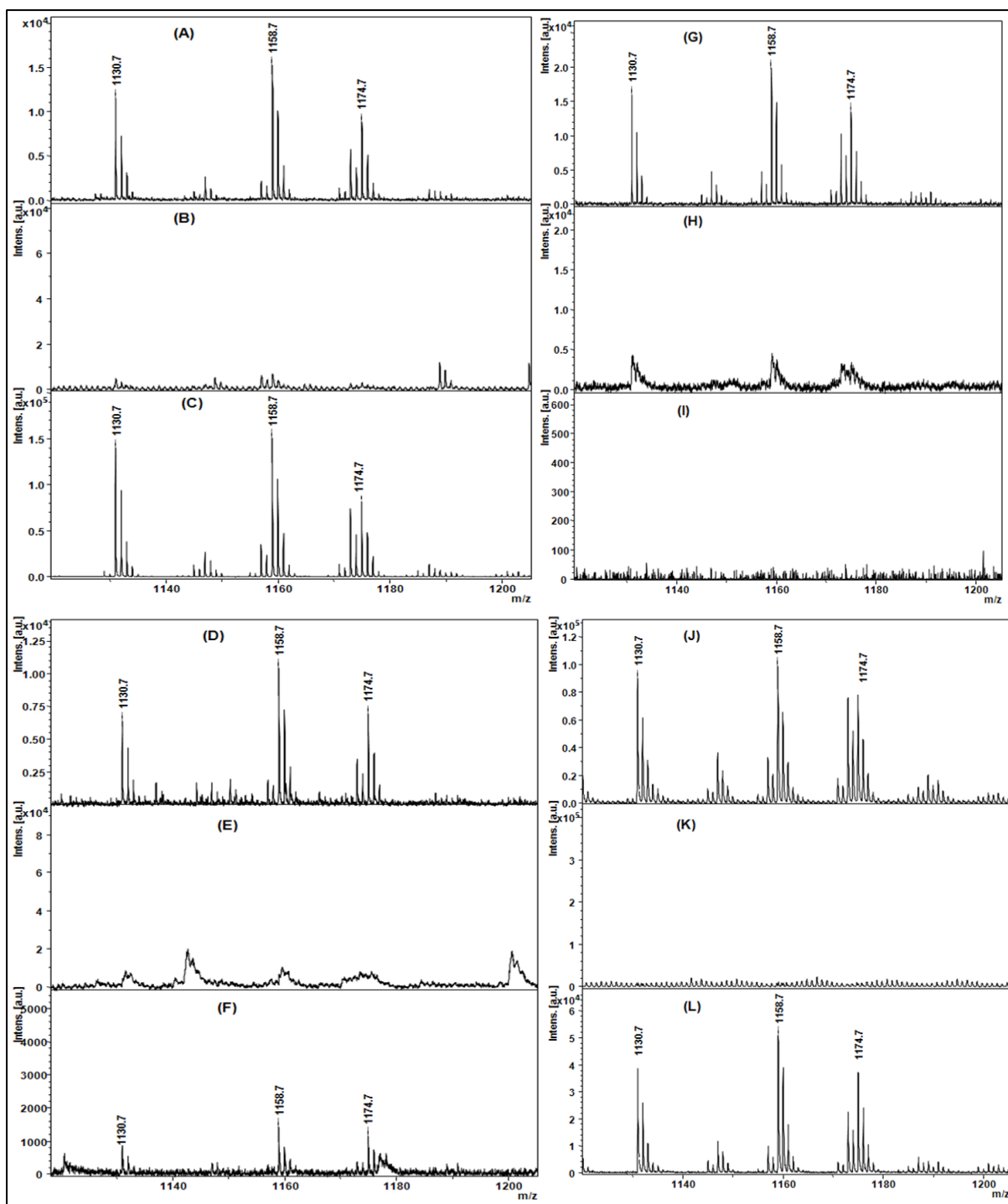


Figure 6.5 Evaluation of different matrix compounds: MALDI-TOF spectra of porcine Gb3 generated using 12 different matrix compounds prepared as saturated solutions in 50% MeOH are shown. The m/z range covers the mass range of the most intense 3 peaks of Gb3 (m/z 1158.7, 1174.7 & 1130.7). These matrix compounds used were: (A) 2-(4-hydroxyphenylazo) benzoic acid, (B) 2-mercaptobenzothiazole, (C) 5-chloro-2-mercaptobenzothiazole, (D) 2,5-dihydroxy acetophenone, (E) 6-aza-2-thiothymine, (F) 9-aminoacridine hemihydrate, (G) α -cyano-4-hydroxycinnamic acid, (H) 2,5-Dihydroxybenzoic acid (DHB), (I) picolinic acid, (J) super-DHB, (K) sinapic acid, (L) 2,4,6-trihydroxy acetophenone monohydrate, the figure adapted from [1].

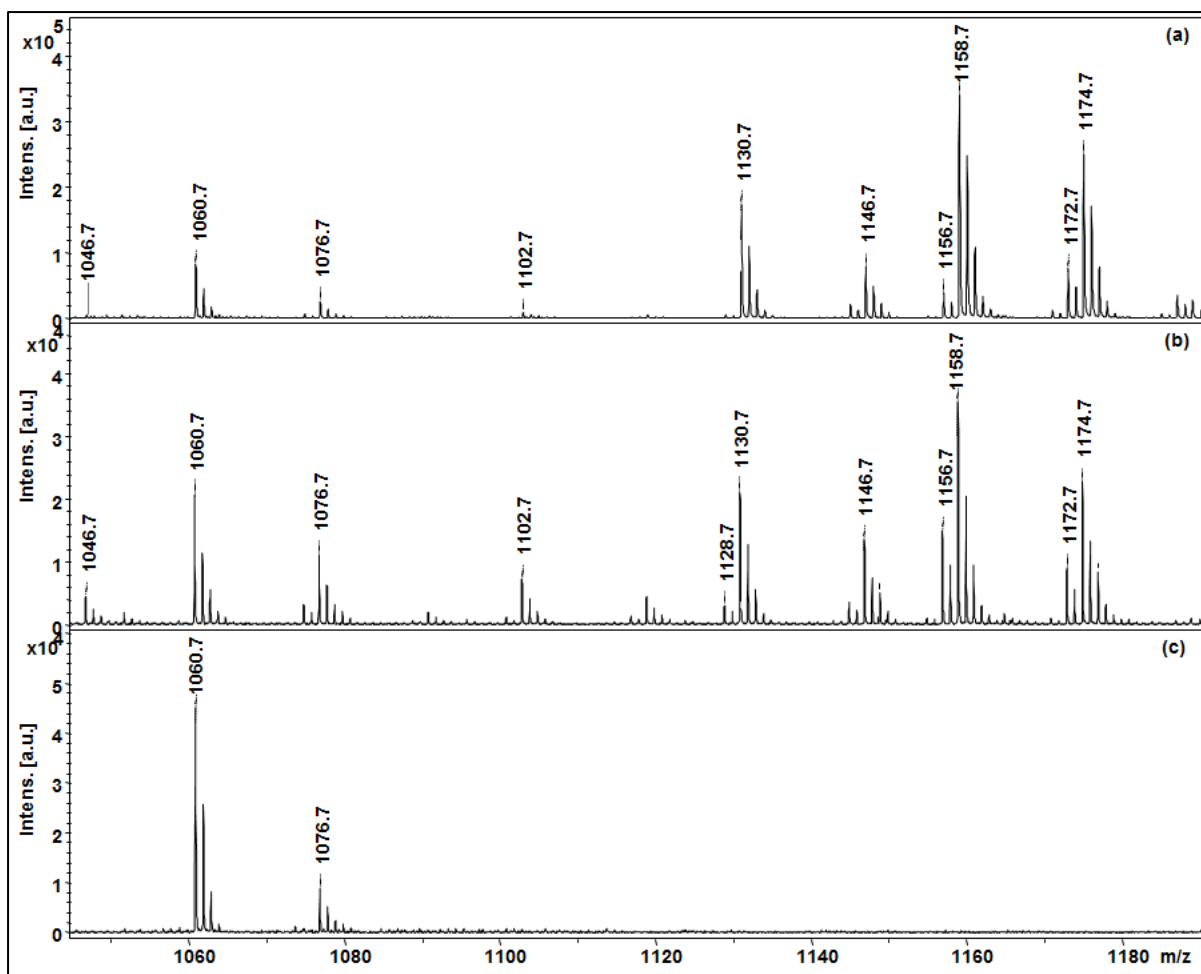


Figure 6.6 Mass spectra of Gb3 and internal standard: MS spectra of Gb3 species and Gb3 internal standard were acquired from MALDI-TOF-MS. (a) Purchased porcine Gb3 standard (b) Urine from untreated classical Fabry male and (c) Healthy control urine, the figure adapted from [1].

6.3.3 MS/MS Confirmation of Gb3 peaks in Fabry patient urine

MS spectra of Fabry patient urine contain peaks with exactly the same m/z values as those in purchased porcine Gb3: m/z 1046.7 (predicted structure C16:0), 1074.7(C18:0), 1102.7(C20:0), 1128.7(C22:1), 1130.7(C22:0), 1146(C22:0-OH), 1156.7(C24:1), 1158.7(C24:0), 1172.7(C24:1-OH) and 1174.7(C24:0-OH). To confirm that the peaks in Fabry patient urine spectra correspond to Gb3 they were subjected to MALDI-TOF/TOF

MS/MS. The MS/MS spectra of the urine peaks show the loss of sugar moieties (Figure 6-7) and confirm that these ions correspond to Gb3 species by the presence of the fragment m/z 264.2 which represents sphingosine (sphingosine-2[H₂O]). The purchased porcine Gb3 generated identical MS/MS spectra.

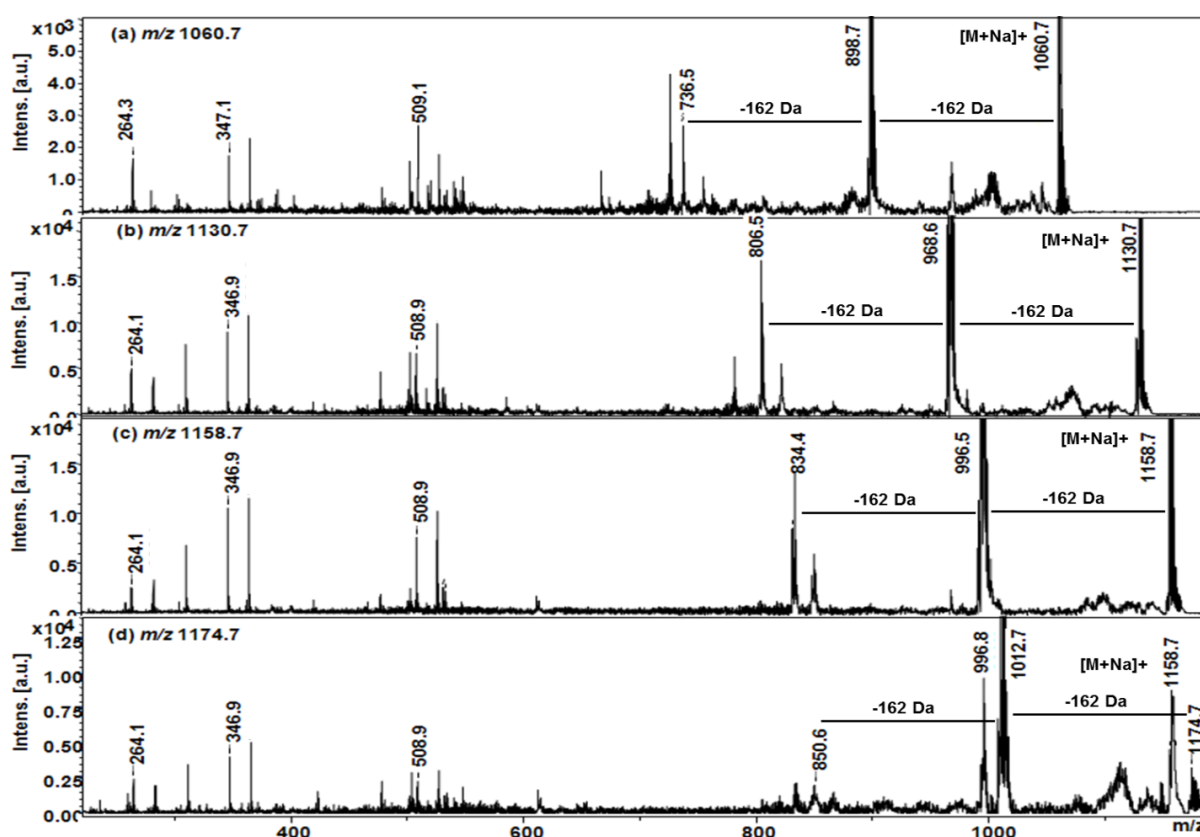


Figure 6.7 MS/MS Confirmation of Gb3 peaks in Fabry patient urine: MALDI-TOF/TOF MS/MS of: a) Gb3-Internal standard m/z 1060.7 (C17:0); and the 3 most abundant Gb3 species in Fabry patient urine: m/z 1130.7 (C22:0), 1158.7 (C24:0) and 1174.7 (C24:0-OH). The peaks corresponding to ions generated by the loss of 1 or 2 sugars (-162 and -324 Da respectively) have different m/z values for each species whereas the fragments corresponding to sphingosine -2H₂O, (m/z 264.1) and di- and tri-saccharide ions (m/z 347 and 509) are invariant, the figure adapted from [1].

6.3.4 Quantitative measurement of urinary Gb3 by MALDI-TOF-MS

To correct for variations in extraction and ionisation efficiencies an internal standard was added to all urine samples prior to extraction. The internal standard appears as a peak at m/z 1060.7 (C17:0) (plus a secondary peak at m/z 1076.7(C17:0-OH)). No peaks were seen at these m/z values in the MALDI spectra of any of the extracted urines used in this study in the absence of internal standard (Figure 6.8) (also, no contaminants with the same masses as the Gb3 peaks were detected). Gb3 concentrations were calculated from the (total Gb3/internal standard) area ratio as described in Methods and using the calibration curve shown in Figure 6.9. Inter-assay and intra-assay variability were measured to assess the reliability of the method. Ten healthy controls urine samples were used. Each sample was spiked with 2.5 μ L of total Gb3 standard [200ng/ μ L] generating a final concentration of 5ng/ μ L. Each sample was processed as 3 independent technical replicates. Each extract was spotted onto the MALDI target in triplicate giving 9 readings for each sample. The same procedure was repeated on 3 different days resulting in each sample been analysed 27 times. The results showed high reproducibility and reliability of the method with an intra-assay coefficient of variation of 9.9% and inter-assay of 13.7%. The lower limit of detection was 0.15 ng/ μ L and limit of quantitation was 0.30 ng/ μ L. The assay was linear up to the highest Gb3 concentration investigated (40 ng/ μ L). Gb3 generates multiple peaks in mass spectra. The total area of the 4 most intense peaks (m/z 1130.7 (C22:0), 1146.7 (C22:0-OH), 1158.7 (C24:0) and 1174.7(C24:0-OH)) was used to measure the ratio of Gb3 to the internal standard. These 4 peaks were selected because they have the highest intensities and they were detected in all patients while the other peaks (other Gb3 species) were not always detected. We found that the ratio of these 4 peaks to one another is constant across individuals i.e. classical Fabry disease results in accumulation of all 4 Gb3 species (Figure 6.10). Although we have used the sum of the 4 main peaks for quantitation (considering that this might be the

most robust approach) we also found that any one of the four peaks in alone would yield the same results.

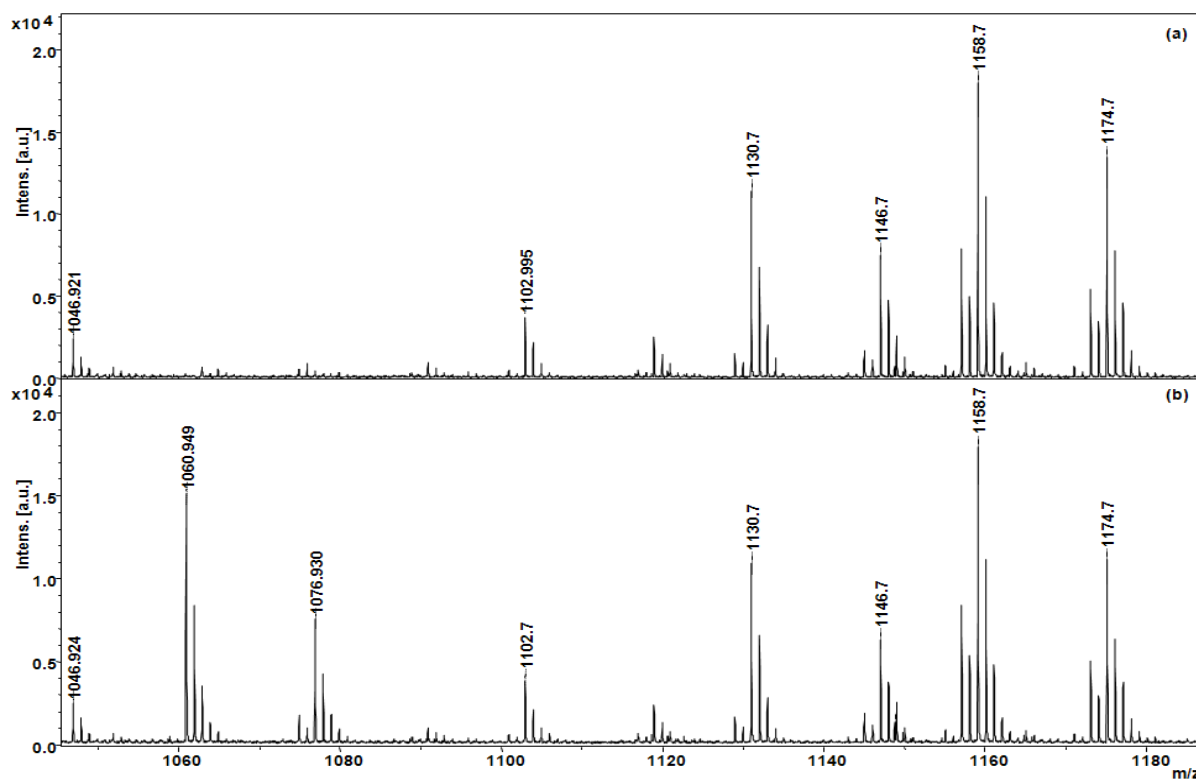


Figure 6.8 Gb3 internal standard in human urine: The presence of Gb3 internal standard peaks in human urine has been tested: (a) Fabry urine without adding internal standard. (b) Fabry urine spiked with Gb3 internal standard, the figure adapted from [1].

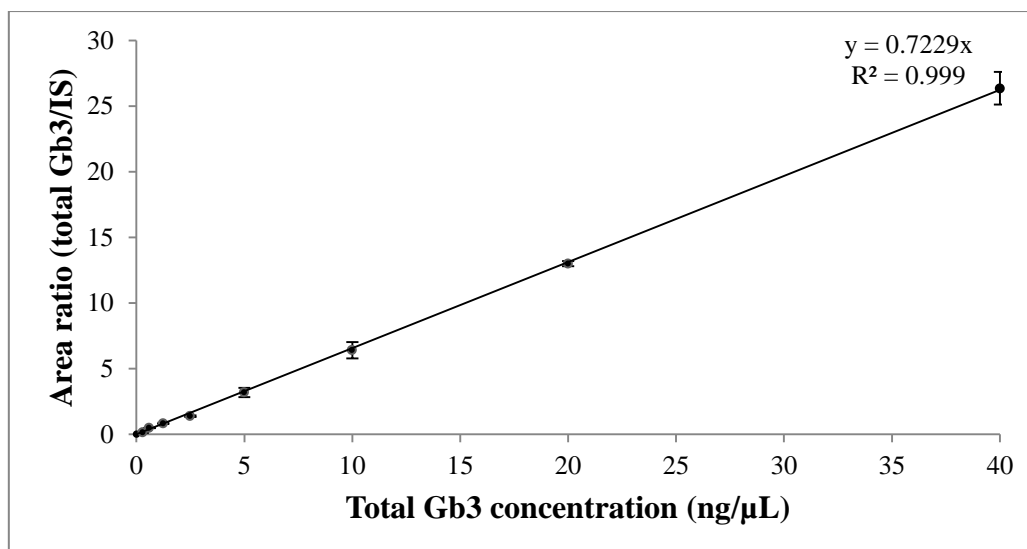


Figure 6.9 Urinary Gb3 standard curve: Increasing amounts of porcine Gb3 standard covering the reported clinical range and a constant amount of Gb3 internal standard were spiked into depleted urine from a healthy control subject. Thereafter, the lipids were extracted and analysed by MALDI-TOF MS, figure adapted from [1].

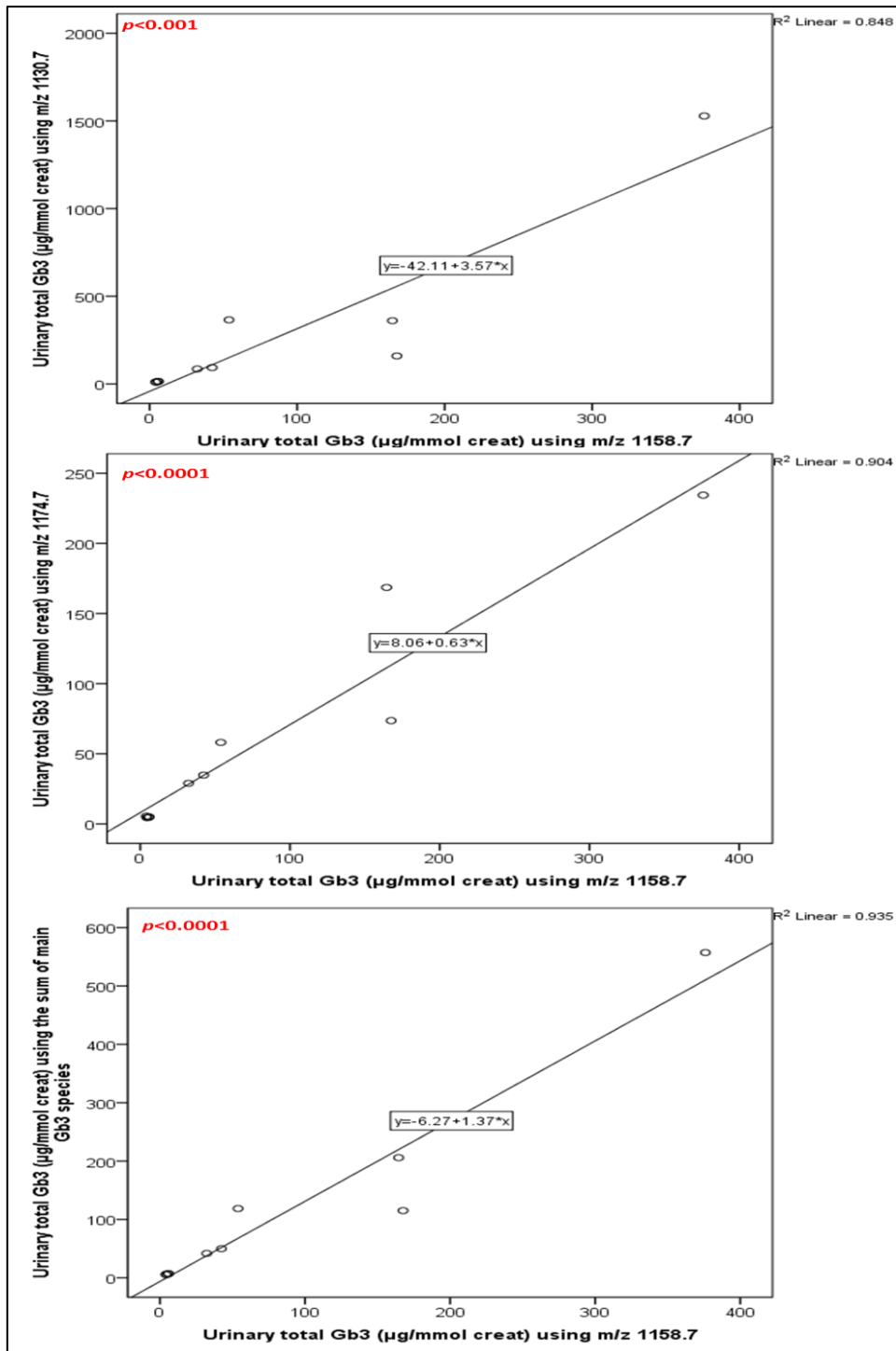


Figure 6.10 Correlation between urinary Gb3 species: The figure shows that there is a correlation between the main urinary Gb3 species (m/z 1130.7, 1158.7 and 1174.7). Using any one of these Gb3 species individually or using the sum of these species yields similar results.

6.3.5 Urinary total Gb3 levels in classical Fabry patients

The levels of urinary total Gb3 in classical Fabry patients (n=19) and healthy controls (n=47) were measured. Prior to statistical comparison the urinary Gb3 concentrations were normalised to urinary creatinine as shown in Figure 6.11. The mean normalised concentration in the classical Fabry males (n=12, mean = 280.9 $\mu\text{g}/\text{mmole creatinine}$) was significantly higher than in control males (n=22, mean = 8.0 $\mu\text{g}/\text{mmole creatinine}$), ($p < 0.0001$). The normalised mean concentration of classical Fabry females (n=7, mean = 48.3 $\mu\text{g}/\text{mmole creatinine}$) was significantly higher than in control females (n=25, mean = 16.4 $\mu\text{g}/\text{mmole creatinine}$), ($p < 0.05$). However, the normalised mean concentration in the classical Fabry males was higher than in Fabry females, although this did not reach statistical significance ($p > 0.05$).

Using the urinary Gb3 concentrations measured in the 56 individuals (including 5 untreated classical Fabry males, 4 untreated classical Fabry females, 22 control males and 25 control females) in this study to detect FD generated a receiver operator characteristic (ROC) curve as shown in (Figure 6.12).

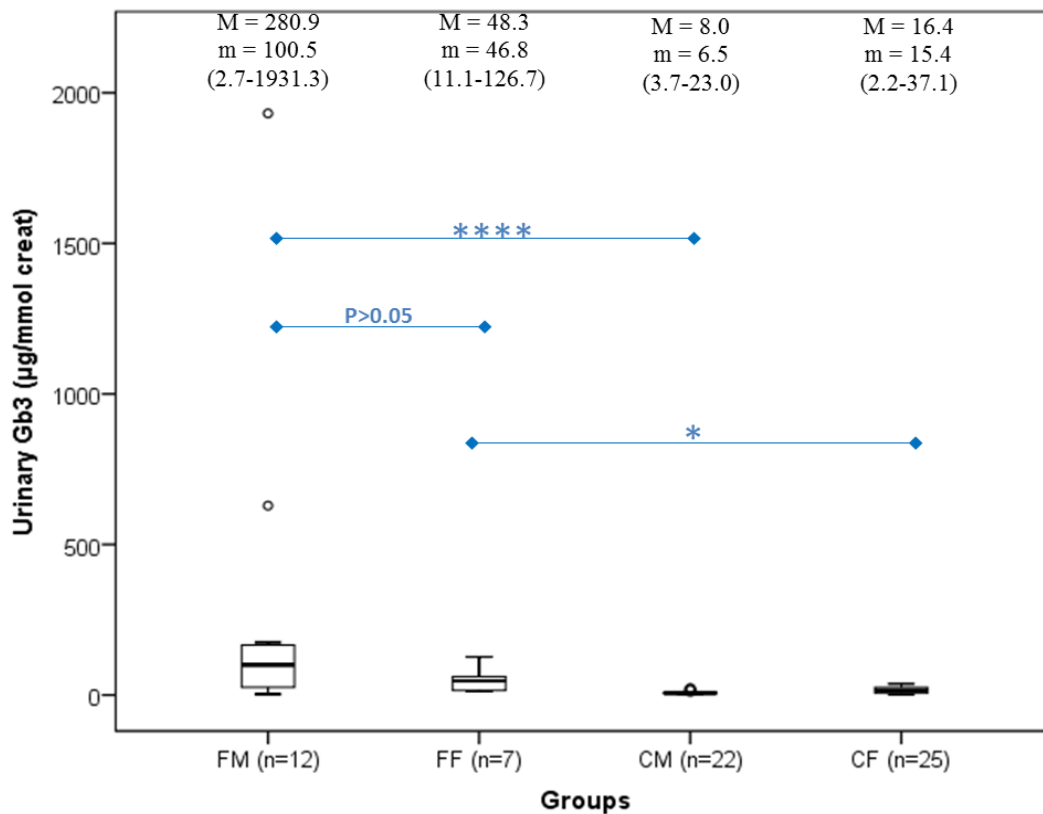


Figure 6.11 Urinary total Gb3 levels in classical Fabry patients: box-and-whiskers plot chart showing urinary Gb3 levels in both genders of classical Fabry patients and control subjects. The concentrations were measured using Gb3 calibration curve. Data were analysed using SPSS. The subjects were divided to 6 groups as follows: Fabry male (FM), Fabry female (FF), control male (CM) and control female (CF). For each group: n = number of subject; M = Mean; m = median; the range of minimum and maximum values in brackets; box= maximum and minimum quartiles; line inside the box = median; whiskers = maximum and minimum non-outlier values; o = outlier values. Various groups were compared against each other using Mann-Whitney U tests. The differences were considered statistically significant if the p value < 0.05 (**= $p < 0.01$, ***= $p < 0.001$ and ****= $p < 0.0001$).

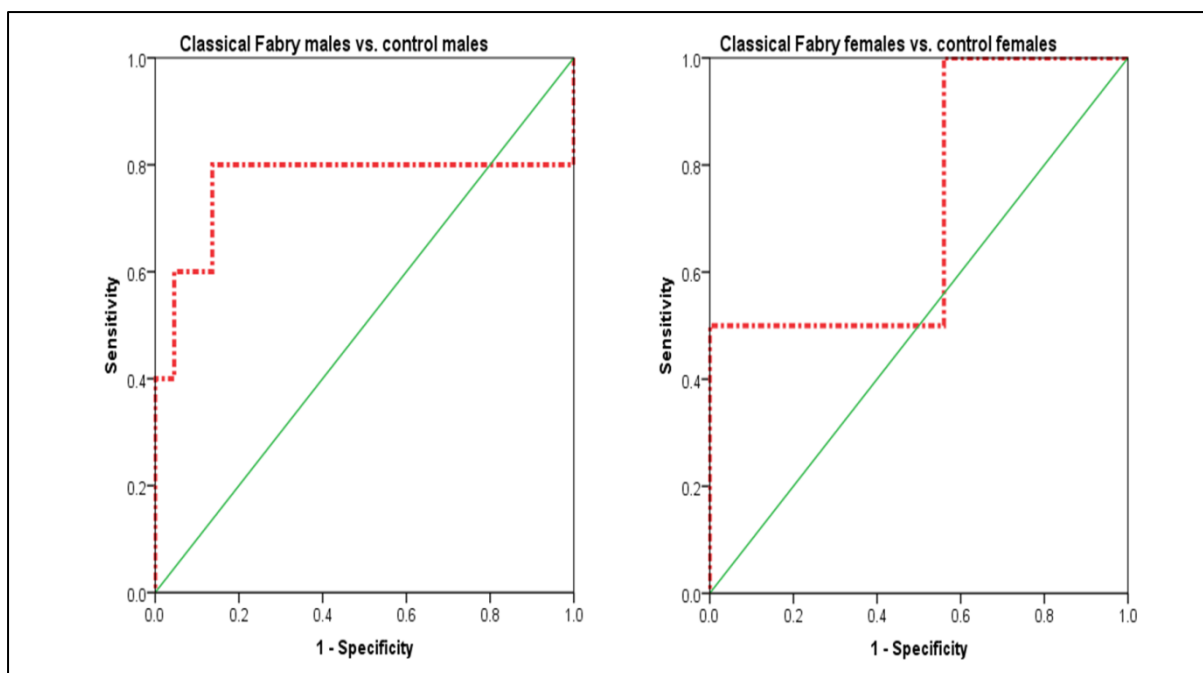


Figure 6.12 ROC analysis for urinary total Gb3 in classical Fabry patients: Receiver operator characteristic (ROC) curve of Urinary total Gb3 in classical Fabry males vs. control males (left panel) and in classical Fabry females vs. control females (right panel).

6.3.6 Urinary total Gb3 levels in cardiac variant Fabry patients:

The levels of urinary total Gb3 in cardiac variant Fabry patients (n=21) and healthy controls (n=47) were measured. Prior to statistical comparison the urinary Gb3 concentrations were normalised to urinary creatinine (Figure 6.13). The mean normalised concentration in the cardiac variant Fabry males (n=11, mean = 15.1 $\mu\text{g}/\text{mmole}$ creatinine) was higher than in control males (n=22, mean = 8.0 $\mu\text{g}/\text{mmole}$ creatinine) but did not reach the statistical significance ($p>0.05$). Nevertheless, the normalised mean concentration of cardiac variant Fabry females (n=10, mean = 21.3 $\mu\text{g}/\text{mmole}$ creatinine) was significantly higher than in control females (n=25, mean = 16.4 $\mu\text{g}/\text{mmole}$ creatinine), ($p<0.05$). However, variation in the normalised mean concentration of total urinary Gb3 between the two genders of cardiac variant Fabry patients was statistically not significant ($p>0.05$).

Using the urinary Gb3 concentrations measured in the 59 individuals (including 6 untreated cardiac variant Fabry males, 6 untreated cardiac variant Fabry females, 22 control males and 25 control females) in this study to detect FD generated a receiver operator characteristic (ROC) curve as shown in (Figure 6.14).

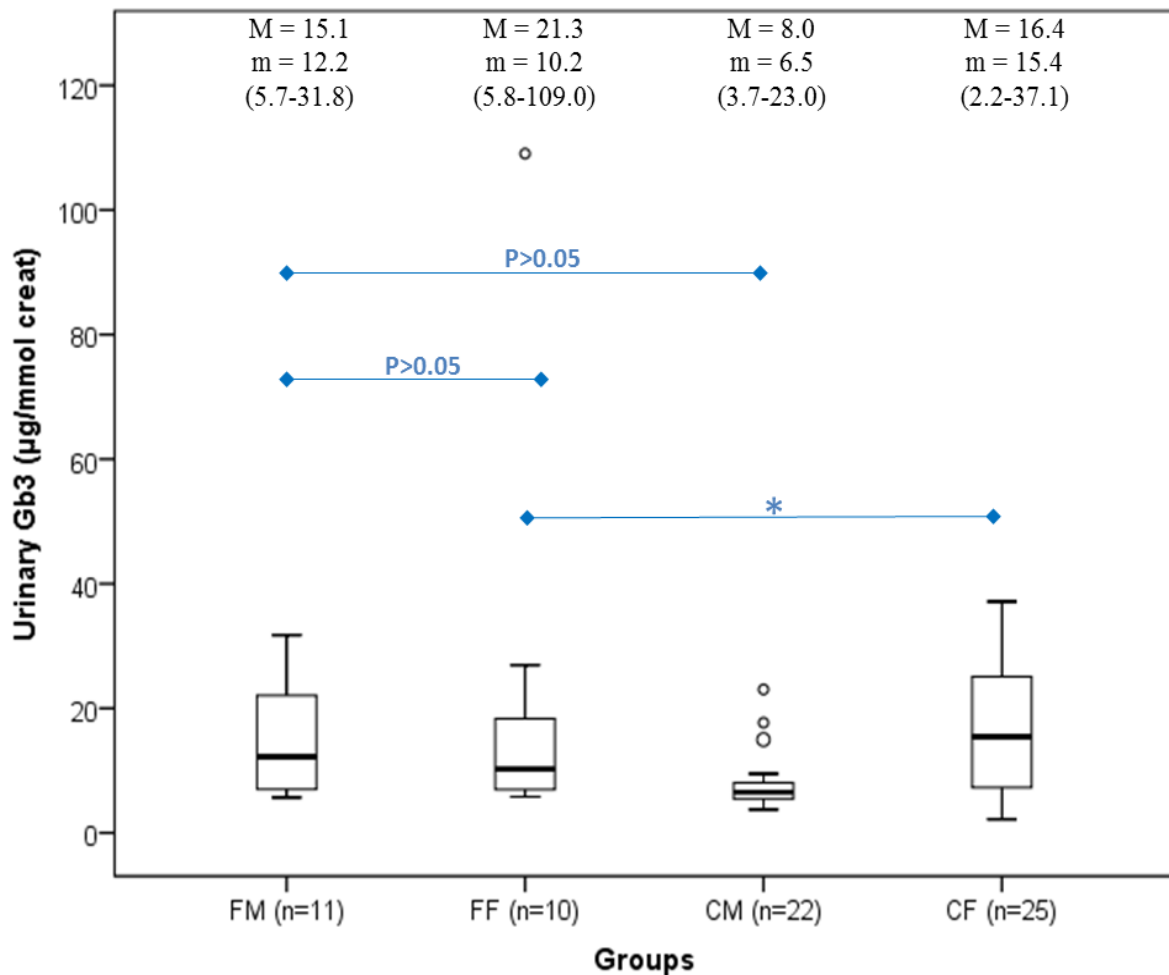


Figure 6.13 Urinary total Gb3 levels in cardiac variant Fabry patients: box-and-whiskers plot chart showing urinary Gb3 levels in both genders of cardiac variant Fabry patients and control subjects. The concentrations were measured using Gb3 calibration curve. Data were analysed using SPSS. The subjects were divided to 6 groups as follows: Fabry male (FM), Fabry female (FF), control male (CM) and control female (CF). For each group: n = number of subject; M = Mean; m = median; the range of minimum and maximum values in brackets; box= maximum and minimum quartiles; line inside the box = median; whiskers = maximum and minimum non-outlier values; o = outlier values. Various groups were compared against each other using Mann-Whitney U tests. The differences were considered statistically significant if the p value < 0.05 (**= $p < 0.01$, ***= $p < 0.001$ and ****= $p < 0.0001$).

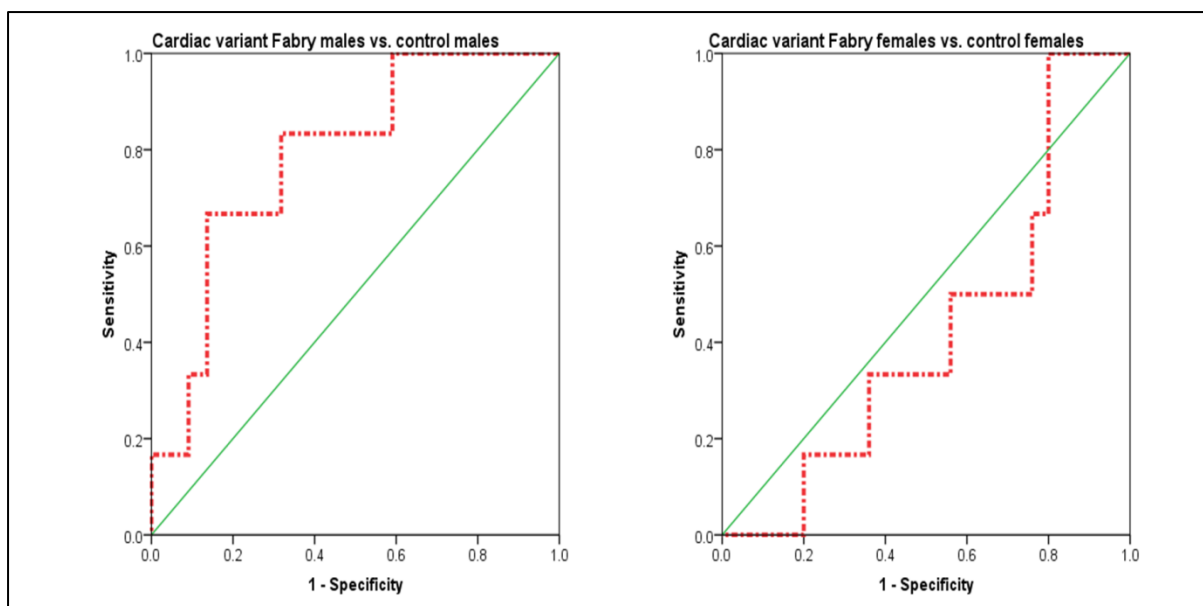


Figure 6.14 ROC analysis for Urinary Gb3 in cardiac variant Fabry patients: Receiver operator characteristic (ROC) curve of Urinary total Gb3 in cardiac variant Fabry males vs. control males (**left panel**) and in cardiac variant Fabry females vs. control females (**right panel**).

6.3.7 Correlation between urinary Gb3 levels and Lyso-Gb3 levels in Fabry patients:

The correlation between the levels of urinary total Gb3 in Fabry patients and the levels of urinary Lyso-Gb3 has been tested in this work. Figure 6.15 shows that urinary total Gb3 is correlated with urinary Lyso-Gb3 in Fabry patients with Pearson Chi-Square p value <0.0001 . Moreover, urinary total Gb3 is correlated with the total levels of urinary Lyso-Gb3 and its analogues in Fabry patients with Pearson Chi-Square p value of <0.01 (Figure 6.15).

Based on outcomes of this study supported with similar findings in previous studies, we can say that urinary total Gb3 could be a useful biomarker for classical hemizygous Fabry males but it is not the best choice for symptomatic heterozygous Fabry females and both genders with atypical cardiac variant FD. As it has been postulated in Chapter 4 and 5, Lyso-Gb3 in

plasma and urine showed better utility in diagnose and management FD more than urinary total Gb3.

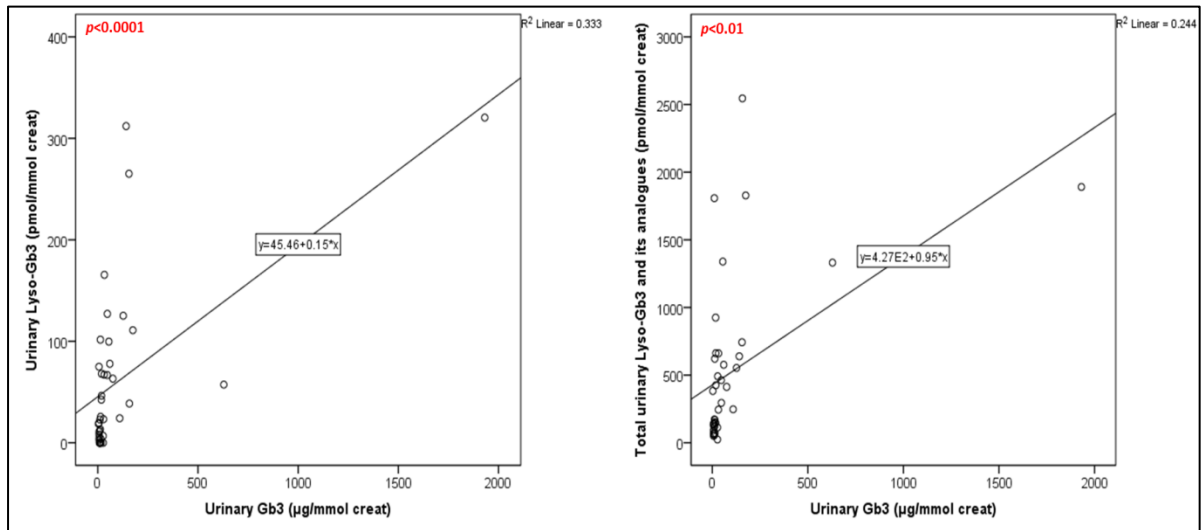


Figure 6.15 Correlation between urinary total Gb3 and urinary-Lyso-Gb3 in Fabry patients: the figure shows the correlation between urinary Gb3 and urinary Lyso-Gb3 (left panel) and total urinary Lyso-Gb3 and its analogues (right panel).

6.4 DISCUSSION

By coupling a novel liquid-liquid extraction and MALDI-TOF-MS with an internal standard we have been able to develop a rapid (<15 minutes from start to finish) method for measuring Gb3 in urine. Considerable optimisation of the Gb3 extraction and MS analysis has been performed to produce an assay with good reproducibility, sensitivity and specificity sufficient to easily detect the elevated levels of urinary Gb3 that are expected in classical Fabry patients.

N- Heptadecanoyl ceramide trihexoside has been used as an internal standard in this study because it has very similar chemical properties to human Gb3 but cannot be synthesised by humans (due to the odd number of carbon atoms). The internal standard generates a main peak at m/z 1060.7 (C17:0) and a minor peak at m/z 1076.7 (C17:0-OH) (most likely due to oxidation, both peaks were combined in calculations). Following liquid-liquid extraction human urine is devoid of peaks at these m/z values unless the internal standard is added. Including the internal standard in our assay has enabled a high degree of reducibility and reliability to be reached with correspondingly low intra-assay and inter-assay coefficients of variation. The assay is based on MS, however, MS/MS was used to verify the identity of the Gb3 peaks: the most abundant peaks at m/z 1130.7 (C22:0), 1146.7 (C22:0-OH), 1158.7 (C24:0) and 1174.7 (C24:0-OH) all show identical fragmentation pattern by losing sugar groups (-162Da) and generating the di-dehydrated sphingosine moiety (m/z 264.2) as previously reported for Gb3 [201, 269-272].

In one study, Branton and colleagues [273] have shown that individual presenting with a conservative amino acid change display a significantly delayed onset of symptoms when compared to those with non-conservative amino acid substitutions. However, a study by Vedder and colleagues [89] did not find any correlation between the levels of plasma or urinary Gb3 and clinical symptoms supporting that the better utility of Lyso-Gb3 as a potential biomarker for FD, over its acylated precursor molecule Gb3. A similar study also failed to identify any correlation between urinary Gb3 levels in Fabry patients and the disease severity or treatment response, suggesting that the measurement of plasma or urinary Gb3 could be an unreliable biomarker specially in hemizygous Fabry females [160].

In conclusion, our method significantly reduces the number of steps and time required to complete the test compared with the current standard methods. The method could be easily

implemented in any laboratory with access to a MALDI mass spectrometer and used for non-invasive cost-effective detection of FD.

CHAPTER-7
GENERAL DISCUSSION

Fabry disease (FD, OMIM 301500) is an *X-linked* LSD caused by a deficiency of α -gal-A resulting in accumulation of GSLs, mainly Gb3 and its derivatives, in the walls of small blood vessels, cardiomyocytes, renal glomerular and tubular epithelial cells, neurons of the autonomic nervous system, small unmyelinated nerve fibers and to some extent in all other tissues with lysosomes [17, 25, 27-30, 80]. Affected males typically present in childhood with episodes of severe neuropathic pain in the hands and feet. This is followed by the development of angiokeratoma, proteinuria, progressive renal impairment, cardiomyopathy, and cerebrovascular events, culminating in major disability and death, generally before age 60 years [80]. According to the Human Gene Mutation Database (www.hgmd.cf.ac.uk) (last accessed at www.hgmd.cf.ac.uk, May, 23, 2016), more than 800 mutations in the *GLA* gene have been described and most are private mutation. Estimated birth prevalence range between 1:40,000 and 1:110,000 [47-50]. However, recent new-born screening data and targeted screening suggest a higher prevalence up to 1:3000 [48]. Most individuals identified through screening lack characteristic classical Fabry signs or symptoms such as neuropathic pain, angiokeratoma or cornea verticillata, and the disease pursue a different clinical course limited to a single organ system and called Fabry variant to distinguish them from classical Fabry with typical signs and symptoms. One subgroup of these subjects, are the “cardiac variants”, who are typically free of the usual clinical manifestations of the disease for most of their lives, then present with progressive LVH and conduction abnormalities, which often require cardiac intervention and leads to premature death.

Because of founder effect, in England, there is large cohort of cardiac variant with the N215S mutation. The pathogenicity of N215S mutations is well described as a disease causing mutation. However, like other atypical Fabry patients, most male have significant residual α -gal-A enzyme activity, in contrast to the very low or absent enzyme activity in classical Fabry males. The atypical late presentation, presence of residual enzyme in a variant Fabry and the

increasing number of *GLA* mutation of unknown significance led to diagnostic challenge in Fabry expert centres. In a day to day practice this phenotypic and genotypic heterogeneity resulted in over diagnosis of Fabry with the consequence of stigmatising patient and subjecting them to cumbersome and high cost ERT.

A reliable diagnosis in affected classical males can be made by the measurement of α -gal-A activity in plasma or peripheral blood leukocytes. The diagnosis of the affected cardiac variant male and both classical and variant female will require *GLA* mutation analysis. In some cases, mutation analysis may not be sufficient. Accumulating evidence suggest that Lyso-Gb3 is significantly elevated in the plasma of all male patients with classical FD and to a lesser extent in symptomatic female patients with excellent sensitivity and specificity. Recent consensus guidelines acknowledged the use of Lyso-Gb3 as an important diagnostic marker for FD [224, 258, 259] but there is limited data about its usefulness to assess disease severity and response to ERT and chaperon [165, 274-281].

FD is not only heterogenic in terms of the spectrum of gene variations or mutations that can occur, but is also heterophenotypic in terms of its clinical manifestations and the array of symptoms that affected individual present with. However, in most instances patients typically present with the involvement of three organs: heart, kidney and nervous system. Disease progression ultimately leads to heart failure, renal failure or stroke.

Although patients diagnosed with classical FD present with multiple tissue and organ involvement, some patients develop complications in only a single organ. These are referred to as cardiac or renal variants, often presenting as clinically relevant late onset variants FD [224, 282, 283]. Additional variants of the *GLA* gene have been identified; termed genetic variants of unknown significance [259], where patients present with nonspecific features (e.g.

LVH) or are asymptomatic and detected during screening. These patients pose a particular diagnostic challenge as a misdiagnosis can impose significant lifetime burden of investigation, monitoring and treatment. Further, atypical variants and classical Fabry females may exhibit the same symptoms and severe organ involvement as classical Fabry males, even though α -gal-A enzyme activity in these patients is often only slightly reduced.

While the levels of plasma or urinary Gb3 have utility in diagnosing classical Fabry hemizygotes, its measurement is not helpful to identify clinical manifestation or disease severity in either hemizygous males or heterozygous females [46, 152].

In our study, the deacylated form (a degradation product) of the accumulating Gb3, Lyso-Gb3 appeared to be a better predictor of *GLA* mutation, than measurement of α -gal-A enzymatic activity in classical Fabry heterozygotes and atypical variants Fabry patients. Moreover, the study hypothesized that assessing Lyso-Gb3 can play an important role in the diagnosis and monitoring of classical and clinically relevant cardiac variant FD. The study evaluated the sensitivity and specificity of plasma and urinary Lyso-Gb3 levels in subjects with classical and cardiac variant mutation. Furthermore, the study analysed plasma Lyso-Gb3 levels in Fabry patients' pre- and post-ERT and evaluated plasma and urinary Lyso-Gb3 change with time.

In Chapter 3 we were able to develop reliable and robust SPE extraction and HPLC-MS/MS based methods to extract and analyse Lyso-Gb3 and its associated analogues from plasma and urine. All methodologies were subject to further improvement of the various techniques. For example, we found that SPE was superior to liquid-liquid extraction due to the latter requiring the use of halogenated solvents (chloroform); however, the standard guidelines of clinical laboratories avoid the use of this type of solvent for environmental reasons.

Moreover, SPE is a fairly simple and rapid protocol that does not require many steps of sonication and centrifugation as is required in liquid-liquid extraction.

Internal standards are fundamental in the quantitative mass spectrometry methodologies. Generally, an internal standard is a chemical substance characterized by being similar to the measured analyte in terms of chemical structure, processing and analysis behaviour. No isotopic version of Lyso-Gb3 is commercially available. Moreover, the preparation of isotopic labelled Lyso-Gb3 in-house is a complex, laborious process and requires specialist knowledge. So we focused on the use of the plant-derived GSG as an internal standard for the whole study of Lyso-Gb3 analyses in plasma and urine. GSG behaves exactly like Lyso-Gb3 in terms of MCX extraction, HPLC separation and MS/MS fragmentation. Bovine GSG, which was employed initially, and Lyso-Gb3 give rise to exactly the same fragment m/z 282.2 which could cause carry over and/or confusion. Therefore, I have tested the suitability of using plant-derived GSG which contains an extra double-bond in the sphingosine moiety giving a fragment of m/z 280.3. The use of plant-derived GSG removes any potential carry over, and was used in the whole study of Lyso-Gb3 analyses in plasma and urine.

Likewise, to optimise the analytical part of the process, we tested the newly developed nano-Viper fittings - HPLC connection tubing and found it to be superior to the standard fittings regarding quantitation of Lyso-Gb3 and related metabolites.

As this project deals with clinical samples, it was critical to ensure that the instrument performance is stable between runs. We prepared our own in-house Lyso-Gb3 QCs to monitor the performance of the instrument. Overall, the MS performance was highly stable giving acceptable Intra- and inter-assay variability. Moreover, the MS calibration and tuning

were performed before each run of clinical samples and the results recorded in order to follow up the instrument performance over the duration of the project.

As outlined in Chapter 4, using a reliable and robust LC-MS/MS methodology, coupled with the use of an inexpensive and commercially available internal standard, GSG, we were able to confirm that the levels of Lyso-Gb3 in plasma samples can be used as a reliable diagnostic biomarker for FD. Plasma Lyso-Gb3 can easily discern classical hemizygous Fabry males and classical heterozygous Fabry females from control subjects. These findings are consistent with previous studies using HPLC assays [46] or LC-MS/MS methodologies [204, 215, 224, 258, 284].

In our cohort of Fabry patients, the levels of plasma Lyso-Gb3 in classical Fabry males (the lowest value = 45.3 nM) were much higher than the levels observed in control male subjects (the highest value = 2.1 nM). This 20 fold difference confirms that plasma Lyso-Gb3 is a strong and a reliable diagnostic tool for diagnosing classical Fabry males. In this respect, our findings are in keeping with those of Smid and co-workers [258] who, using an LC-MS/MS-based approach, also found that the classical Fabry males enrolled in their study had levels of plasma Lyso-Gb3 that exceeded a threshold of 45 nM (range = 45-150 nM) and far exceeded those of normal healthy male control subjects (range = 0.3-0.6 nM). On the other hand, despite the lower levels of plasma Lyso-Gb3 in classical Fabry females (values ranging from 3-43.9 nM) compared to classical Fabry males (range = 45.3-210.8 nM), the low levels of plasma Lyso-Gb3 found in healthy female control subjects (range 0.3-2.3nM), makes a diagnosis of classical FD females also possible using this biomarker. Essentially similar findings were observed by Smid and co-workers [258], who also found that the levels of plasma Lyso-Gb3 in classical Fabry females were considerably lower than those observed in classical Fabry male subjects (range 1.5-41.5nM vs 45-150nM), yet were sufficiently high to make a diagnosis of FD.

In the current study, we also examined the utility of measuring levels of plasma Lyso-Gb3 for the diagnosis of non-classical FD patients, in this case, 38 patients who had been diagnosed as cardiac variant FD. The majority of this group carried the N215S mutation ($\approx 90\%$).

Although the total amounts of plasma Lyso-Gb3 were considerably lower in both male and female cardiac variant Fabry patients (5.7-19.5 nM vs 1.2-12.6 nM), compared to classical Fabry males and females (range = 45.3-210.8 nM and 3.0-43.9 nM, respectively) the levels were sufficiently high to differentiate these patients from healthy male and female control subjects (0.3-2.1 nM and 0.3-2.3 nM, respectively). While a certain degree of caution should be taken with cardiac variant Fabry female, only a patient in this subgroup showed an overlap with controls. Despite the significant variation in levels of plasma Lyso-Gb3 between classical and cardiac variant Fabry patients, the findings of our study confirm that plasma Lyso-Gb3 can be used as a potentially reliable biomarker for the diagnosis of cardiac variant Fabry males. In their study, Smid and co-workers [258] also found that non classical Fabry males and females displayed elevated levels of plasma Lyso-Gb3 compared to healthy male and female control subjects. Thus, despite certain instances where cardiac variant Fabry females displayed a degree of overlap in their plasma Lyso-Gb3 levels when compared to control females, plasma Lyso-Gb3 still appears to have utility as a diagnostic biomarker for cardiac variant Fabry females.

In their study, Smid and co-workers could not ascribe a “cut-off” to classical Fabry females, due to a slight overlap in the levels of plasma Lyso-Gb3 in non-classical Fabry females [258]; however, they also concluded that Lyso-Gb3 was a reliable diagnostic tool for identifying FD in classical females.

Niemann and colleagues [224] have suggested that classical Fabry patients and non-classical Fabry patients (such as those with carrying the cardiac variant), could be differentiated on the

basis of their plasma Lyso-Gb3 levels. Niemann et al [224] recommended that the plasma Lyso-Gb3 can be used to differentiate between classical Fabry patients and non-classical (e.g. cardiac variant) Fabry patients. The findings from our study identified significant variation between plasma Lyso-Gb3 levels in the two aforementioned FD phenotypes. Moreover, it proved relatively straightforward to distinguish classical Fabry patients from cardiac variant Fabry patients based on their levels of plasma Lyso-Gb3 where the lowest observed level in classical Fabry males was more than 2 fold higher than the highest observed level in cardiac variant males (45.3nM vs 19.5 nM) respectively. Hence, plasma Lyso-Gb3 is a potentially useful diagnostic biomarker for FD, as it can be used to categorise and distinguish different phenotypes of FD patients.

In the current study, we observed that the levels of plasma Lyso-Gb3 in three cardiac variant Fabry males showed a slight increase over time and this increase may represent disease progression. However, Smid and colleagues [258] found that the levels of plasma Lyso-Gb3 did not increase appreciably over time in adult patients. Thus, due to lack of long term follow up, it is very difficult to determine whether this slight increase in plasma Lyso-Gb3 levels in our untreated Fabry patients, was simply related to age or it had any prognostic implication. Interestingly, a total of eight cardiac variant patients on ERT showed a trend towards falling Lyso-Gb3 levels. Moreover, in a group of twelve classical Fabry patients on ERT, trend toward falling Lyso-Gb3 levels was noted. The falling Lyso-Gb3 levels of patients on ERT suggest that Lyso-Gb3 has a role in monitoring FD.

Numerous recent publications advocate the use of Lyso-Gb3 rather than Gb3 as a diagnostic biomarker for FD [46, 204, 215, 222]. The concentrations of plasma Lyso-Gb3 have shown useful in diagnosing FD, and at least in one study, shown to correlate with disease severity [215]. Findings presented in Chapter 4 of this thesis further substantiate these claims and support the utility of measuring plasma Lyso-Gb3 levels in the diagnosis of classical and

cardiac variant forms of the disease. Moreover, in agreement with a study by van Breemen and colleagues [165], I have shown that the levels of plasma Lyso-Gb3 are reduced in classical male Fabry patients receiving ERT and may, therefore, have a potential utility in monitoring patients' response to ERT.

In the current study, outlined in Chapter 6, we were able to establish a novel MALDI-TOF-MS based method to measure total urinary Gb3 in Fabry patients. The method is simple, rapid and straightforward. It could easily be implemented in any clinical laboratory which has access to a MALDI-MS and, furthermore it is noninvasive and cost-effective procedure for the detection of classical FD.

The total amounts of urinary Gb3 were sufficiently high in classical FD males (range = 2.7-1931.3 $\mu\text{g}/\text{mmol}$ creatinine) to differentiate them from control males (range = 3.7-23 $\mu\text{g}/\text{mmol}$ creatinine) despite an overlap in small number of subjects in the two groups. Urinary Gb3 levels in classical Fabry females (range = 11.1-126.7 $\mu\text{g}/\text{mmol}$ creatinine) overlapped with that of control females (range 2.2-37.1 $\mu\text{g}/\text{mmol}$ creatinine). Similar results were found for male and female cardiac variant Fabry patients (5.7-31.8 $\mu\text{g}/\text{mmol}$ creatinine vs 5.8-109 $\mu\text{g}/\text{mmol}$ creatinine), compared to classical FD males. The levels in cardiac variant Fabry males and females overlapped with control males and females (3.7-23 $\mu\text{g}/\text{mmol}$ creatinine and 2.2-37.1 $\mu\text{g}/\text{mmol}$ creatinine, respectively). We found that urinary Gb3 can be a potential diagnostic tool for the diagnosis of classical Fabry hemizygotes only but cannot identify classical Fabry heterozygotes or non-classical FD patients carrying cardiac variants. These findings are in keeping with a study by Smid et al [258], where specific emphasis was given to measuring the levels of plasma Lyso-Gb3 in patients with non-classical FD and with *GLA* mutations of unknown significance, who were classified as variants FD, uncertain or as not having FD. Using Gb3 as a biomarker, they failed to

differentiate between these groups, with Gb3 measurements taken from plasma samples only identifying classical male Fabry patients.

As a minimally-invasive procedure, the use of biological fluids circumvents the need for taking biopsies from various tissues and organs. Recently, a number of groups have developed metabolomics-based technologies to measure Lyso-Gb3 and its various analogues in urine samples from Fabry patients [85, 201, 225].

Using the sensitive HPLC-MS/MS protocol developed during the course of this study, we proceeded to quantify the levels of Lyso-Gb3 and its associated analogues in urine samples taken from classical and cardiac variant Fabry patients. Again, urine samples from healthy control subjects were included as a reference. Using this technique, we had proven that it was possible to quantify the amounts of Lyso-Gb3 in urine samples from both classical and a less extent in cardiac variant Fabry patients. Compared to plasma, the concentration of Lyso-Gb3 in urine samples from classical Fabry hemizygotas and heterozygotas were an order of magnitude lower. Also, in contrast to plasma, the presence of various Lyso-Gb3 analogues in urine constituted the bulk of the total pool of Lyso-Gb3, with specific Lyso-Gb3 analogues constituting the major component. The protocol was sufficiently specific and sensitive enough to differentiate both classical and asymptomatic cardiac variant Fabry patients from control subjects. Findings generated as part of my study are in general agreement with those of Auray-Blais and colleagues [85, 225] who used similar approaches to quantify Lyso-Gb3 and associated analogues as a disease-specific biomarker in FD.

Although the levels of urinary Lyso-Gb3 were substantially different between classical and cardiac variant Fabry patients, the levels in cardiac variant Fabry patients were sufficiently

high enough to differentiate these patients from healthy controls. The lack of detectable urinary Lyso-Gb3 in healthy male and female control subjects revealed that urinary Lyso-Gb3 could be used to diagnose both male and female patients with either classical or cardiac variants forms of the disease.

In previous studies, Auray-Blais and colleagues [85] measured the levels of urinary Lyso-Gb3, which, when normalized to the levels of creatinine, were found to correlate significantly with sex, treatment, age and the types of mutations observed in Fabry patients. The relationship between genotype and phenotype has been studied in Fabry patients. In one study, Branton and colleagues have shown that individual presenting with a conservative amino acid change display a significantly delayed onset of symptoms when compared to those with non-conservative amino acid substitutions [273]. However, another study [89], showed that there is no correlation between the levels of plasma or urinary Gb3 and disease severity, clinical presentations in Fabry patients and ERT response [160]. The aforementioned evidence enhances the theory that the measurement of plasma or urinary Gb3 could be an unreliable biomarker for Fabry patients especially in classical Fabry heterozygotes and patients with atypical FD forms.

Lyso-Gb3 constitutes the major component of the total Lyso-Gb3 pool (Lyso-Gb3 and its related analogues) in plasma samples from both classical Fabry hemizygotes and heterozygotes (>80%). Nevertheless, the situation was reversed in urine samples compared to plasma, where urinary Lyso-Gb3 level was reduced to represent approximately (<10%) of the total urinary Lyso-Gb3 pool in urine samples. In urine samples, the sum concentration of the various Lyso-Gb3 analogues constituted approximately 90% of the total Lyso-Gb3 pool compared to approximately 20% in plasma samples. This finding suggests that unlike plasma, measurement of the total pool of Lyso-Gb3 and associated analogues in urine samples constitutes a better analytical tool for diagnosis.

Using a time-of-flight metabolomics approach, Canadian researchers [206, 226] analysed the presence of various Lyso-Gb3 analogues from the plasma of Fabry patients in an attempt to identify “more specific” biomarkers for this disease. Their studies identified six Lyso-Gb3 analogues at m/z values of 758 (-28 Da), 784 (-2 Da), 802 (+16 Da), 804 (+18 Da), 820 (+34 Da), and 836 (+50 Da) whose expression was increased in classical Fabry hemizygotes compared to classical Fabry heterozygotes, but was absent from age-matched controls. My analysis of Lyso-Gb3 and associated analogues revealed the presence of distinct species in the urine from both genders of classical Fabry patients, to varying extents, with Lyso-Gb3 (-28 Da), Lyso-Gb3 (+14 Da), Lyso-Gb3 (+34 Da) and Lyso-Gb3 (+50 Da), constituting the most abundant analogues. Again, in all cases, the amounts of various analogues were always higher in Fabry males compared to Fabry females. In all instances, these comparisons were found to be statistically highly significant. My findings are also in broad agreement with a recent study by Lavoie and colleagues [225], who identified the existence of seven novel urinary Lyso-Gb3 analogues with m/z values of 758 (-28 Da), 774 (-12 Da), 784 (-2 Da), 800 (+14 Da), 802 (+16 Da), 820 (+34 Da), and 836 (+50 Da) in classical Fabry patients.

Similar trends were observed in cardiac variant Fabry patients, where distinct analogues were detected in both male and female patients, with Lyso-Gb3 (-28 Da), Lyso-Gb3 (-2 Da), Lyso-Gb3 (+16 Da), Lyso-Gb3 (+34 Da) and Lyso-Gb3 (+50 Da), being the most abundant analogues. In all cases, the levels of specific analogues were higher in samples from male patients compared to females with the exception of Lyso-Gb3 (+50 Da), where broadly similar concentrations were detected. In all cases, these comparisons were found to be highly statistically significant.

Given the low abundance of Lyso-Gb3 and associated analogues in urine compared to plasma, we quantified the total pool of Lyso-Gb3 and associated analogues in both classical Fabry and cardiac variant Fabry patients. In both cases, the combined total of Lyso-Gb3 plus

analogues was significantly higher in Fabry patients compared to control subjects. To my knowledge, these analyses reveal for the first time, that measurement of the total pool of Lyso-Gb3 and analogues in urine samples is not only sufficiently specific, but also sensitive enough to make an initial diagnosis of classical and cardiac variant forms of FD given the complete absence of Lyso-Gb3 in the urine of control subjects. Measurement of the total pool of urinary Lyso-Gb3 overcomes problems associated with the identification and quantification the expected small amounts of urinary Lyso-Gb3 and its related analogues.

It is important to underscore the importance of measuring the total levels of Lyso-Gb3 and its associated analogues in urine samples from FD patients, as it proved to be a highly sensitive and highly specific analytical tool in aiding FD diagnosis. This sensitivity was due to the complete absence of Lyso-Gb3 and its various related analogues in urine samples taken from healthy control subjects. Similar levels in control subjects were shown in urine samples by Auray-Blais and co-workers [202] and in plasma samples by Aerts and collaborators [46]. Moreover, these findings demonstrate that the measurement of urinary Lyso-Gb3 is superior to the measurement of urinary Gb3, as the latter has been shown to be found in control subjects which expected to be due to the presence of the normally accruing Gb3 isoform (C18:0) [201].

Smid et al [259] reported that in some cases of non-classical FD phenotype, the diagnosis cannot be confirmed definitely by using genetic or enzymatic tests. In their consensus recommendation they suggested that using the histological tests of the heart can be used as a useful diagnostic tool for FD in non-classical Fabry patients (such as cardiac variant phenotype). The study [259] recommended two exit criteria (tests which can be used to exclude FD). The first criterion is the abnormal low ECG voltage and the second criterion is the severe LVH (>15 mm) at young age (<20 years). Nevertheless, the characteristic storage in the endomyocardial biopsy on electron microscopy can be used as a gold criterion for

definite diagnosis of FD [259]. In my study we have demonstrated that a positive Lyso-Gb3 level can support the diagnosis of FD in such situations and a negative Lyso-Gb3 along with criteria above can be of help in excluding FD.

Limitations of the study

A major limitation of this study was the general lack of availability of clinical specimens (blood and urine) from both classical and non-classical FD patients. This was inevitable due to the rarity of the disease. Approximately 100 samples were collected from clinics run at the Northern Hospital in Sheffield, the Royal Free hospital in London, and the Queen Elizabeth hospital in Birmingham - UK. While 100 cases is a respectable number, given the rarity of the disease, multicenter collaborations would generate a larger number of samples and provide better statistical evaluation of the newly derived methodologies and its clinical utility for assessing disease severity and treatment monitoring.

Despite the positive findings of our study, the lack of long term-follow up samples is a limitation of this study. It is likely that if patients were followed up over a longer duration of time, then any change in Lyso-Gb3 in ERT naïve and patients on ERT would be more evident.

While urinary Lyso-Gb3 appears to be a fairly stable compound, the various analogues are quite labile. While the urine samples were snap frozen on collection immediately in liquid nitrogen, any such testing of urinary samples in the future should take this fact into account and ensure that all samples are processed immediately.

Contributions to the field

Our study subsequently led to development of regional laboratory service for testing Lyso-Gb3 in Queen Elizabeth Hospital, Birmingham, UK. This service is now open to Fabry patients across England. Although it is a regional service, it is open to all Fabry patients across UK. We feel this will translate to improved care for Fabry patients. Having a service now in place puts us in a situation to monitor Lyso-Gb3 levels long term.

During the course of this study, a novel rapid MALDI-TOF-MS based method has been developed for measuring urinary total Gb3 in Fabry patients. This work has been published in the American Journal of Mass Spectrometry [1] [Appendix-6].

An important discovery from this research project was to identify the utility of measuring the total levels of Lyso-Gb3 and associated analogues in the urine of Fabry patients. The highly specific and sensitive nature of the LC-MS/MS protocol was able to differentiate clearly between classical Fabry and non-classical Fabry patients. This will not only aid diagnosis but may also have utility in the therapeutic monitoring of patients receiving ERT.

A major strength of this study is the inclusion of a large number of Fabry patients diagnosed as having the cardiac variant forms of FD mainly the mutation N215S. To my knowledge, this is the only study that has examined these patients Lyso-Gb3 profile in detail.

Future work

- Future work will focus on recruiting large number of Fabry patients to see if Lys-Gb3 can be used as disease severity assessment tool and treatment monitoring.
- Measurement and study of the cofactor saposin and its isoforms and study if there is a role in pathogenicity and diagnosis and monitoring of FD.

- To extend the study to include larger numbers of Fabry patients who present with an uncertain genetic diagnosis (as defined by the “Hamlet study: Fabry or not Fabry, Valorization of clinical and laboratory assessments for improved diagnosis of Fabry disease”), to evaluate the utility of urinary Lyso-Gb3 and metabolite measurements in unclear cases of FD mainly patients with cardiac variant and renal variant mutations.
- The identification of other novel related analogues and/or isoforms of Gb3 and Lyso-Gb3 as potential biomarkers for the diagnosis and monitoring of Fabry patients.

In conclusion, the use of biological fluids circumvents the need for taking biopsies from various tissues and organs. Lyso-Gb3, a deacylated form of Gb3 has been identified as a storage product in FD. Interestingly, in addition to Lyso-Gb3, at least six other Lyso-Gb3 related analogues in plasma and seven related analogues in urine with varying base composition have been identified. While the relative distribution of Lyso-Gb3 analogues in urine is often higher than Lyso-Gb3 itself, the relative distribution of these metabolites in plasma has shown that Lyso-Gb3 is the major metabolite present. Plasma Lyso-Gb3 is a sensitive marker for FD and its concentration in plasma is much higher than in Urine. Despite its high water solubility, Lyso-Gb3 is not readily cleared by the kidney. Although male Patients with FD presenting with a classical phenotype can be identified by their very high levels of plasma Lyso-Gb3, the diagnostic value of smaller increases of plasma Lyso-Gb3 levels in atypical late onset cardiac variant patients seems to be attainable.

Lyso-Gb3 is a reliable diagnostic tool to discern classical Fabry males and females from control subjects. The current study suggests that the same applies to patients with cardiac variant phenotype. Lyso-Gb3 values of Fabry females overlap with controls. Consequently, in uncertain cases, increased Lyso-Gb3 values are suggestive of FD, but normal values cannot exclude FD. Confirmation in larger cohorts and data on the specificity of small Lyso-Gb3 increases are necessary.

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APPENDICES

Clinical end points

Blood Pressure: Systolic: (mm Hg), Diastolic:mm Hg

1) Neurological Involvement:

Anhidrosis/hypohidrosis	Yes	No
Pain attacks	Yes	No
Heat intolerance	Yes	No
White Matter Lesions	Yes	No
Stroke	Yes	No
Transient Ischemic Attack (TIA):	Yes	No

(2) Cardiac Involvement:

LVM index (g/m ²)	
Arrhythmia	Yes	No
Myocardial Infarction	Yes	No
Abnormal ECG	Yes	No
Any cardiac intervention	Yes	No

(3) Renal Involvement:

Hypertension	Yes	No
Dialysis	Yes	No
kidney Transplant	Yes	No

(4) Ear:

Tinnitus	Yes	No
Hearing impairment	Yes	No
(pure tone average >25 dB)	Yes	No
Vertigo	Yes	No

(5) Eye:

Tortuous vessels	Yes	No
Cornea verticillata	Yes	No

(6) Skin and GI:

Angiokeratomas or telangiectasia	Yes	No
GI symptoms	Yes	No

Additional comment:

Treatment (complete at 1st patient visit)

Enzyme Replacement Therapy: Yes No

If Yes, Drug: agalsidase alfa agalsidase beta

Dose (mg/kg): **Frequency:**

Date of ERT initiation: **Date of last infusion:**

Medications:

Angiotensin-converting enzyme (ACE) Inhibitors and/or Angiotensin receptor blockers (ARBs):

Yes No

Asprin: Yes No

Statins: Yes No

Appendix-2 Fabry patients consent form



DONATION OF HUMAN TISSUE FOR RESEARCH

Patient Consent Form

Please initial each box if you agree with the statement and then sign the bottom of the form

I have received the Information Sheet entitled 'DONATION OF HUMAN TISSUE FOR RESEARCH' dated 26-Jul-2010 (Version 3).

Please initial

I consent to the storage of my tissues in the Human Biomaterials Resource Centre and their use for ethically approved research projects, including genetic studies.

Please initial

I understand that donated tissue samples may sometimes be used in ethically approved medical research which uses animals, but only when this is absolutely necessary and no alternative approach is available.

Please initial

I understand that giving my tissue for research is completely voluntary and that I am free to withdraw my consent at any time without giving a reason, and without my medical care being affected.

Please initial

I understand that my health records may be accessed for research but that all extracted information will be anonymised.

Please initial

I understand that my tissues may be used by researchers in Birmingham and elsewhere.

Please initial

Name of Patient	Date	Signature

Name of Person taking Consent	Role	Date	Signature

Version 3
26-Jul-2010

*Pink copy to be given to the patient
Top white copy to go in patient's notes
Yellow copy to be retained by the Human Biomaterials Resource Centre*

Appendix-3

The Scientific Contribution and Participation

- I have been trained in mass spectrometry in the Waters-CHUS Expertise Centre (Sherbrooke - Canada) in Clinical Mass Spectrometry 19–23 November 2012.
- Cancer and Genomic Sciences postgraduate researchers Festival, Birmingham-UK, 5–7 June 2013 (Oral presentation).
- Cancer and Genomic Sciences postgraduate researchers Festival, Birmingham-UK, 5–7 June 2014 (Oral presentation).
- Medical and Dental Sciences College Postgraduate Festival 30 March 2015 (poster presentation).
- Analytical tools for cutting-edge metabolomics a joint meeting of the analytical division of the royal society of chemistry and the international metabolomics society - London, UK on 30th April 2014 (Poster presentation).
- Society for the Study of Inborn Errors of Metabolism (SSIEM) 2014 Annual Symposium, Innsbruck, Austria, 2–5 September 2014 (Poster presentation).
- Society for the Study of Inborn Errors of Metabolism (SSIEM) 2015 Annual Symposium, Lyon, France, 1–4 September 2014 (Poster presentation).
- 4th Update on Fabry Nephropathy: Biomarkers, Progression and Treatment Opportunities, Manchester, UK, 1–2 June 2015 (Poster Presentation).
- Fabry disease & the institute of translational medicine (metabolic, renal & cardiac perspectives), Birmingham, UK, 25 November 2015 (slides presentations).

Appendix-4 Training certificate



Faculté de médecine et des sciences de la santé
3001, 12^e avenue Nord, Sherbrooke, J1H 5N4

Sherbrooke, April 19 2016

To whom it may concern,

I would like to mention that Mr Fahad Al-Harbi has been trained in mass spectrometry in the Waters-CHUS Expertise Centre in Clinical Mass Spectrometry, for which I am the Scientific Director, for one week from November 19 to 23 in 2012.

He was quite quick to learn different methodologies for the study of glycosphingolipids for lysosomal storage disorders, mainly for Fabry disease. He was able to perform the preparation of samples, extraction and analysis on tandem mass spectrometry instruments.

He was very dedicated and interested to learn as much as possible in this short period of time. He asked keen questions during his stay in order to be efficient with mass spectrometry technologies during his studies.

Please do not hesitate to contact me if you have questions.

Kind regards,

A handwritten signature in blue ink that reads 'Christiane Auray-Blais'.

Christiane Auray-Blais, LL.M., Ph.D.
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Appendix-5

Publication a raised from this Thesis



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RESEARCH ARTICLE

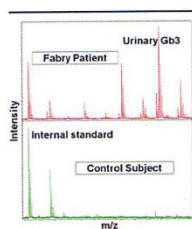
A Novel Rapid MALDI-TOF-MS-Based Method for Measuring Urinary Globotriaosylceramide in Fabry Patients

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Abstract. Fabry disease is an X-linked lysosomal storage disorder caused by deficiency of α -galactosidase A, resulting in the accumulation of glycosphingolipids in various organs. Globotriaosylceramide (Gb3) and its isoforms and analogues have been identified and quantified as biomarkers of disease severity and treatment efficacy. The current study aimed to establish rapid methods for urinary Gb3 extraction and quantitation. Urine samples from 15 Fabry patients and 21 healthy control subjects were processed to extract Gb3 by mixing equal volumes of urine, methanol containing an internal standard, and chloroform followed by sonication and centrifugation. Thereafter, the lower phase was analyzed by MALDI-TOF MS and the relative peak areas of the internal standard and four major species of Gb3 determined. The

results showed high reproducibility with intra- and inter-assay coefficients variation of 9.9% and 13.7%, respectively. The limit of detection was 0.15 ng/ μ L and the limit of quantitation was 0.30 ng/ μ L. Total urinary Gb3 levels in both genders of classic Fabry patients were significantly higher than in healthy controls ($p < 0.0001$). Gb3 levels in Fabry males were higher than in Fabry females ($p = 0.08$). We have established a novel assay for urinary total Gb3 that takes less than 15 min from start to finish.

Keywords: MALDI-TOF-MS, Fabry disease, Biomarker, Gb3

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Introduction

Fabry disease (FD) is a rare multi-systemic X-linked lysosomal storage disorder. This inborn error of glycosphingolipid metabolism results from the absence or deficiency of alpha-galactosidase A (α -GAL A, OMIM 301500), which is responsible for hydrolytic cleavage between the galactose residues in globotriaosylceramide (Gb3). The unmetabolized glycosphingolipids accumulate in various organs, resulting in a range of pathologies in the kidneys, heart, brain, eye, peripheral nervous system, and skin. Male patients show symptoms at an early age whereas females display symptoms when older. The precise incidence of FD is not known: the reported figures vary widely. The rarity of the disease and wide

spectrum of symptoms displayed by Fabry patients make diagnosis based on clinical manifestation problematic. FD is currently diagnosed by measuring the activity of α -GAL A and/or by mutation analysis of the α -GAL A gene where enzyme analysis may not be informative in females [1–7].

Enzyme replacement therapy (ERT), using recombinant α -GAL A has become the standard treatment for symptomatic Fabry patients since 2001 [8–10]. However, the clinical benefit of this expensive treatment is limited, depending on the clinical stage at which therapy is initiated. Gb3 levels in plasma or urine can be used to monitor the efficacy of ERT and as a diagnostic biomarker although with limited sensitivity [11–17]. Gb3 levels in biological samples decrease considerably within 2 wk of starting ERT but later increase in some patients probably due to development of α -GAL A antibodies and do not show a good correlation with effects on clinical symptoms or organ function [11, 12].

Gb3 and its isoforms/analogues have been detected at elevated levels in plasma and urine of Fabry patients using mass spectrometric approaches [14, 15, 18–21]. Several analytical

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methods have been reported for urinary Gb3 extraction and analysis. Auray-Blais and co-workers originally used filter paper discs saturated with urine, which were dried and Gb3 extracted with methanol and analyzed by LC-MS/MS [14]. Later, Krüger and colleagues [15] combined liquid extraction/protein precipitation and solid phase extraction for urinary Gb3 extraction before LC-MS/MS analysis. Despite being fairly complex and time-consuming, these methods generated invaluable data on urinary Gb3 levels in FD. Recently, an advanced multiplex method has been established by Auray-Blais team where they were successful in measuring the levels of different Gb3 isoforms and analogues using liquid-liquid extraction and LC-MS/MS [19, 20].

In this study, we report the development of a rapid, robust assay for Gb3 based on a novel liquid-liquid extraction followed by MALDI-TOF MS, and validate it using urine from patients with classic FD and healthy control subjects.

Materials and Methods

Patient Samples

Fabry patients with diagnoses confirmed by mutation analysis and enzyme activity test were enrolled in the study after giving written informed consent (ethical approval reference: 09/H1010/75). Urine samples were collected from 15 classic Fabry patients and 21 age- and gender-matched healthy controls (patient details are shown in Supplemental Information Table S1). Urine samples were stored at -80°C until further processing without any centrifugation or filtration. Classic Fabry patients are defined as having typical Fabry disease manifestations and known classic mutations with the deficiency of α -GAL A enzyme activity (<1%).

Chemicals

HPLC grade water and acetonitrile (ACN) were purchased from (VWR International-UK). Acetone was purchased from (Fisher Scientific, Loughborough, UK). HPLC grade methanol (MeOH, $\geq 99.9\%$), HPLC grade chloroform (CHCl_3 , $\geq 99.9\%$), 5-chloro-2-mercaptobenzothiazole (5C2M), 2-mercaptobenzothiazole, 6-aza-2-thiothymine, 2-(4-hydroxyphenylazo) benzoic acid, 2,5-dihydroxy benzoic acid (DHB), super-DHB, sinapic acid, 2,4,6-trihydroxy acetophenone monohydrate, 2,5-dihydroxy acetophenone, α -cyano-4-hydroxycinnamic acid, picolinic acid, and 9-aminoacridine hemihydrate were purchased from (Sigma Aldrich, Gillingham, UK). Porcine Gb3 standard and *N*-heptadecanoyl ceramide trihexoside (C17:0) internal standard were purchased from Matreya (Pleasant Gap, PA, USA).

Total Gb3 Standard

Total Gb3 (source: porcine RBC) was used for method development and standard curve purposes. Total Gb3 stock solution (200 ng/ μL) was prepared by dissolving 10 mg of Gb3 standard in 50 mL of MeOH, then stored at -20°C .

Gb3 Internal Standard

N-heptadecanoyl ceramide trihexoside internal standard (0.5 mg) was dissolved in 1 mL of MeOH: CHCl_3 (2:1) and then 9 mL of MeOH was added to generate 10 mL of internal standard at 50 ng/ μL . This solution was stored at -20°C in glass until used as 2 ng/ μL .

Extraction of Urinary Gb3

One hundred μL each of urine, MeOH containing 200 ng of Gb3 internal standard, and CHCl_3 were mixed and incubated in a sonicating water bath for 5 min at room temperature. The mixture was then centrifuged at 13,000 rpm for 5 min and 1 μL of the lower (CHCl_3) layer spotted directly onto a stainless steel 384-position MALDI target in triplicate.

MALDI-TOF-MS

Once dry, the samples were overlaid with 1 μL of 5C2M matrix (saturated solution in 50%MeOH). MS and MS/MS (using laser induced dissociation in LIFT mode) were acquired on a Bruker Ultraflex extreme TOF instrument equipped with a 1 kHz laser in positive ion mode. The MS spectra used for quantitation were the sum of 20,000 laser shots acquired in a random walk pattern. The peak areas used for Gb3 quantitation were extracted from the spectra using ClinproTools software (Bruker).

Gb3 Internal Standard and Assay Calibration

N-heptadecanoyl ceramide trihexoside was used as the internal standard throughout this study. A standard curve was produced by extracting a series of Gb3 dilutions ranging from (0 to 40) ng/ μL made in depleted urine from a healthy individual containing 2 ng/ μL internal standard. The depleted urine was prepared by passing through a C18 cartridge and effective removal of Gb3 confirmed by parallel depletion of urine from a classic Fabry patient analyzed by MALDI-MS (Supplemental Information, Figure S-1). The four most abundant Gb3 species have been used to calculate the urinary total Gb3 level. The sodiated molecular ions of Gb3 species are at mass-to-charge ratios (m/z) of: 1130.7 (C22:0), 1146.7 (C22:0-OH), 1158.7 (C24:0), and 1174.7 (C24:0-OH). The main peak of the Gb3 internal standard is at m/z 1060.7 (C17:0) with a secondary peak at m/z 1076.7 (C17:0-OH). The total area of both internal standard peaks and all four Gb3 species were used to determine the response ratio (Equation 1) and used in conjunction with a calibration curve to determine urinary Gb3 concentrations.

$$\text{Response ratio} = \frac{\text{total area of the 4 most abundant Gb3 species}}{\text{area of Gb3 internal standard}} \quad (1)$$

The limit of detection (LOD) and the limit of quantitation of total Gb3 in urine were determined using (Equation 2) and (Equation 3) respectively, where $F = 3.3$ and 10 for LOD and

LOQ respectively, $stdev$ = standard deviation and b : slope of the regression line (Supplemental Information Figure S-2).

$$LOD = \frac{F \times stdev}{b} \quad (2)$$

$$LOQ = \frac{F \times stdev}{b} \quad (3)$$

Results and Discussion

Optimization of Gb3 Extraction from Urine

Depleted urine was spiked with 2.5 μL of porcine total Gb3 standard [200 ng/ μL] generating a final concentration of 5 ng/ μL , and the performance of various extraction procedures involving different volumes of MeOH, acetone, and CHCl_3 were assessed by MALDI-TOF-MS. We found using 1:1:1 (v:v:v) of urine, MeOH, and CHCl_3 to be the optimal condition for Gb3 extraction into the hydrophobic layer (CHCl_3 layer) based on peak intensity and signal-to-noise ratio in the mass spectra (Figure 1) with no Gb3 detectable in the aqueous layer (Supplemental Information Figure S-3).

Optimization of MALDI-TOF-MS

We acquired and compared the MALDI spectra for depleted urine spiked with 5 ng/ μL Gb3 using 12 different matrix compounds (spectra shown in Supplemental Information Figure S-4). The matrices were used as saturated solutions both in 50% MeOH and 50% ACN. Spectra obtained with 5C2M in 50% MeOH provided the best signal intensity and signal-to-noise ratio. Example MS spectra showing Gb3 peaks in the urine of a Fabry patient, porcine Gb3 standard, and a healthy control subject are shown in (Figure 2).

MS/MS Confirmation of Gb3 Peaks in Fabry Patient Urine

MS spectra of Fabry patient urine contain peaks with exactly the same m/z values as those in purchased porcine Gb3: m/z 1046.7 (predicted structure C16:0), 1074.7 (C18:0), 1102.7 (C20:0), 1128.7 (C22:1), 1130.7 (C22:0), 1146 (C22:0-OH), 1156.7 (C24:1), 1158.7 (C24:0), 1172.7 (C24:1-OH:), and 1174.7 (C24:0-OH). To confirm that the peaks in Fabry patient urine spectra correspond to Gb3, they were subjected to MALDI-TOF/TOF MS/MS. The MS/MS spectra of the urine peaks show the loss of sugar moieties (Figure 3) and confirm

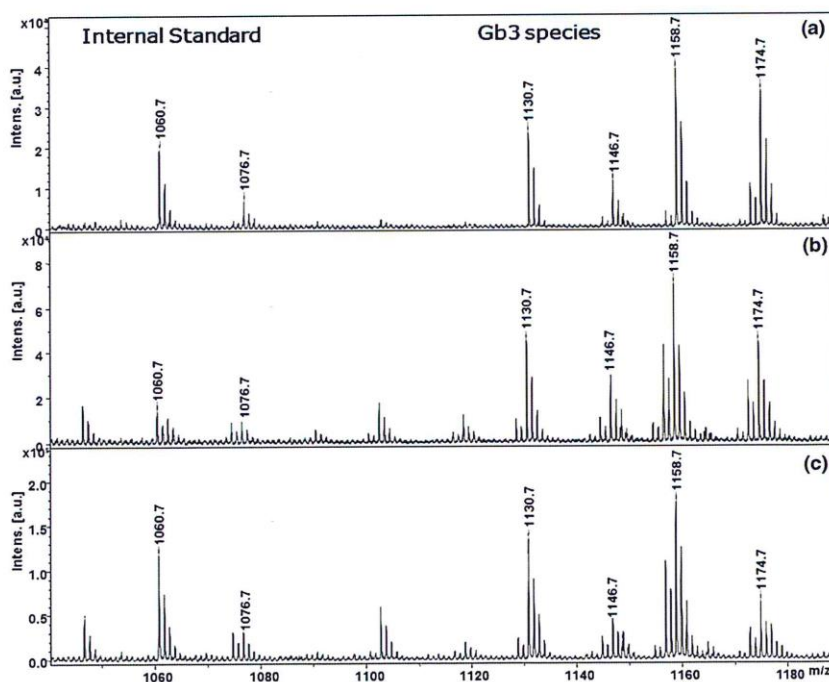


Figure 1. Validation of liquid-liquid extraction using different solvents: (a) acetone: MeOH:H₂O, (45:45:10) (b) MeOH:CHCl₃, (2:1) (c) MeOH:CHCl₃ (1:1)

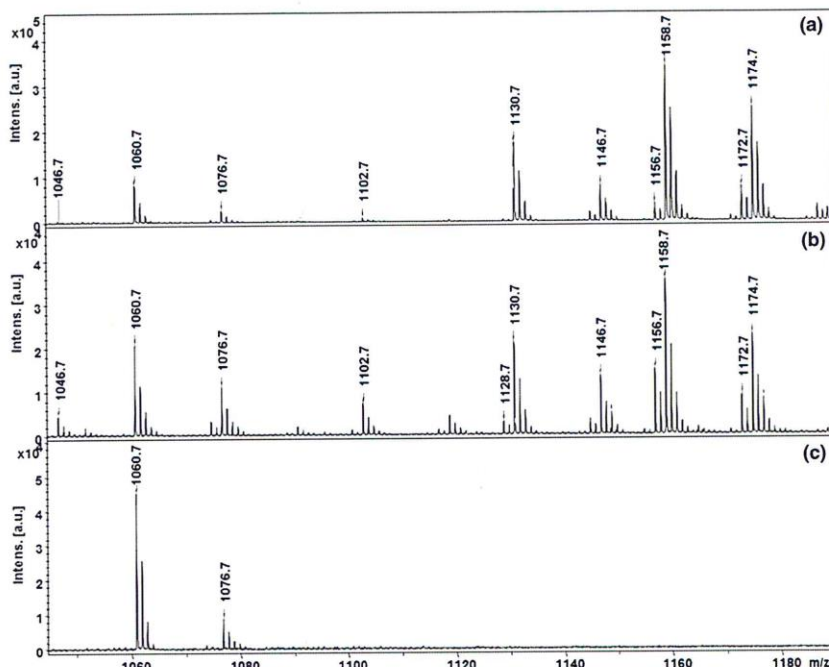


Figure 2. MS spectra of Gb3 species and Gb3 internal standard in (a) purchased porcine Gb3 standard, (b) urine from an untreated classic Fabry male, and (c) healthy control urine

that these ions correspond to Gb3 species by the presence of the fragment m/z 264.2, which represents sphingosine (sphingosine-2[H₂O]). The purchased porcine Gb3 generated identical MS/MS spectra (data not shown).

Quantitative Measurement of Urinary Gb3 by MALDI-TOF-MS

To correct for variations in extraction and ionization efficiencies an internal standard was added to all urine samples prior to extraction. The internal standard appears as a peak at m/z 1060.7 (C17:0) (plus a secondary peak at m/z 1076.7(C17:0-OH)). No peaks were seen at these m/z values in the MALDI spectra of any of the extracted urines used in this study in the absence of internal standard (Supplemental Information Figure S-5) (also, no contaminants with the same masses as the Gb3 peaks were detected). Gb3 concentrations were calculated from the (total Gb3/internal standard) area ratio as described in Methods and using the calibration curve shown in Figure 4. Inter-assay and intra-assay variability were measured to assess the reliability of the method. Urine samples of 10 healthy controls were used. Each sample was spiked with 2.5 μ L of total Gb3 standard [200 ng/ μ L] generating a final concentration of 5 ng/ μ L. Each sample was processed as three

independent technical replicates. Each extract was spotted onto the MALDI target in triplicate giving nine readings for each sample. The same procedure was repeated on three different days resulting in each sample being analyzed 27 times. The results showed high reproducibility and reliability of the method with an intra-assay coefficient of variation of 9.9% and inter-assay of 13.7%. The lower limit of detection was 0.15 ng/ μ L, and limit of quantitation was 0.30 ng/ μ L. The assay was linear up to the highest Gb3 concentration investigated (40 ng/ μ L).

Urinary Gb3 Levels in Fabry Patients

We measured the levels of urinary Gb3 in classical Fabry patients ($n = 15$) and healthy controls ($n = 21$) as shown in Figure 5. The mean Gb3 concentrations in classic patients and healthy controls were 1.35 ng/ μ L and 0.08 ng/ μ L, respectively. Prior to statistical comparison, the urinary Gb3 concentrations were normalized to urinary creatinine. Overall, the mean normalized concentration in the classic Fabry patients was significantly higher than healthy controls ($n = 21$, mean = 6.7 μ g/mmol creat, ($p < 0.0001$). The normalized mean concentration in the classic Fabry males was higher than in Fabry females (171.7 v 57.9 μ g/mmol creatinine) although this did not reach statistical significance ($p = 0.08$), and

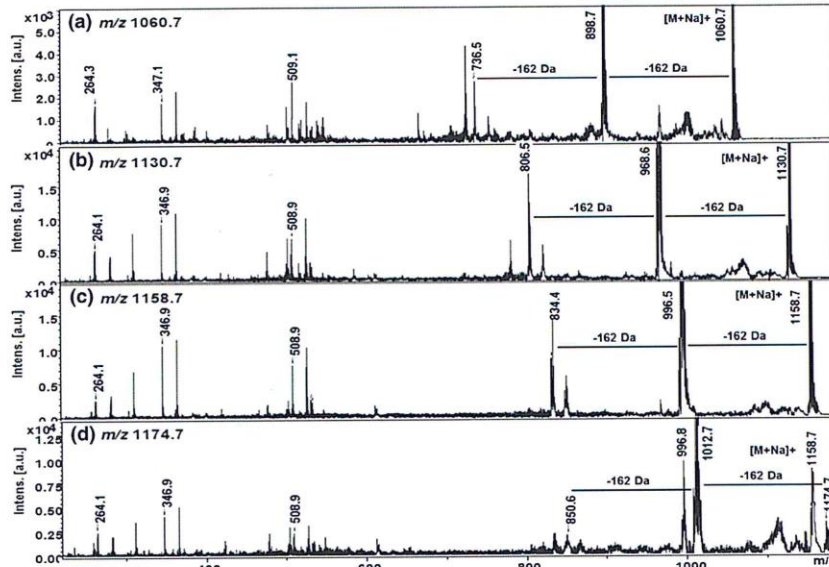


Figure 3. MALDI-TOF/TOF MS/MS of (a) Gb3-internal standard m/z 1060.7 (C17:0); and the three most abundant Gb3 species in Fabry patient urine: m/z 1130.7 (C22:0), 1158.7 (C24:0), and 1174.7 (C24:0-OH). The peaks corresponding to ions generated by the loss of 1 or 2 sugars (-162 and -324 Da, respectively) have different m/z values for each species, whereas the fragments corresponding to sphingosine -2H₂O, (m/z 264.1) and di- and tri-saccharide ions (m/z 347 and 509) are invariant

urinary Gb3 was significantly higher in both male and female FD patients than in healthy controls ($p < 0.0001$ in both cases). Using the urinary Gb3 concentrations measured in the 36 individuals in this study to detect Fabry disease generated a receiver operator characteristic (ROC) curve with an area of 0.92 (95% CI 0.79–1.00) and, using a threshold of 25 $\mu\text{g}/\text{mmol}$ creatinine gave a sensitivity of 80% (95% CI 52–96) at 100% specificity (95% CI 84–100), (Supplemental Information Figure S-6).

Gb3 generates multiple peaks in mass spectra. The total area of the four most intense peaks [m/z 1130.7 (C22:0), 1146.7 (C22:0-

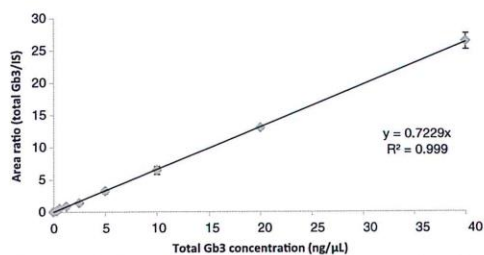


Figure 4. Gb3 standard curve. Increasing amounts of porcine Gb3 standard covering the reported clinical range and a constant amount of Gb3 internal standard were spiked into depleted urine from a healthy control subject. Thereafter, the lipids were extracted and analysed by MALDI-TOF MS

OH), 1158.7 (C24:0), and 1174.7 (C24:0-OH)] was used to measure the ratio of Gb3 to the internal standard. These four peaks were selected because they have the highest intensities, and they were detected in all patients whereas the other peaks (other Gb3 species) were not always detected. We found that the ratio of these four peaks to one another is constant across individuals (i.e.

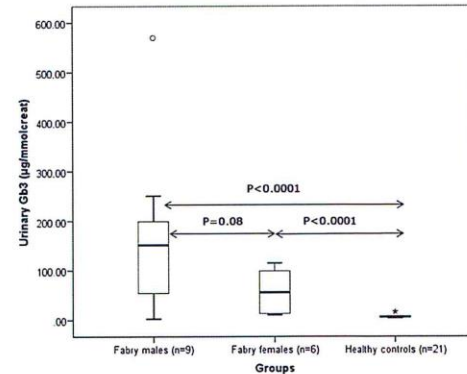


Figure 5. Urinary total Gb3 levels: box-and-whiskers plot showing urinary Gb3 levels in both genders of classical Fabry patients and healthy control subjects (P values obtained using t -tests)

classic FD results in accumulation of all 4 Gb3 species). Although we have used the sum of the four main peaks for quantitation (considering that this might be the most robust approach), we also found that any one of the four peaks alone would yield the same results (data not shown).

Discussion

By coupling a novel liquid–liquid extraction and MALDI-TOF-MS with an internal standard, we have been able to develop a rapid (<15 min from start to finish) method for measuring Gb3 in urine. Considerable optimization of the Gb3 extraction and MS analysis has been performed to produce an assay with good reproducibility, sensitivity, and specificity sufficient to easily detect the elevated levels of urinary Gb3 that are expected in classic Fabry patients. *N*-heptadecanoyl ceramide trihexoside has been used as an internal standard in this study because it has very similar chemical properties to human Gb3 but cannot be synthesized by humans (due to the odd number of carbon atoms). The internal standard generates a main peak at *m/z* 1060.7 (C17:0) and a minor peak at *m/z* 1076.7 (C17:0-OH) (most likely due to oxidation, both peaks were combined in calculations). Following liquid–liquid extraction human urine is devoid of peaks at these *m/z* values unless the internal standard is added (Supplemental Information Figure S-5). Including the internal standard in our assay has enabled a high degree of reducibility and reliability to be reached with correspondingly low intra-assay and inter-assay coefficients of variation. The assay is based on MS; however, MS/MS was used to verify the identity of the Gb3 peaks: the most abundant peaks at *m/z* 1130.7 (C22:0), 1146.7 (C22:0-OH), 1158.7 (C24:0), and 1174.7 (C24:0-OH) all show identical fragmentation pattern by losing sugar groups (–162 Da) and generating the di-dehydrated sphingosine moiety (*m/z* 264.2) as previously reported for Gb3 [13, 14, 16, 18, 22].

Conclusion

Our method significantly reduces the number of steps and time required to complete the test compared with the current standard methods. The method could be easily implemented in any laboratory with access to a MALDI mass spectrometer and used for noninvasive, cost-effective detection of Fabry disease.

Acknowledgments

The authors would like to say a special thank you to all Fabry patients and healthy volunteers who participated in this study. This work was supported by Prince Sultan Military Medical City, Riyadh, Kingdom of Saudi Arabia (Grant Agreement no: JAM2254).

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Appendix-6

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S146

J Inherit Metab Dis (2014) 37 (Suppl 1):S27–S185

Background: Niemann-Pick type C (NPC) is a neurodegenerative lysosomal storage disease caused by incorrect intracellular lipid trafficking. Mutations in two genes are responsible of NPC: NPC1 (95 % cases) and NPC2 (5 % cases). Filipin staining in cultured fibroblasts and chitotriosidase activity are biochemical markers commonly used in clinical practice in diagnostic algorithm of NPC, apart from DNA analysis. **Objective:** Molecular genetic analysis of 19 patients with clinically diagnosed NPC disease.

Results: 19 NPC patients with genetically confirmed diagnosis were studied. Clinically 14 patients presented with early infantile onset, 6 with juvenile onset NPC disease. Direct sequencing of genomic DNA was used to identify NPC1/NPC2 mutations. All identified mutations were in the NPC1 gene. 26 different disease-causing mutations were found: 7 deletions and 19 missense mutations. 12 mutations have not been previously described. The mutation S954L was observed in 4 patients and was associated with a juvenile onset form. The mutation 2316_2317insT was found in 4 patients (3 juvenile and 1 early onset form).

Conclusions: Direct sequencing of the NPC1/NPC2 genes allows accurate diagnosis of NPC. Our study indicates possible genotype-phenotype correlations in NPC. Further study are needed

P-392

Is NPC2 more common than NPC1?

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Background: Niemann Pick type C (NPC) disease is a lysosomal storage disorder affecting the intracellular trafficking of unesterified cholesterol. So far it was shown that 95 % of NPC patients show mutations in the NPC1 and only 5 % in the NPC2 gene.

Case report: We identified an 18 year old girl with a typical NPC phenotype with first neurological symptoms at the age of 12y. Symptoms included decline of neurocognitive and motoric performance, vertical gaze palsy, weight loss >12 kg in one year, dysarthria and dysphagia.

Results: Biomarkers for NPC (cholestan-3 β ,5 α ,6 β -triol and chitotriosidase activity) were normal. A genetic analysis revealed a homozygous splice mutation in NPC2, with an allele frequency of 0.9 % (<http://evs.gs.washington.edu/EVS/>) and a homozygosity frequency of 1:12.000. Three different abnormal protein variants are synthesized. At least one of those proteins is expected to provide residual function. A filipin staining in fibroblasts showed a cholesterol accumulation typical for NPC disease.

Conclusion: The frequency of this mutation indicates that NPC is ten times more frequent than currently estimated and that NPC2 disease is more frequent than NPC1. Although an early therapy allows slowing of disease progression, insufficient knowledge of the disease and normal biomarkers prevents early recognition of this treatable metabolic disorder. Conflict of Interest declared.

P-393

Unusual bone presentation in a 4 years old female with Gaucher disease (GD) type 3

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Background: Bone manifestations are common in GD patients but only few cases showed involvement of short bone. We present a new case of vertebral body involvement in a child with GD 3.

Case Report: S.T., female, was diagnosed with sickle cell trait at 9 m because of microcytic anemia. At age 3y splenomegaly was identified which was ascribed to CMV infection. After 1y, because of hepatosplenomegaly, anemia and thrombocytopenia, bone marrow aspiration was performed showing foam cells leading to the diagnosis of GD (high ACE, glucocerebrosidase absent on DBS). Molecular analysis confirmed GD3 (Rec Ncil+N188S). Two days before ERT, fourteen after diagnosis, she developed intensive back pain and deambulation refuse without fever, normal cell count and CRP. Lumbar MRI during acute episode showed signal hyperintensity (T1 and T2) of the entire L1 vertebral body. She needed analgesic drugs iv for 5d. ERT was started. After 3 months the area of hyperintensity was still present on MRI but reduced without collapse.

Conclusions: We present a peculiar case of haemorrhagic infarct of a short bone in a 4y old child with a recent diagnosis of GD 3. We highlight the importance of early diagnosis because ERT could prevent bone crises but cannot reverse skeletal changes.

P-394

HPLC-MS method development for Fabry disease biomarkers analysis

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Background and objectives: Fabry disease is an X-linked lysosomal storage disease caused by the deficiency of α -galactosidase A, resulting in glycosphingolipids accumulation in body fluids and different organs. Globotriaosylceramide (Gb3), globotriaosylsphingosine (Lyso-Gb3) and their analogues have been identified and quantified as biomarkers for the disease severity and treatment efficacy. The current study aimed to develop HPLC-MS methods in order to identify and quantify FD biomarkers.

Materials and Methods: Human Fabry patients' plasma and urine samples were processed using solid phase extraction. The samples were then analysed for levels of Lyso-Gb3 and its analogues using HPLC-ESI-MS.

Results: Extraction recovery was 90 % for urine and 70 % for plasma. Reverse phase-HPLC methods were optimised with an isocratic elution of (0.1 % formic acid/50 % acetonitrile) and flow rate of 3 μ L/min. A multiple reaction monitoring mode MS method was optimised for identification and quantification of metabolites showing limit of detection of 10fmoles Lyso-Gb3. Lyso-Gb3 and 7 analogues were detected showing comparable fragments. These analogues vary from Lyso-Gb3 due to a modification in the sphingosine moiety.

Conclusion: We have established an HPLC-ESI-MS approach for analysis of Lyso-Gb3 and its analogues. Pilot data shows low levels of these biomarkers are quantified in urine and plasma.

P-395

Biological study of pediatric forms of Gaucher disease in Moroccan patients

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Appendix-7

SSIEM 2015 Annual Symposium: Lyon, France, 2–5 September 2015
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J Inherit Metab Dis (2015) 38 (Suppl 1):S35–S378

S267

diagnosis, followed by thrombocytopenia and anaemia. As the main cause of diagnostic delay, 8/12 experts stated lack of awareness of GD or misdiagnosis; 4/12 stated phenotypic heterogeneity, non-specific clinical presentation or mild symptomatology; and 1/12 stated outsourced testing.

Conclusion: Trends from a physician survey suggest that haematology/haem-oncology is the main specialty to which GD patients first present, and splenomegaly is the main presenting feature at GD diagnosis. There is a need to increase awareness of LSDs across a range of specialties.

Conflict of Interest declared.

P-532

Insight into the pre-diagnosis period of patients with Gaucher disease: results of the OnePath® US patient survey

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Objective: To examine the pre-diagnosis period of patients with Type 1 Gaucher disease (GD1).

Methods: Consenting GD1 patients registered in the US OnePath® patient support system completed a survey with 13 questions related to their pre-diagnosis journey.

Results: 212/576 invited patients responded. An enlarged stomach/abdomen was cited most as the first symptom or sign of GD that patients recall experiencing, although an abnormal blood test result was the most cited final GD-related problem that prompted patients to seek further medical advice. Paediatrics, haematology/haem-oncology and primary care were the most cited specialties to which patients first presented regarding their health problems, with haematology/haem-oncology being the main specialty to make the final GD diagnosis (70/116 patients, 60%). 78/208 patients (38%) were diagnosed before the age of 10 years. Most patients (112/154, 73%) were diagnosed ≤1 year after first seeing a doctor, but for others (22/154, 14%), diagnosis took ≥7 years. Commenting on their path to diagnosis, many patients described experiences with physicians lacking awareness of GD and previous misdiagnoses.

Conclusion: An enlarged stomach/abdomen was the most common first GD-related problem. Haematology/haem-oncology was the main specialty involved in making a GD diagnosis, although it could take more than 7 years from earliest symptoms to eventual diagnosis.

Conflict of Interest declared.

P-533

Plasma and urinary levels of glycosphingolipids in cardiac variant (N215S) Fabry patients

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Introduction: Fabry disease is an X-linked lysosomal storage disease caused by defective α -galactosidase A. This defect leads to accumulation of glycosphingolipids in various organs and body fluids. Globotriaosylceramide (Gb3), globotriaosylsphingosine (lyso-Gb3) and related isoforms/ analogues have been investigated as biomarkers for Fabry disease severity and treatment efficacy. We now report the levels of glycosphingolipids in the plasma and urine of cardiac variant patients with the N215S mutation.

Methods: Plasma and urine were collected from 40 cardiac variant Fabry patients, 73 classical Fabry patients and 34 age/gender matched healthy controls. Lyso-Gb3 and 8 related analogues were enriched by solid phase extraction and analysed by LC-MS/MS. Urinary Gb3 was measured using a novel rapid method utilising liquid-liquid extraction and MALDI-TOF-MS.

Results: Plasma and urine levels of lyso-Gb3 and several of its analogues were significantly higher in N215S male patients than in healthy male controls but significantly lower than in classical male Fabry patients. These glycosphingolipids were also significantly elevated in the plasma of N215S female patients but did not reach statistical significance in urine. Urinary Gb3 was not significantly elevated in N215S patients.

Discussion: This study shows that plasma levels of lyso-Gb3 and its analogues may prove useful in monitoring cardiac variant Fabry disease.

P-534

Prenatal diagnosis of Gaucher disease using next generation sequencing technology

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Background and purpose: Gaucher disease (GD) shows severe progressive neurological manifestations. To analyze genetic

Appendix-8

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6

WSI Quantitative (BLISS) and Semiquantitative (FSS) Assessment for Cortical PTC Gb3 Inclusions in Fabry Disease Patients Following IV Administration of Plant Derived Alpha-GAL-A Enzyme (PRX-102)

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Introduction: Previous studies on Fabry disease patients receiving enzyme replacement therapy (Fabrazyme) showed reduction of Gb3 inclusions in renal peritubular capillaries (PTC) endothelium by using a semiquantitative approach (Fabrazyme Scoring System – FSS). A subsequent study with male and female Fabry patients evaluating the efficacy of a pharmacological chaperone (AT1001) demonstrated that the application on annotated whole slide images (WSI) of a quantitative scoring methodology (Barisoni Lipid Inclusion Scoring System – BLISS) increases sensitivity, accuracy and reproducibility. The aim of this study is to evaluate the efficacy of a novel therapeutic option for Fabry disease, a plant derived α galactosidase A enzyme (PRX-102), by applying both BLISS and FSS on annotated WSI.

Methods: 5 symptomatic Fabry patients (2/3, F/M), either naïve or free from ERT in the last 6 months, with negative anti PRX-102 antibodies, and with eGFR ≥ 60 ml/min/1.73 m² received IV-PRX-102 (0.2 mg/kg every 2 weeks). Plastic embedded 1 μ thick sections stained with toluidine blue from kidney biopsies obtained prior the first IV treatment and at 6 months of treatment, were scanned into WSI (Aperio) at 100x. 300 PTC/biopsy were first annotated by one renal pathologist and then scored by 2 different renal pathologists, using BLISS and FSS in a random and blinded

manner. Individual PTCs with discrepant score were rescored for adjudication by the annotator.

Results: Substantial reduction of Gb3 inclusions was noted in PTC using both BLISS and FSS (figure 1).

Conclusions: The application of BLISS and FSS on annotated WSI to assess PRX-102 IV infusion efficacy shows significant reduction of PRC Gb3 renal PTC inclusions after 6 months of therapy.

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7

Validation of HPLC-MS Approach for Analysis of Fabry Disease Biomarkers

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Introduction: Fabry disease (FD) is an X-linked lysosomal storage disease caused by the deficiency of α -galactosidase A, resulting in the accumulation of glycosphingolipids in body fluids and different organs. Globotriaosylceramide (Gb3), globotriaosylsphingosine (Lyso-Gb3) and their analogues have been identified and quantified as biomarkers for the disease severity and treatment efficacy. The current study aimed to develop HPLC-MS methods in order to identify and quantify FD biomarkers.

Methods: Human plasma and urine samples were collected from healthy controls, Fabry patients and patients of other 5 disorders including (renal, adrenal, vascular, liver autoimmune and inflammatory bowel disorders). The samples were processed using

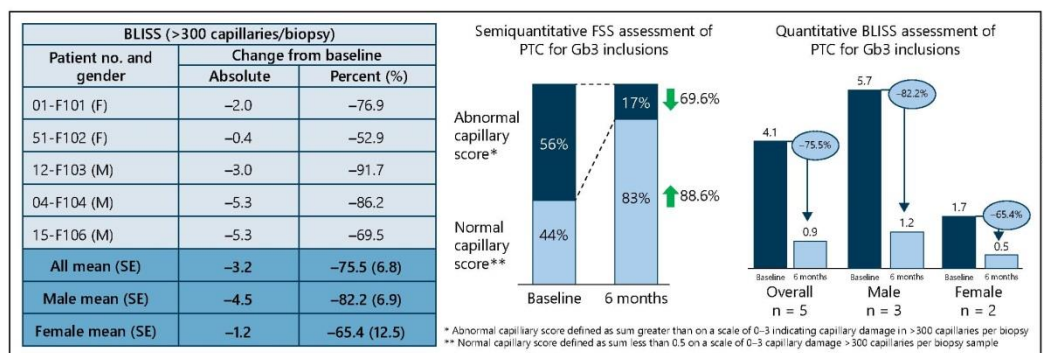


Fig. 1. (for Abstract 6).

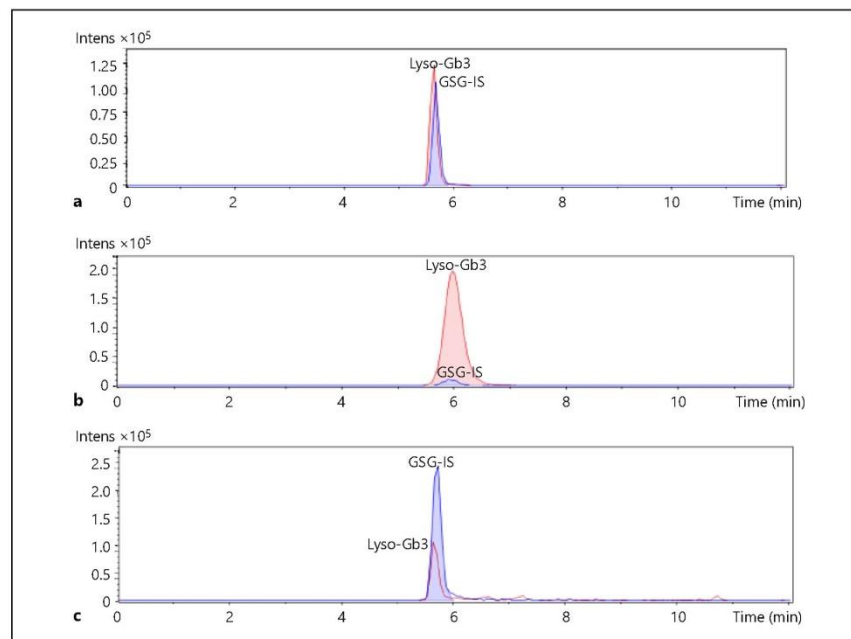


Fig. 1. Extracted ion chromatogram of (a) Lyso-Gb3 standard (made in-house), (b) Lyso-Gb3 in a plasma sample from a classical Fabry male, (c) Lyso-Gb3 in a urine sample from a classical Fabry male (for Abstract 7).

solid phase extraction before being analysed for Lyso-Gb3 and its analogues levels using HPLC-ESI-microTOFMS.

Results: Lipids extraction from urine and plasma samples showed a recovery of 90% & 70% respectively. Reverse phase-HPLC methods were optimised with an isocratic elution of (0.1% formic acid/40% acetonitrile) and flow rate of 3 μ L/min. A multiple reaction monitoring mode MS method was optimised for metabolites analysis showing limit of detection and quantification of 10 & 20 fmoles Lyso-Gb3 respectively. Lyso-Gb3 and its analogues levels and their correlations with vital factors were studied by details.

Conclusion: We have developed an HPLC-ESI-MS approach for analysis of Lyso-Gb3 and its analogues. Pilot data shows low levels of these biomarkers are quantified in urine and plasma.

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8

Fabry Disease Diagnosed in Living Donor Kidney Transplant Biopsy

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Introduction: A 33 year old woman donated a kidney to her 52 year old brother, a hemodialysis patient with presumed IgA nephropathy, not biopsied. Comorbid conditions included a diagnosis of multiple sclerosis, atrial fibrillation, left ventricular hypertrophy and a transitory ischemic attack. The transplanted kidney was biopsied shortly after transplantation due to proteinuria. Fabry disease was diagnosed based on GL3 deposits in the podocytes, and the recipient was started on Agalsidase Beta 1.0 mg/kg/eow one year

Appendix-9
HPLC-MS instruments utilised in the study

