AN INVESTIGATION INTO THE ASSOCIATION BETWEEN FABRY DISEASE, ITS CLINICAL MANIFESTATIONS AND CHRONIC RENAL FAILURE IN PATIENTS ATTENDING PUBLIC HOSPITALS IN KWAZULU-NATAL

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Submitted in fulfilment of the academic requirements for the degree of

MASTERS IN HEALTH SCIENCES

in the

Department of Clinical Technology

Faculty of Health Sciences,

Durban University of Technology, Durban

AUGUST 2022

AUTHOR'S DECLARATION

I, Jillian Singh, confirm that the work presented in this dissertation titled "**AN INVESTIGATION INTO THE ASSOCIATION BETWEEN FABRY DISEASE, ITS CLINICAL MANIFESTATIONS, AND CHRONIC RENAL FAILURE IN PATIENTS ATTENDING PUBLIC HOSPITALS IN KWAZULU- NATAL**", is my own. It has not been submitted to any other tertiary institution. Any information which has been derived from other sources has been acknowledged in the text.

The research described in this dissertation was carried out in the Department of Clinical Technology, Faculty of Health Sciences, Durban University of Technology, under the supervision of Dr SC Benjamin and the Nelson R Mandela School of Medicine, under the supervision of Professor AGH Assounga.

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I hereby certify that the above statement is correct.

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PROFESSOR AGH ASSOUNGA_____

ABSTRACT

Fabry disease is characterized as a genetic, progressive, lysosomal storage disorder. It is inherited in an X-linked manner in which the mutated gene inhibits the functioning of the alpha-Galactosidase-A enzyme causing a deficiency or absence of the enzyme. This results in the accumulation of glycolipids, particularly globotriaosylceramide (Gb3) in the lysosomes causing progressive damage to tissues and major organs. Fabry nephropathy is progressive and is one of the major organ complications after cardiovascular manifestations caused by Fabry disease. Left untreated, Fabry nephropathy can result in end-stage kidney disease.

To our knowledge, no research has been conducted to determine the association between Fabry disease, its clinical manifestations, and chronic kidney disease in KwaZulu-Natal.

Methods: This study was a prospective, quantitative study. A total of 200 male patients with chronic kidney disease (CKD stage 2-5D) were enrolled in three dialysis clinics at Inkosi Albert Luthuli Central Hospital, Addington Hospital and St Aidan's Hospital in KwaZulu-Natal. A control group of 14 healthy males was also enrolled for this study. The ELISA technique was employed to determine the alpha Gal-A enzyme concentration levels in the plasma. A questionnaire using the MSSI scoring system was presented to the participants to identify clinical manifestations.

Results: A cut-off value for the alpha Gal-A enzyme concentration levels of <500pg/ml was calculated using the standard deviation and mean. A total of 17 participants from the patient group (n=11) and the control group (n=6) displayed alpha-Gal-A enzyme levels <500pg/ml. A *p*-value of <0.05 was considered to be statistically significant. A statistically significance result was exhibited between alpha-Gal levels of <500pg/ml and demographic parameters such as age (p=0.007), where the mean age was 30.5 years. Clinical parameters such as heat or cold intolerance, MSSI scores and hypertension also displayed significance. Heat and cold intolerance displayed a *p*-value of 0.049, where 2 patients reported the manifestation. MSSI scores displayed a negative association where p=0.001. Low MSSI scores should correlate with high alpha-Gal levels, however, in this study, all the patients displayed low MSSI scores between 9 and 12.5 with low alpha-Gal

levels. Hypertension also presented with a significance of $p<0.001$. A total of 4 patients were diagnosed with hypertension.

Conclusion: Fabry disease is suspected in a total of 17 participants with alpha-Gal levels of <500pg/ml. The cause of CKD nephropathy raises interest as conditions such as FSGS have been associated with FD. The low levels of the alpha-Gal enzyme and presentation of the clinical manifestations can be used as preliminary findings. It is recommended that confirmatory tests such as DNA analysis or Gb3 and GL3 analysis should be performed to confirm the diagnosis.

DEDICATIONS

The completion of this thesis would not have been possible without the guidance of the amazing people who stood by me and believed in me. I dedicate this work to:
The Divine above for always guiding me even in the most challenging times. I am eternally grateful for the lessons that were presented to me. I have grown emotionally and spiritually through this.

My husband, Atish Singh for being my pillar of strength and never allowing me to give up. You have taught me to be patient and trust divine timing. Thank you for taking on the responsibilities of the home and persuading me to complete this work. Your constant encouragement motivated me to continue and complete this task. A big thank you for always being there for my emotional rollercoasters and putting up with my moods. You are truly the best husband. To my two precious daughters Adthi and Nidhi, I cannot begin to express my gratitude for all your patience and understanding during these years. I am so proud to have such compassionate, caring, kind, and understanding girls.

To my Mum and Dad. Thank you for all your support and motivation. Even in the most challenging of times, you encouraged me to recognise my potential. Your advice and guidance were and will always be priceless to me. To my siblings, Yuna, Leena, and Ashley. Thank you for all your assistance : the advice, the chauffeuring, and going the extra mile to assist me. I am forever grateful. To my nieces and nephews for always enquiring about my work and taking an interest in everything I do. You are all amazing.

ACKNOWLEDGEMENTS

I would like to offer my sincere gratitude to the following:
My supervisor, Dr. SC Benjamin, for her guidance, willingness to assist, knowledge and motivation.

My clinical supervisor, Prof AGH Assounga for his invaluable knowledge and expertise, his patience, guidance, motivating talks, and encouragement.

The Durban University of Technology (DUT) for allowing me the opportunity to undertake and complete the degree. I thank the staff of the Faculty of Health Sciences and the Department of Biomedical and Clinical Technology for their assistance.

The KwaZulu-Natal Kidney Association for awarding me the David Hepburn Study Award which contributed to the research and the consumables thereof.

The Department of Health for permitting me to recruit patients at the provincial hospitals for my research.

The hospital managers of Inkosi Albert Central Hospital, Addington, and St Aidan's for allowing me to recruit patients and trusting me to enter the dialysis units during the COVID-19 pandemic.

The unit managers Sr E Jacobus, Sr K Kasiga, Sr M Green, Sr T Maseko, and the staff of all the units for their assistance and time.

The Secretary of the Nephrology Department at IALCH, Ms. Jane Padayachee for her willingness to assist me and the time-consuming phone calls and emails in her effort to assist me.

The Principal Technician of the SCM Lab at the Nelson R Mandela School of Medicine, Ms. Fazana Dessai for teaching me the ELISA technique, and the operations of the lab and offering her time to ensure my research testing was successful.

Mr. Daya Rampersad, COO of Lab 24, for generously sponsoring the kidney function tests for the control group. I am truly grateful for the gesture offered.

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ABBREVIATIONS

AAV	-	Adeno-associated Virus
ALPHA GAL A-		Alpha- Galactosidase A
ACD	-	Angiokeratoma corporis diffusum
ADA	-	Anti-drug antibody
Aδ fibres	-	A Delta fibres
BP	-	Base pairs
CWMH	-	Chronic White Matter Hyperintensities
C fibres	-	Transmits diffuse pain signals from the periphery to the spinal cord
CV	-	Cardiovascular
CKD	-	chronic kidney disease
CRRT	-	chronic renal replacement therapy
CWMH	-	chronic white matter hyperintensities
CNS	-	central nervous system
CV	-	cornea verticillata
CsC	-	Cystatin-C
Gal2 Cer	-	digalactosylceramide
DNA	-	Deoxyribonucleic acid
DBS	-	dried blood spot
ECG	-	electrocardiogram
EDTA	-	Ethylenediamine tetra acetic acid
eGFR	-	estimated glomerular filtration rate
ELISA	-	Enzyme-Linked Immunosorbent Assay
ESRD	-	end-stage renal disease
ERT	-	enzyme replacement therapy
EBPG	-	European Best Practice Guidelines
FD	-	Fabry disease
DS3	-	Fabry Disease Severity Scoring System
FOS	-	Fabry Outcome Survey
FOS-MSSI	-	Fabry Outcome Survey Mainz Severity Score Index
FASTEX	-	Fabry Stabilization Index
FDA	-	Food and Drug Administration
FMD	-	fibromuscular dysplasia

FSGS	-	Focal segmental glomerulosclerosis
GI	-	Gastrointestinal
GLA GENE	-	alpha galactosidase A gene
Gb3	-	Globotriaosylceramide
LYSO-Gb3	-	Globotriaosylsphingosine
GHF	-	Glomerular hyperfiltration
GlcCer	-	glucosylceramide
GCS	-	glucosylceramide synthetase
HSPC	-	hematopoietic stem/ progenitor cells
HGMD	-	Human Gene Mutation Database
iADA	-	inhibiting anti-drug antibodies
ICD	-	implantable cardioverter defibrillators
IVCM	-	in vivo corneal confocal microscopy
IMT	-	intima-media thickness
KDIGO	-	Kidney Disease Improving Global Outcomes
LNPs	-	lipid nano-particles
LVH	-	left ventricular hypertrophy
LSD	-	Lysosomal storage disorders
MSSI	-	Mainz Severity Score Index
M6p	-	mannose-6-phosphate
MRI	-	magnetic resonance imaging
mRNA	-	messenger RNA
OD	-	optical density
OMIM	-	Online Mendelian Inheritance in Man
PC	-	parapelvic cysts
PD	-	Parkinson’s disease
PEG	-	Polyethylene Glycol
QoL	-	Quality of Life
RNA	-	Ribonucleic acid
RS	-	Raw Score
REM	-	Rapid eye movement
RAAS	-	Renin-Angiotensin-Aldosterone-System
RVH	-	Right ventricular hypertrophy

SFN	-	Small Fibre Neuropathy
SRT	-	Substrate reduction therapy
SST	-	Serum Separator Tube
T2 FLAIR	-	T2 weighted Fluid Attenuated Inversion Recovery
TIA	-	Transient Ischemic Attack
TMAO	-	Trimethylamine N-oxide
UTI	-	Urinary Trypsin Inhibitor
WS	-	Weighted Score
WML	-	White matter lesions

CHAPTER ONE: INTRODUCTION

1.1 Background

Fabry disease (Online Mendelian Inheritance in Man #301500) (FD, OMIM) is classified as a lysosomal storage disorder. Lysosomal storage disorders (LSD) are a group of disorders that are inherited or acquired. The disruption of the primary function of the recycling and disposal centres of the lysosome due to errors with the encoding of different lysosomal proteins, lysosomal enzymes, and lysosomal membrane proteins is the main feature of LSD (Platt *et al.* 2018). Fabry disease is defined as a complex multisystem disease with non-specific signs and symptoms and is the second most frequent disorder of this type after Gaucher's disease (Colpart and Felix 2017). The characteristic feature of Gaucher's disease is the presence of lipid-laden reticuloendothelial cells present in the spleen, liver, and bone marrow contributing to symptoms such as hepatosplenomegaly and pancytopenia (Kok *et al.* 2021). Fabry disease, also known as Alpha- Galactosidase A Deficiency or Anderson- Fabry disease is the deficiency of the alpha galactosidase-A (α- Gal A) enzyme. The monogenic disease is inherited in an X-linked manner. The alpha-galactosidase A (GLA) gene is situated on the Xq22.1 position on the X chromosome, affecting all hemizygous males, their daughters become heterozygous carriers and their sons are non-carriers and remain unaffected (Di Toro, Favalli and Arbustini 2018). An incomplete functioning or deficiency of the alpha-galactosidase A enzyme results in a systemic, intracellular accumulation of complex glycosphingolipids, mainly globotriaosylceramide (Gb3) or the water-soluble deacylated Gb3 known as globotriaosylsphingosine (lyso-Gb3) causing progressive damage to tissues and major organs, including amongst others, the heart, brain, vascular endothelium and kidneys leading to end-stage renal disease (Perretta 2018). Ethnic preference has not been observed in FD. Due to the low incidence rate of the condition, the prevalence rate can only be estimated as ranging from 1:40 000 men to 1:117 000 live births (Perretta 2018). The prevalence in live births has significantly increased with atypical mutations of the disease included, with statistics varying between 1:2900 to 1:3900 (Giugliani et al. 2016).

1.2 Manifestation and symptoms of Fabry Disease

Early symptoms manifesting during childhood and adolescence include pain in the extremities, angiokeratomas, tinnitus, and anhidrosis. Disease progression ultimately results in left ventricular hypertrophy (LVH), stroke, proteinuria, and renal failure. Complications of the heart, cerebrovascular, or kidneys become pronounced after the age of 30 reducing mortality by 20 years (Germain 2010). Under- or misdiagnosis of the disease during later manifestations is common due to the non-specific nature and the mimicking of symptoms associated with diabetes and hypertension (Terryn et al. 2013).

1.3 Diagnosing and reporting of Fabry Disease

Diagnosis of FD in males requires observing low levels or absence of the alpha-galactosidase-A enzyme activity in leukocytes, plasma, or fibroblasts and increased levels of Gb3 and lyso-Gb3 concentrations in plasma and urine. Pathogenic mutations can be assessed by genetic analysis (Yenicerioglu *et al.* 2017). The absence of residual enzyme activity in males is categorized as a severe classical phenotype, where characteristic FD symptoms manifest and progress into more severe symptoms later in life and affect multiple organs. The non-classical phenotype is milder in males where residual enzyme activity is evident. Patients are less severely affected and only one organ is affected later in life. Heterozygous females can present with normal alpha-galactosidase levels due to skewed X-inactivation (as per Lyons hypothesis whereby the process of the X chromosome is rendered inactive) and therefore are not reliably diagnosed by enzymatic assay. In such cases, molecular analysis is required (Curiati et al. 2017).

The Fabry Outcome Survey (FOS) established in 2001, is an international database designed to enhance the clinical management of patients diagnosed with Fabry disease (Giugliani *et al.* 2016). The data collected on the FOS provides information on the safety and efficacy of enzyme replacement therapy as well as the natural history of Fabry disease. The FOS patient report confirmed that as of January 2019, 3855 patients were enrolled in the FOS from 26 countries, which is a 10% increase from January 2018.

Diagnosing Fabry disease in its early stages has proved to be challenging due to the variability of symptoms, thereby increasing the frequency of organ involvement in the later stages of the disease. Chronic kidney disease (CKD) remains as one of the main characteristics of Fabry disease. The deposition and accumulation of Gb3 occur first in the glomeruli and progress to various areas of the nephron including the mesangial, and interstitial cells, podocytes, cells of the proximal and distal tubules, and loop of Henle, and the vascular endothelial cells including the smooth muscle cells (Levstek, Vujkovac and Trebusak Podkrajsek 2020). Proteinuria is an early indication in detection of the disease in both males and females and is usually a significant clinical manifestation of renal involvement (Mena Rodríguez *et al.* 2018). The appearance of proteinuria occurs mainly during the second to third decades of life, however, it is also shown to be evident in male and female adolescents as well as boys as young as six years old (Mena Rodríguez *et al.* 2018).

1.4 Testing and Treatment

The Kidney Disease Improving Global Outcomes (KDIGO) foundation recommends testing patients with chronic kidney disease when biopsies are not performed and there is no definitive cause of nephropathy (Schiffmann *et al.* 2017). The European Best Practice Guidelines (EBPG) recommend testing males under 50 years of age with chronic kidney disease with no definitive diagnosis (Terryn *et al.* 2013). Although there have been documented cases of Fabry disease in South Africa, the prevalence has not been established.

Treatment for Fabry disease currently comprises two forms of recombinant enzyme replacement therapy (ERT): Agalsidase-alpha produced in human fibroblasts and agalsidase- beta produced in Chinese hamster ovary cells (van der Veen et al. 2020a). Both forms of treatment with enzyme replacement require biweekly administration. Oral chaperone therapy is a relatively new form of treatment, however, is effective in patients with specific mutations of the disease (Lenders and Brand 2021). Novel gene therapies are in the clinical trial phase and are producing promising results through in vivo and ex vivo procedures (Domm et al. 2021). Although reports have shown that after a short course of enzyme replacement therapy patients showed improvements in pain relief,

ability to sweat, and quality of life, there were no improvements to the cerebrovascular and renal damage (Abensur and Reis 2016; Schiffmann et al. 2017).

1.5 Rationale of the Study

In this prospective, quantitative study 200 male patients with chronic renal failure were tested for low levels or absence of the alpha- Galactosidase- A enzyme. Clinical manifestations were assessed and scored using the Fabry Outcome Survey Mainz Severity Score Index (FOS-MSSI). Results displaying low levels or absence of the Alpha-Galactosidase-A enzyme in conjunction with high scores in the assessment of their clinical manifestation will establish an association between Fabry disease and chronic renal failure. Therefore, this study aims to demonstrate that early testing for Fabry disease is required when the diagnosis for chronic kidney disease is unconfirmed. This can ultimately retard or prevent damage to all major organs.

CHAPTER TWO: LITERATURE REVIEW

2.1 Lysosomal Storage Disorders

Lysosomes are membrane-bound cell organelles containing a variety of hydrolases. The hydrolytic enzymes within the lysosome are responsible for the breakdown of biological polymers, including proteins, nucleic acids, carbohydrates, and lipids (Bonam, Wang and Muller 2019). Disintegrating and recycling of cellular waste, cellular signalling, and energy metabolism are known functions of the lysosome. Recent research has recognized the lysosome for its further involvement in the process of degradation, innate and adaptive immunity, and nutrient sensing (Zhang *et al.* 2021).

Lysosomal storage disorders (LSD) are characterized by dysfunctional lysosomes due to the interruption or cessation of the recycling process of complex molecules and cellular structures (Parenti, Medina and Ballabio 2021). Incorrect encoding of the lysosome due to mutated genes result in the disruption of the enzymatic function. This causes an accumulation of ungraded substrate that disrupts cellular functions, extracellular inflammatory responses, tissue damage, and organ dysfunction. Clinical manifestations are therefore diverse depending on the substrate and site of the accumulation (Sun 2018). The accumulated substrate, the underlying mechanism, or the defective enzyme are indicators used to classify LSD, while the age of onset is used to establish the subgroups (Tanpaiboon and Ferreira 2020). There have been over fifty-one genetically established LSD documented. These can be classified as inherited or acquired disorders. The majority of LSD is inherited in an autosomal recessive manner, of which, nine different mechanisms have been established. Inhibition of alpha-mannosidase II by ingestion of specific plants and treatment with drugs such as amphophilic cationic drugs, amiodarone, and chloroquine results in the acquired form of the disorder (Alroy and Lyons 2014).

2.2 The Association with Fabry Disease and Lysosomal Storage Disorders

Fabry disease is an inherited, monogenic lysosomal disorder linked to the X chromosome. The inborn error of glycosphingolipid catabolism results from the lysosomal deficiency of hydrolase alpha-galactosidase- A in tissues and fluids (Ferreira and Gahl 2017). The mannose-6-phosphate (M6P) pathway is responsible for the delivery of the alpha-Gal-A enzyme into the lysosome. Breakdown of glycolipids (complex sugar-lipid molecules), specifically globotriaosylceramide (GL-3 or Gb3), its deacylated form Lyso-GL-3/Gb3 and related glycolipids, is broken down by the alpha-Gal-A enzyme. This occurs by the removal of the terminal galactose sugar from the extremities of the glycolipid molecules (Ferreira and Gahl 2017). Due to the deficiency of the alpha-Gal-A enzyme, GL3 and other glycosphingolipids are unable to break down to be recycled by the body. Accumulation of GL3 occurs in the lysosomes resulting in a wide range of symptoms.

2.3 Historical Background of Fabry Disease

Angiokeratoma corporis diffusum was the first characteristic sign of Fabry disease, discovered in 1898 by two dermatologists Johannes Fabry and William Anderson working independently of each other (Bartolotta *et al.* 2015). The 13-year-old patient of Johannes Fabry presented with angiokeratoma corporis diffusum which is now recognized as a characteristic sign of Fabry disease. William Anderson reported the case of a 39-year-old patient in the same year. The patient presented with angiokeratomas, proteinuria, deformity of the fingers, and lymphedema. Dr. M Ruiter, a Dutch dermatologist, concluded in 1939 that angiokeratoma corporis diffusum was the dermal manifestation of systemic disease. The lipid character of the storage material was confirmed by Scriba in 1951. The importance of the lysosome as an important cellular organelle was described by Nobel laureate Christian de Dauve in 1959, providing insight into the concept of lysosomal storage disorders and the possibility of enzyme replacement therapy as a form of treatment. The discovery in 1963 by Dr. CC Sweeley (Professor of Biochemistry) and Klionsky of the lipid material globotriaosylceramide which accumulates in the lysosomes as a result of the deficiency of the alpha-galactosidase-A enzyme provided evidence that the major constituent of the glycolipid fraction is a trihexoside composed of sphingosine, glucose, and galactose at a molar ratio of 1:1:2 (Gaggl *et al.* 2016). The underlying cause

of Fabry disease was established in 1967, by Dr. Roscoe Brady, an American biochemist, as the result of the deficiency of the alpha galactosidase-A enzyme (Gaggl *et al.* 2016). Desnick et al. cloned the GLA gene in 1985. This allowed for molecular genetic diagnosis and specific therapy.

Medical research and discoveries made from far back as the late 1800s have allowed further research of Fabry disease to continue and grow especially in the field of genetics. With the prevalence of the disease increasing, awareness is crucial to prevent misdiagnosis and therefore further research is imperative.

2.3.1 Outcomes of the Earliest Renal Biopsies and Electron Microscopy Procedures

Proteinuria and albuminuria were noted in the first documented cases even though the central manifestation was angiokeratoma corporis diffusum. Proteinuria or abnormal urinary sedimentation was later frequently indicated in patients presenting with angiokeratoma (Gaggl *et al.* 2016). Abnormal vacuoles in the blood vessels were discovered in 1947, during autopsies on patients with renal insufficiency classifying the disease as a generalized storage disorder (Martins *et al.* 2011). The first renal needle biopsy was performed in 1958 on two patients presenting with angiokeratomas. Distinct vacuolation and distention of the cells of the glomerular tufts and distal tubules were observed on both biopsies (Gaggl *et al.* 2016). The inability to concentrate urine was evident in both cases, however, in one case the glomerular filtration rate remained normal while the other displayed moderate impairment. Upon re-examination of the kidney of a deceased female relative of the two patients revealed similar lesions on the kidney, however, no indication of characteristic skin lesions was noted.

2.4 Fabry Disease and the Associated Subtypes

Alpha-Gal-A is an enzyme found in the lysosome of the cell with its primary function being the breakdown of the globotriaosylceramide (Gb3) lipid. Mutation of the GLA gene causes the underproduction of the alpha Gal-A enzyme causing the progressive build-up of Gb3 in the lysosomes of the cells, body fluids including blood plasma, and urine initiating the symptoms of Fabry disease (Curiati *et al.* 2017). Some GLA gene mutations are benign and the production of the alpha Gal-A enzyme is not affected (Curiati *et al.* 2017). These benign mutations do not cause Fabry's disease. The two main subtypes of Fabry disease identified are classic FD and later onset or non-classic FD.

2.4.1 Classic Fabry disease

Classic FD in males occurs when it is minimal or no production of the Alpha Gal-A enzyme and the mean normal is < 1% (Ortiz *et al.* 2018b). Mutations responsible for the minimal to no production of the alpha Gal-A enzyme are the nonsense, consensus splice site most frameshift mutations (Ortiz *et al.* 2018b). Significant accumulation of Gb3 commences in utero and deposition has been found in placental tissue (Perretta, Antongiovanni and Jaurretche 2017). Symptoms manifest in early childhood or early adolescence. Childhood symptoms include anhidrosis, burning pain in the extremities, hearing loss, angiokeratoma, and digestive problems. Major organ involvement including kidney and cardiac complications begin in the second decade of life and strokes present after the third decade of life (Arends *et al.* 2017). In females, the severity of symptoms can range from having no symptoms throughout their lifetime to being as severe as their affected male relatives.

2.4.2 Late-Onset Fabry Disease

Missense mutations and cryptic splicing mutations can encode enzymes and produce residual, varying levels of alpha-Gal-A activity with reduced Gb3 accumulation, thereby slowing down the progression of the symptoms. This leads to later onset FD experienced in males (Ortiz *et al.* 2018b). Symptoms do not manifest during childhood and organ involvement is usually limited to one organ, characterizing the disease as oligosymptomatic (Atiskova *et al.* 2019). Kidney and cardiac complications manifest between the third and sixth decades of life. In females, there is limited information

regarding the manifestation of symptoms, although typically symptoms are absent until later in life (Arends *et al.* 2017).

Manifestation of symptoms in females occurs late in life with slow disease progression resulting in an increased life expectancy of approximately seventy years (Viggiano and Politano 2021). In both subtypes, the severity of symptoms in females depends on the number of cells having the mutated gene after random X chromosomal inactivation. When most cells have normal GLA genes (that are actively producing the alpha Gal-A enzyme), as compared to the mutated GLA genes, then symptoms may be less severe or completely asymptomatic. Conversely, symptoms will be more severe when most cells have mutated genes than normal GLA genes which are inactive and unable to actively produce the alpha-Gal enzyme (Viggiano and Politano 2021).

2.5 Genetic Correlation with Fabry Disease

Fabry disease is a genetic condition inherited by means of the X chromosome. The alpha Gal-A enzyme is a protein composed of two polypeptide chains identical in the order, number, and kind of their amino acid residues, thus making it a homodimer glycoprotein. The enzyme hydrolyses the terminal alpha-galactosyl moieties from glycolipids and glycoproteins (Kok *et al.* 2021). The GLA gene is found on the long arm of the X chromosome (Xq22.1). The coding part of the gene comprises 1290 base pairs (bp) and is divided into seven exons (Varela, Caldas and Pesquero 2019). It comprises a polypeptide of 429 amino acids. The incomplete functioning or deficiency of the alpha-Gal-A enzyme leads to the accumulation of globotriaosylceramide (Gb3) in major organs. A significant number of studies have shown outcomes of several recorded mutations of the GLA gene, however, there are outcomes of mutations that remain unclear. Reported cases of alpha-Gal-A mutations of unknown significance have shown reduced levels of alpha-Gal-A enzyme activity but no association with Fabry disease has been established (Kok *et al.* 2021).

2.5.1 Inheritance of the Mutated Gene

Males have XY chromosomes and females have XX chromosomes. In males affected by Fabry disease, the X chromosome is mutated and since there is no copy of the GLA gene on the Y chromosome, there is no functioning gene. In females, if the one X chromosome has an affected GLA gene, production of the alpha-Gal-A enzyme is still possible by the normal functioning gene on the second X chromosome of the pair (Figure 1). These females are the heterozygote carriers (Rajeshwari 2016).

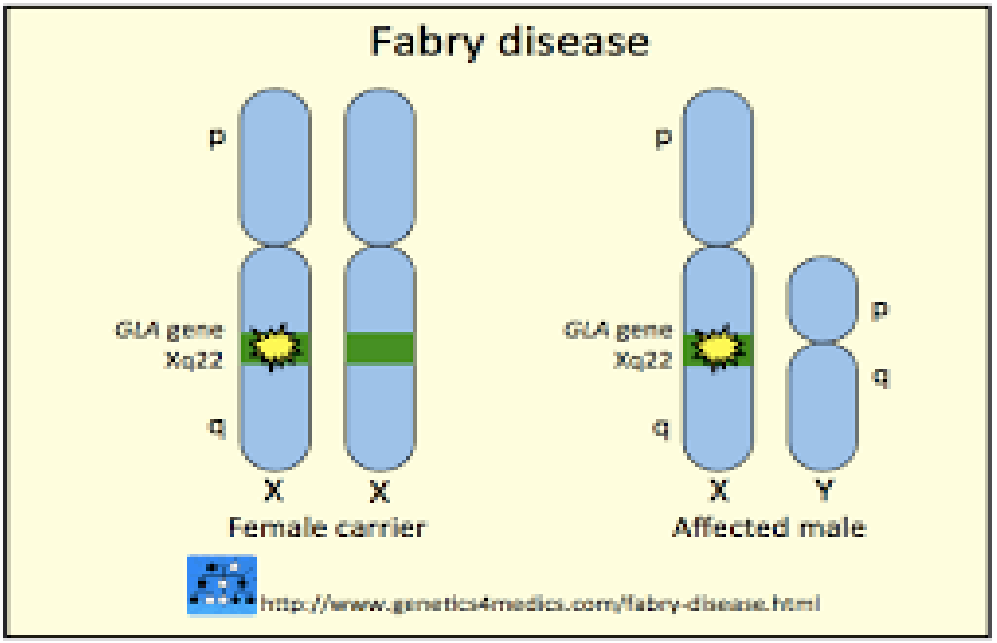


Figure 1: Inheritance of affected gene(Vujkovac 2017)

In pregnancies where the male is affected and the female is unaffected, there will be a 50% chance of the affected X chromosome will be passed. In this case, the foetus will be a girl and a heterozygous carrier. The Y chromosome also has a 50% possibility of being passed. In this case, the foetus will be a boy and unaffected as the GLA gene is not present on the Y chromosome. In pregnancies where the male is unaffected but the female is a heterozygous carrier, there is a 50% chance the female passes the mutated X chromosome and 50% that the unaffected X chromosome will be passed. There is a further 50% chance that the male passes the unaffected X chromosome and a 50% chance he passes the Y chromosome. This case, therefore, has 4 possible outcomes (Figure 2) where there is a 25% chance of having a heterozygous daughter, a 25% chance

of having a son who is affected, a 25% chance of having an unaffected daughter, a 25% chance of having an unaffected son (Doheny 2016).

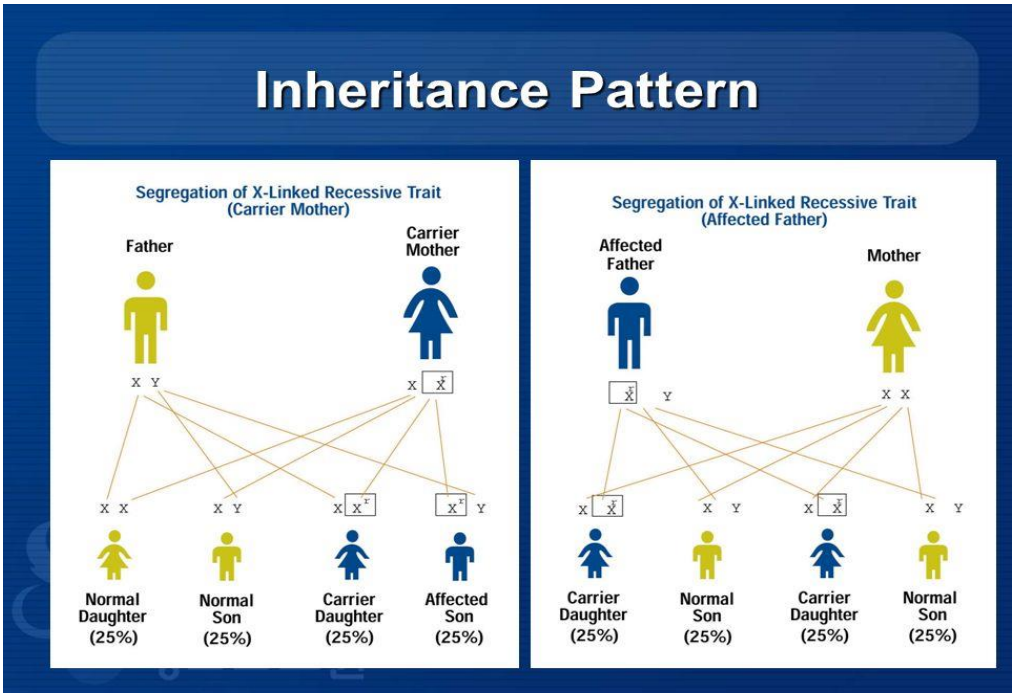


Figure 2: X- Linked Inheritance Pattern (Monserrat et al. 2007)

2.5.2 Mutations Associated with Fabry Disease

Currently, over one thousand disease-associated gene variants have been reported in the Human Gene Mutation Database (HGMD). Approximately 75% of the mutations are point mutations primarily consisting of the missense mutations, 14% of the total comprising of the nonsense mutations, and splicing region mutations making up only 4% of the gene variants (Varela, Caldas and Pesquero 2019). In a study conducted in Russia by Moiseev, et.al. (2019), 5572 dialysis patients were tested for Fabry disease by evaluating alpha-Gal-A enzyme activity using the dried blood spot test method (DBS). Confirmatory genetic testing was performed on patients with low levels of Alpha-Gal-A results. Fabry disease was confirmed in 20 patients. Genetic testing revealed 17 different mutations in the GLA gene, of which 12 were missense mutations and 5 nonsense mutations (Moiseev *et al.* 2019). The missense, nonsense, consensus splice site, cryptic splicing, and frameshift mutations are gene mutations primarily associated with FD. Mutations resulting in residual or no production of alpha-Gal-A activity are commonly the

nonsense, consensus splice site, and frameshift mutations. This is categorized as a classic Fabry disease. Non-classical or late-onset Fabry disease is associated with a part of the missense and rare cryptic splicing mutations where enzymes are encoded with residual alpha-Gal-A activity (Ortiz *et al.* 2018b).

2.5.3 Missense Mutation

Missense mutations occur when an alteration of a single base pair causes the substitution of a separate amino acid in the resulting protein. The substitution may produce either no effect or it can render the protein non-functional. There have been over 350 missense mutations identified in Fabry disease (Schiffmann 2015). Ge *et.al*.(2017), identified a missense mutation when the 47th amino acid tryptophan (W47) was replaced with arginine (W47R), leading to reduced Alpha-Gal-A activity in 293T cells (Ge *et al.* 2017).

2.5.4 Nonsense Mutation

Nonsense mutations are associated with the classic form of FD, accounting for approximately 11% of all disease-causing gene lesions (Lombardi *et al.* 2020). This follows a substitution of a single base pair leading to the appearance of a stop codon, resulting in premature termination of translation of truncated polypeptides. The manifestation of this stop codon results in the production of a shortened and non-functional protein (Lenders *et al.* 2016). Nonsense mutations usually occur within the tryptophan amino acid (Ge *et al.* 2017).

2.5.5 Frameshift Mutations

Frameshift mutations are caused by a deletion or insertion in a DNA sequence that shifts the manner in which the sequence is read. In a study conducted by Kim *et.al.* (2013), a hemizygous male patient diagnosed with Fabry disease presented with only dermatological symptoms involving angiokeratoma and anhidrosis. Genetic testing revealed a frameshift mutation (Kim *et al.* 2013). In recent studies, a significant association was recognised between FD patients with frameshift mutations and inhibiting anti-drug antibodies (iADA). Patients displaying iADA typically were male patients with the classic form of FD with frameshift or nonsense mutations and had commenced

agalsidase beta with higher levels of the disease biomarker (lysoGb3) in plasma before treatment (van der Veen *et al.* 2020b).

2.5.6 Pre- mRNA Splicing

The mRNA is formed by 1290bp encoding 429 residues. The signal peptide is formed by the first 31 amino acids. The mature enzyme is formed when the signal peptide is removed (Varela, Caldas and Pesquero 2019). Pre- mRNA genes are formed after the transcription process of DNA. Pre-mRNA splicing is a process to produce a functional mRNA molecule by removing intronic sequences from eukaryotic pre-mRNA transcripts and joining together exons (Li *et al.* 2019). Spliceosomes are composed by ribonucleoprotein complexes and allow the splicing process to occur. The spliceosome machine can identify specific regions of the pre-RNA, that require the deletion of the intron and connection of exons (Varela, Caldas and Pesquero 2019). There are a reported 43 variants of Fabry disease which affect the splicing process. The late-onset cardiac form of FD has been associated with the c.639+919G>A mutation and is prevalent in the Asian population (Dardis and Buratti 2018).

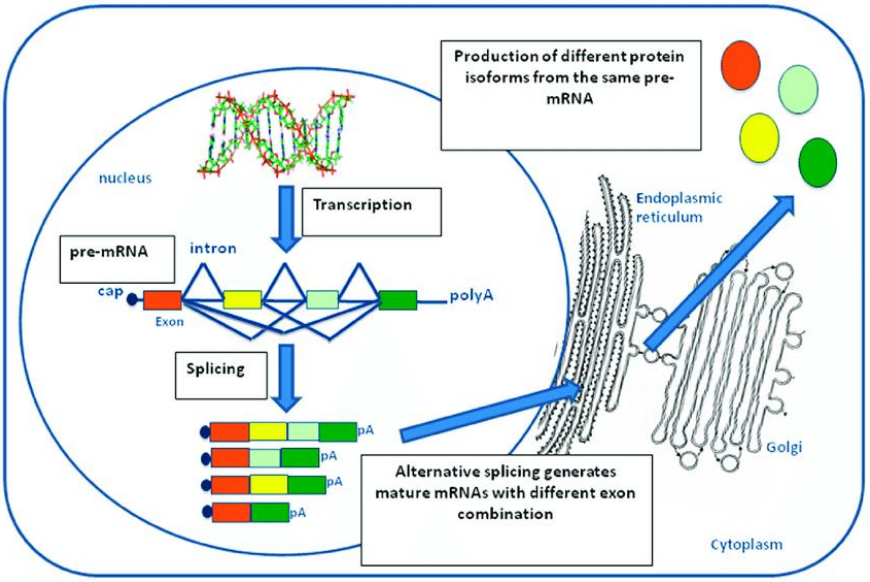


Figure 3: Schematic representation of the splicing process. (Dardis and Buratti 2018).

2.6 Alpha Galactosidase-A Enzyme

The Alpha Gal-A enzyme is a lysosomal enzyme and the main function is the elimination of terminal α 1.3 and α 1.4- linked galactosyl residues through hydrolysis from various glycoconjugates within the lysosomes. The conversion of the human blood group B to blood group O is an additional function of the alpha-Gal A enzyme (Guce *et al.* 2010). Gb3 is the main substrate during this process. The deficiency of the alpha-Gal enzyme leads to the accumulation of Gb3 in the tissues and organs. Other substrates include deacylated Gb3 or globotriaosylspingosine (lyso-Gb3), digalactosylceramide (Gal2 Cer), and blood group B and P1 glycosphingolipids. Therefore, patients with blood groups AB and B can exhibit more severe manifestations of the disease due to an additional accumulation of glycosphingolipids in the membrane of the erythrocytes of blood group B[25] (Bernardes, Foresto and Kirsztajn 2020). Gb3 is a glycosphingolipid. Further names for Gb3 include ceramide trihexoside, CD77, pk blood group antigen and Burkitt lymphoma antigen. They are defined as the building blocks of the outer leaflet of the cell membrane and synthesis occurs in the Golgi. Recycling of the Gb3 occurs by fragmentation inside the lysosomes by glycosidases (Aerts *et al.* 2019). Gb3 localizes to the outer leaflet of the plasma membrane once synthesis takes place. Clusters are formed in the lipid rafts with their glycan portion facing the extracellular environment (Aerts *et al.* 2019). Gb3 synthase deficiency also plays a role in the risk of miscarriages (Miller, Kanack and Dahms 2020). The kidneys and the heart are most frequently affected in patients with Fabry disease as a result of the abundance of Gb3 synthase mRNA present in these organs. Cell surface Gb3 has also been associated with infectious processes. The Shiga toxin family uses the Gb3 cell surface receptor as entry into the cell. This usually results in haemolytic-uremic syndrome (Lingwood and Simons 2010). Lyso- Gb3 is a minor Alpha-Gal-A substrate and accumulates to a lesser extent than Gb3, however, they are used as biomarkers for monitoring therapeutic efficacy and disease progression (Miller, Kanack and Dahms 2020). In females, enzyme assays are not a reliable method in diagnosing FD due to alpha-Gal-A levels usually being low to normal ranges and there is a weak association between Gb3 and alpha-Gal-A enzyme activity. Therefore, Gb3 is not ideal as a diagnostic marker (Ouyang *et al.* 2018).

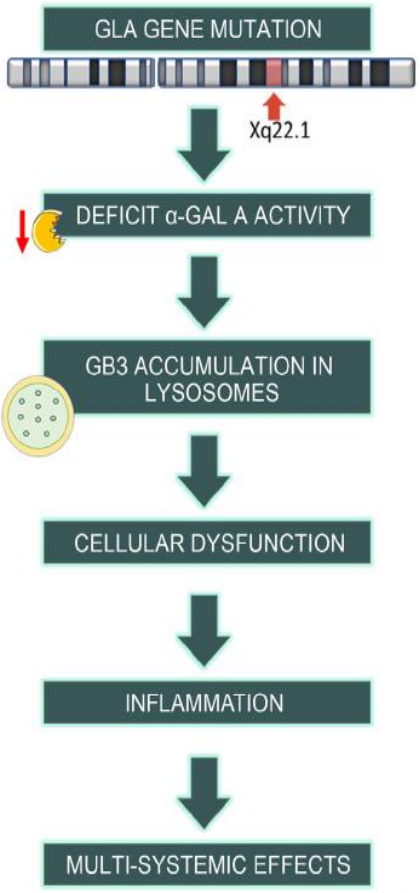


Figure 4: Representation of the pathogenic mechanism occurring in Fabry disease. GB3, globotriaosylceramide; GLA, Alpha-Galactosidase A (Castelli et al. *2021)*

2.7 The Presentation of Fabry Disease in Patients with Blood Groups Ab and B

Clinical manifestations of FD may be more severe in patients with blood groups Ab and B. This is due to the further accumulation of glycosphingolipids in the membrane of erythrocytes of blood group B (Bernardes, Foresto and Kirsztajn 2020). Glycosphingolipids with terminal α-galactosyl moieties, including globotriaosylceramide are natural substrates of the alpha Gal-A enzyme. Additional substrates include the blood group B, B1, and P1 antigens collectively known as the blood group B glycosphingolipids (B-GSLs) (Rybova *et al.* 2018). The B-GSLs are detected mainly in the membranes of erythrocytes, although they have been detected on the surface of other cell types of blood group B individuals. Further accumulation of the B-specific glycosphingolipid with a terminal alpha galactosyl residue will produce an increase in the severity of clinical manifestations in hemizygotes and heterozygotes with blood type B or Ab (Celi *et al.* 2022).

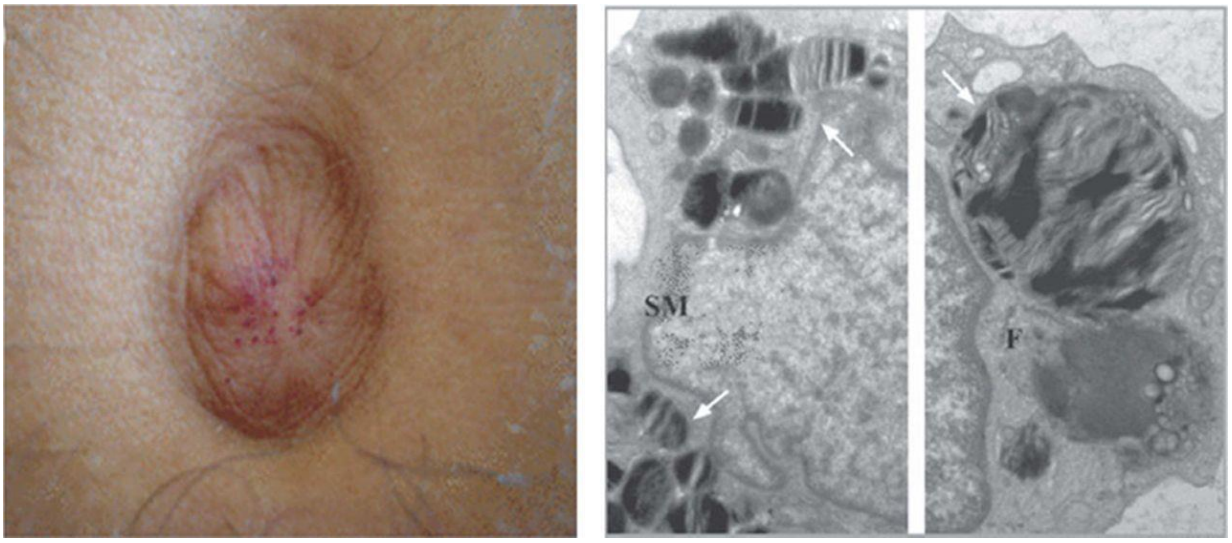
2.8 Clinical Manifestations Associated with Fabry Disease

2.8.1 Angiokeratoma Corporis Diffusum

Angiokeratomas are hyperkeratotic vascular papules that differ in size. They are distinguished histologically by superficial dilated capillaries in the papillary dermis with epidermal proliferation (Vadher *et al.* 2020). Angiokeratomas which are isolated, are usually common and do not require further investigation. The generalized form is known as angiokeratoma corporis diffusum (ACD) and has been associated with lysosomal storage disorders and deficiencies, the most common being Fabry disease. ACD is an early manifestation in the classical phenotype and is present in 66% of affected men and 36% of affected women (Jesus *et al.* 2018). They present as small reddish to black papules (Figure A) with a diameter of 1mm to 5mm and a smooth or keratotic surface. Lesions characteristically localize in the gluteal, genital, and peri-umbilical area, lower abdomen, and upper part of the thighs around the bathing trunk area (Luna, Boggio and Larralde 2016).

2.8.1.1 Pathophysiology and Histology of Angiokeratoma Corporis Diffusum

The lysosomal accumulation of glycosphingolipids, mainly globotriaosylceramide (Gb3), is caused due to the incomplete functioning or complete loss of function of the GLA gene to produce alpha-Gal-A enzyme. The deposition of glycosphingolipids in the lysosomes of endothelial, perithelial, and smooth muscle cells of blood vessels causes blood vessels to narrow and expand. Angiokeratomas form due to ischemia and infarction of these vessels. Zebra-like (lamellar) bodies (Figure 5B) are characteristic manifestations and are distinct under the electron microscope (Luna, Boggio and Larralde 2016).



A B

Figure 5 (A) Clinical manifestation of angiokeratoma corporis diffusum around the umbilicus. (B) Skin biopsy showing zebra bodies (Nagueh 2014)

2.8.2 Small Fibre Neuropathy (Acroparesthesia and Pain)

Small fibre neuropathy (SFN) is recognized as pain in FD. It usually begins in early childhood as one of the symptoms of the classic phenotype of FD. 62- 80% of males and 30- 65.3% of females are affected (Rajan *et al.* 2021). Levels of residual enzymatic activity determine the severity of pain. Two types of pain have been reported in Fabry disease. The first is acute pain known as Fabry or pain crises. These are periods of excruciating, debilitating pain starting in the extremities and spreading over the entire body (Politei, Durand, and Schenone 2016). The second type of pain is chronic pain known as acro-paraesthesia. It is described as painful numbness, burning, and tingling of the extremities and is triggered by physical activity, thermal stimuli, and fever. The thinly myelinated Aδ fibres are the small nerve fibres affected by the damage caused by the accumulation of Gb3. These are predominately responsible for the transmission of mechanical pain sensitivity to pinprick stimuli or unmyelinated C fibres which conduct warm sensation and pain sensitivity to heat (Politei, Durand and Schenone 2016). However, in the early stages of FD, cold perception (Aδ fibres) is primarily affected rather than the warmth perception (C fibres), therefore the Aδ fibres are more susceptible to Gb3 damage (Politei, Durand and Schenone 2016). Neuropathic pain and decreased sensation of temperature variation is due to the accumulation of Gb3 in Schwann cells (Figure 6) and the dorsal root ganglia or the endothelial cells of blood vessels supplying the small nerve fibres (Inan, Mese and

Bicik 2019). The pathological effect on the ganglia is supported by a study conducted by Choi, et.al. where a direct association between lysoGb3, increased intracellular calcium ions levels in peripheral sensory neurons, and pain was shown. In addition, studies have also revealed an association between depression and chronic pain, thereby increasing the risk of psychological disorders such as depression in patients diagnosed with FD (Schuller *et al.* 2016).

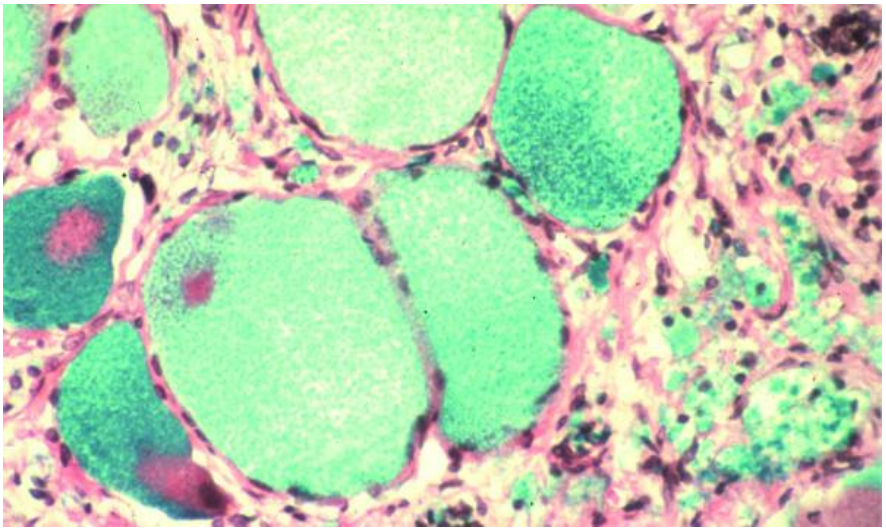


Figure 6: Dorsal root ganglion cells from a Fabry patient revealing accumulation of Gb3 (Burlina et al. 2011)

2.8.3 Anhidrosis

Anhidrosis was thought to occur as a result of autonomic peripheral neuropathy, however, further investigations revealed an accumulation of Gb3 in the sweat glands with sweat gland dysfunction (Nagai-Sangawa *et al.* 2021). Loss of innervation of the sweat glands has also been shown to contribute to anhidrosis. Studies conducted by Cho, et.al. (2021), demonstrated that dysfunction of the preganglionic sudomotor nerves caused anhidrosis in their Fabry patient. Other findings included that due to the accumulation of Gb3 in the CNS, hypohidrosis is initiated due to glycosphingolipid accumulation along the central thermoregulatory sudomotor pathways (Cho *et al.* 2021).

2.8.4 Cornea Verticillata

Ocular manifestations are the most distinctive and common manifestation of FD. Over 90% of hemizygotes present clinically by the age of four and heterozygotes present around the age of ten (Michaud 2019). The early presentation of ocular manifestations can be the key to an early and decisive diagnosis of the disease (Hewavitharana *et al.* 2020). Lens opacities, conjunctival and retinal vessel tortuosity and aneurysmal dilatations are other possible ocular signs presenting with cornea verticillata (CV). Cornea verticillata or vortex keratopathy is caused by deposits on the cornea forming whorl-shaped patterns (Figure 7). Gb3 deposits accumulate at the level of the epithelial basement membrane forming yellowish-brown inclusions radiating outward towards the periphery of the eye. Examinations are usually performed utilizing a slit-lamp, although, limited diagnostic power with the slit-lamp has been noted in comparison to in vivo corneal confocal microscopy (IVCM) (Leonardi *et al.* 2020). A high number of false-negative results was reported with examination using slit-lamp, while the IVCM confirmed corneal microstructural changes in patients with FD (Leonardi *et al.* 2020). Statistics obtained from the Fabry Outcome Survey revealed that the presentation of CV was similar in both males and females, children as young as three years old also presented with CV, and has a higher occurrence in patients with missense mutations (Hewavitharana *et al.* 2020). Further ocular manifestations of FD include vascular tortuosity of the conjunctiva, retina, and upper eyelid. Vascular tortuosity was proven to be unreliable in the diagnosis of FD, as it was a common manifestation among the elderly population and a clinical sign of many other diseases (Wu *et al.* 2020).

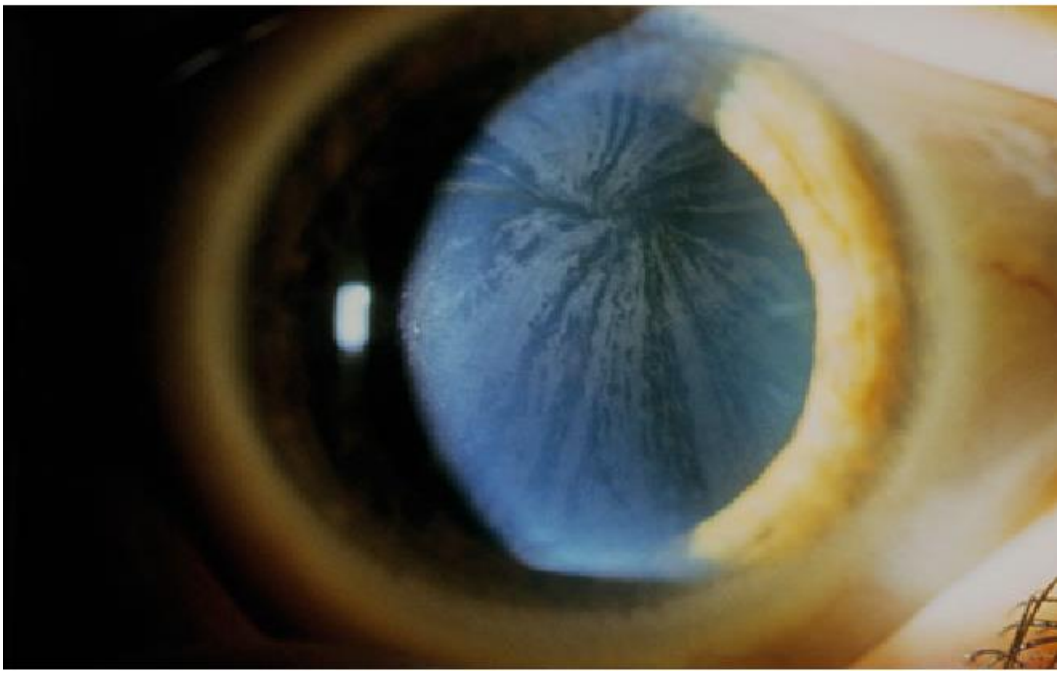


Figure 7:Cornea verticillata. A bilateral, whorl-like pattern of powdery, white, yellow, or brown corneal deposits (Wasielica-Poslednik et al. 2011)

2.8.5 Auditory and Vestibular Symptoms of FD

Otological symptoms of FD include hearing loss, tinnitus, vertigo, fullness of the ear, and dizziness. Hearing loss affects men in the second decade of life, while women are affected in the fourth decade of life (Ciceran and De Maio 2016). Reports have indicated that 66.7% of male patients and 33.3% of female patients with FD with no hearing irregularities presented with tinnitus (Wang *et al.* 2019). Untreated hearing loss has been shown to have significant consequences on a person's quality of life. Moderate hearing loss left untreated has been shown to cause anxiety and impaired memory (Balasubramanian C 2018). Temporal bones of patients with FD were examined seropurulent effusions and hyperplastic mucosa in the middle ears, strial and spiral ligament atrophy in all turns, hair cell loss primarily in the basal turns, and diminished numbers of spiral ganglion cells in the cochlea were identified. All these cases reported having bilateral sensorineural hearing loss (Wang *et al.* 2019). The main arterial responsible for supplying the vestibular system and cochlea arises from the anterior inferior cerebellar artery or can branch directly from the basilar artery. Repeated ischaemic damage to the organ of Corti as a result of stenosis or occlusion of the cochlea small vessels rather than neural hearing pathways can cause sudden hearing loss. This

can also be the cause of this manifestation (Ciceran and De Maio 2016). However, Gb3 accumulation in the audio-vestibular ganglia and vessels of the cochlea can result in progressive hearing loss (Ciceran and De Maio 2016).

Progressive sensorineural hearing loss at high frequencies combined with unexpected deafness has been reported more often in patients with FD than in the general population, though, vestibular involvement has been studied to a lesser extent (Eyermann *et al.* 2019). A correlation between patients with FD presenting with auditory manifestations had a greater mean age and cardiac and cerebrovascular complications were significantly evident. No relation between auditory and renal involvement was determined (Eyermann *et al.* 2019). Patients with sporadic sudden deafness reported a history of stroke and/ or TIA and/ or MRI gaps. Vestibular association was reported in 60% of patients with renal complications, 61.5% having cardiac complications, and 71.4% with cerebrovascular involvement (Eyermann *et al.* 2019).

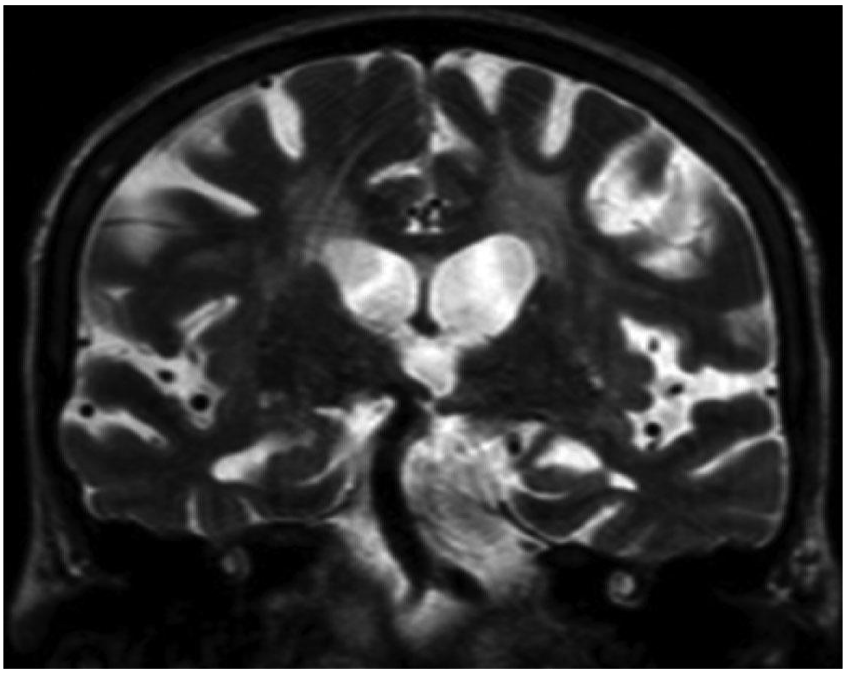


Figure 8: Vertebrobasilar dolichoectasia identified in a 42-year-old male with Fabry disease. (Ciceran and De Maio 2016)

2.8.6 Gastrointestinal Symptoms and Manifestations

Due to the non-specific nature of the symptoms such as abdominal discomfort, diarrhoea, nausea, and vomiting; gastrointestinal (GI) manifestations can often be misdiagnosed as irritable bowel syndrome or inflammatory bowel disease (Hilz *et al.* 2018). In 2018, statistics from the Fabry Outcome Survey indicated that up to 60% of children and females and approximately up to 70% of males presented with GI symptoms upon entry onto the registry (Hilz *et al.* 2018). The aetiology of the GI disturbances, though studied, is yet not well understood; however, autopsy and biopsy investigations have revealed inclusions in the submucosal (Meissner's) plexus and myenteric (Auerbach's) plexus. In investigations with patients with the classic phenotype of FD, the gastric mucosa presented with substrate accumulation in epithelial, vascular, nervous, and interstitial cells (Di Toro *et al.* 2020). This ultimately causes dysfunction of the autonomic nervous system responsible for gut motility, vasculopathy affecting GI circulation, and tissue inflammation related to GL-3 accumulation. Additional histopathological findings show vacuolization of enlarged ganglion cells and surrounding axons (Figure 9), with intracellular glycosphingolipid deposits which are typical of Fabry disease (Lenders and Brand 2022). Consequently, GI symptoms manifest due to a rapid gut transit time, reduced peristalsis, intestinal stasis, bacterial overgrowth, malabsorption of nutrients, pancreatic insufficiency, gastroparesis (delayed emptying), and ischaemic or neuropathic damage. Abdominal pain, gastroparesis, and irregular gut motility are thought to be caused by damage to the enteric neurons by Gb3 deposition (Hilz *et al.* 2018).

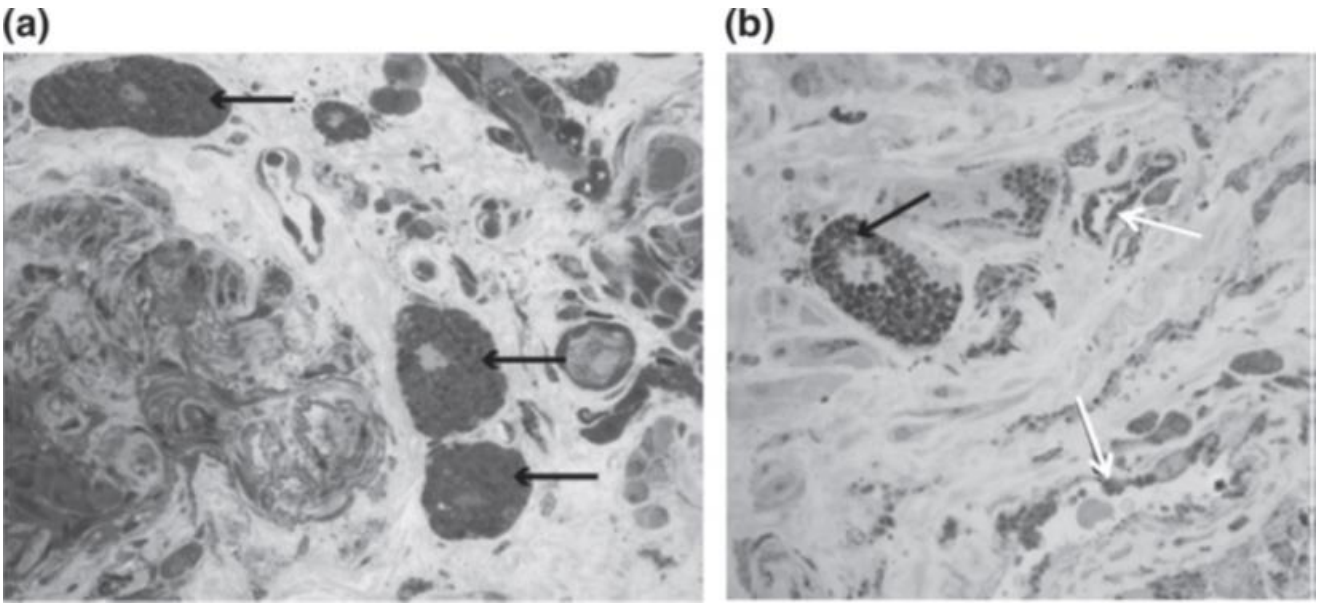


Figure 9: GL3 present in ganglion cells (a) and endothelial cells (b) in the gastrointestinal tract of a Fabry patient (Politei et al. 2016)

2.8.7 Psychological Aspects and Quality of Life (QoL)

A large prevalence of depressive symptoms is indicated in patients with Fabry disease (Korver *et al.* 2020b). Over 46% of Fabry disease cases have reported depressive symptoms, increasing the risk of suicide and increased mortality (Rosa Neto, Bento and Pereira 2020a). In most cases, this is attributable to chronic pain, acroparesthesia, anhidrosis, poor sleep quality, and functionality and quality of life (Rosa Neto, Bento and Pereira 2020a). Socio-economic factors of patients with Fabry disease such as financial status and relationship status have also been associated with depression and a lower quality of life (Ali, Gillespie and Laney 2018). Mood swings as a result of CNS manifestations among children with Fabry disease are common, primarily among teenagers. The secondary manifestation of the psychological disturbances in this age group is largely caused by the impact of living with the disease throughout their childhood (Hopkin et al. 2016). Organ involvement has shown a significantly lower prevalence in its association with depressive symptoms. Patients with depressive symptoms are known to display cognitive impairment, although no definite link has been established between the two (Korver *et al.* 2020a).

2.8.8 Pulmonary Manifestations in Fabry Disease

Pulmonary involvement in FD has not been widely reported over the years and is thought to be underestimated by clinicians. However, detection of inflow limitation in the early stages of life has proven that there is significant pulmonary involvement in patients with FD when compared to the general population, with an 18% prevalence in the Fabry population (Franzen *et al.* 2018). Symptoms such as dyspnoea, dry cough, and bronchospasms are commonly misinterpreted as initial symptoms of cardiac involvement and render the symptoms non-specific. Pulmonary biopsies and investigations using the electron microscope reveal Gb3 accumulation in the pneumocytes, in the muciparous goblet cells, in the bronchial ciliate epithelium, in the bronchial smooth cells, and the pulmonary vessels resulting in small and medium airway narrowing (Faverio *et al.* 2019). A history of smoking and cardiac complications aggravates symptoms that include dyspnoea, a dry cough, and bronchospasm (Regenbogen *et al.* 2021). Pulmonary fibrosis, opacities, and, pulmonary calcifications were further results documented in patients with FD although the findings were inconclusive (Borie *et al.* 2021). Pulmonary involvement has shown to be more evident in males with the classic phenotype of FD and females presenting with both phenotypes (Franzen *et al.* 2018). Enzyme replacement therapy has shown to be efficacious in non-smoking patients with Fabry disease with the p. Arg227Ter mutation, however, further studies are required to validate these results (Pietila-Effati *et al.* 2022).

2.8.9 Rheumatological Manifestations and Inflammatory Pathways

Rheumatoid arthritis is a chronic systemic autoimmune disease of the connective tissue. The aetiology of the disease remains unknown and can be characterized by symmetric polyarthritis and associated extra-articular and systemic manifestations. Numerous studies have revealed an association between autoimmune diseases and Fabry disease (Paim-Marques, de Oliveira and Appenzeller 2022). The abnormally deposited globotriaosylceramide (Gb3) and globotriaosylsphingosine (Lyso-Gb3) within the lysosomes act as damage-associated molecular patterns (DAMPs) or stimulating DAMP production. The activation of an inflammatory pathway as a result of this production, induces apoptosis and a toll-like receptor- 4 (TLR4) mediated innate immune system pro-inflammatory cytokines secretion (IL-1β and TNF-α) (Paim-Marques *et al.* 2020). Invariant

natural killer T cells (iNKTs) are activated due to the Gb3 and Lyso-Gb3 being recognised as antigens. The release of other inflammatory cytokines such as interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), interleukins (IL): IL-4, IL-5, IL-9, IL10, IL13, and IL-17 is initiated by the inducting of the iNKTs. Joint pain, unexplained fever, and elevated inflammatory markers such as erythrocyte sedimentation rate (ESR) and C reactive protein (CRP) can be used as indicators in diagnosing rheumatic disease in FD (Moiseev *et al.* 2020). Of note, minimal research has been conducted to determine the frequency of rheumatological manifestations in FD. Misdiagnosis of FD is still a concern and it has been noted that rheumatological conditions have been frequently overlooked as an indicator of FD (Rosa Neto, Bento and Pereira 2020b).

2.8.10 Low Bone Mineral Density Associated with Fabry Disease

A relatively novel clinical manifestation of FD is a low bone mineral density which is usually diagnosed as osteoporosis or osteopenia. The mechanism of this manifestation is not yet fully understood (Maruyama *et al.* 2020a). Approximately 50% of untreated patients with FD present with osteopenia, while hyperparathyroidism manifests in FD patients which is secondary hyperparathyroidism caused by CKD. Osteoporosis or osteopenia has been reported as significantly higher in males with FD than in the general population, however, external factors such as medications (e.g., anticonvulsants), body mass index (BMI) indicating under-nourishment, intolerance to any form of exercise, or inadequate concentrations of vitamin D has yet to be verified as possible contributing factors (Bruell *et al.* 2022). Calciprotein particles (CPP) are nanoscale particles made up primarily of the circulating glycoprotein fetuin-A bound to ion clusters of calcium and phosphate. CPP pathway involvement in the production and accumulation of the particles is reported to be associated with the development of bone function and remodelling and may be a factor in the inconsistent association of low BMD with higher vascular calcification, predominantly in patients with CKD (Bruell *et al.* 2022).

2.8.11 Cerebrovascular and Neurological Manifestations

The involvement of the central nervous system (CNS) in Fabry disease is a frequent complication of Fabry disease. Manifestations can range from insignificant symptoms to more severe cases including acute cerebrovascular events, occurring prior to diagnosis and in the absence of other clinical symptoms (Cocozza *et al.* 2018; Castro *et al.* 2020). Storage of Gb3 occurs in the endothelium and smooth muscles of brain tissue and is responsible for glial deposition and neuronal ballooning in cortical regions and deep nuclei (Cocozza *et al.* 2018). The risk of transient ischemic attack (TIA) and stroke is higher in patients with Fabry disease in comparison to the general population (Kolodny *et al.* 2015). Data collected from the Fabry registry revealed that 6.9%- 11.1% of males and 4.3%-15.7% of females with Fabry disease developed stroke, while brain haemorrhages accounted for 16.9% of male patients and 6.8% of female patients (Marchesoni *et al.* 2018). The pathophysiological mechanism of stroke has not yet been established. The data to support the manifestation of Fabry disease among stroke patients is controversial although vasospasm, reduced cerebral blood flow velocity, abnormal autoregulation, upregulation of angiotensin II, and accumulation of glycosphingolipids in vascular endothelium are some of the possible causes that have been considered (Ortiz *et al.* 2021).

Cerebrovascular events occur as a result of small vessel ischaemic disease (Castro *et al.* 2020), although increased intima-media thickness (IMT) and impaired fibromuscular dysplasia (FMD) indicate dysfunction of the large arteries as well. White matter lesions (WML) are localized hyperintensities discoverable with the use of brain magnetic resonance imaging (MRI) and are present to a large degree in patients with Fabry disease (Figure 10). Prevalence has been known to increase with age. The effects of WML and brain infarcts in FD patients remain undefined, although there are indications that it is linked to cognitive impairment and clinical stroke. Asymptomatic WML has been documented in children and adolescents with Fabry disease who experienced no prior history of stroke or TIAs, indicating that brain microvascular injury possibly begins early in life and continues in an asymptomatic manner for many years (Olivera-González, Josa-Laorden and Torralba-Cabeza 2018). Females with classic FD have reported being at a higher risk of developing complications in comparison to females with non-classic FD. Documented cases of cerebrovascular, cardiac, or renal events in females have shown

to occur before the seventh decade of life, with strokes or cardiac complications manifesting in the early fifth to mid- sixtieth decades of life.

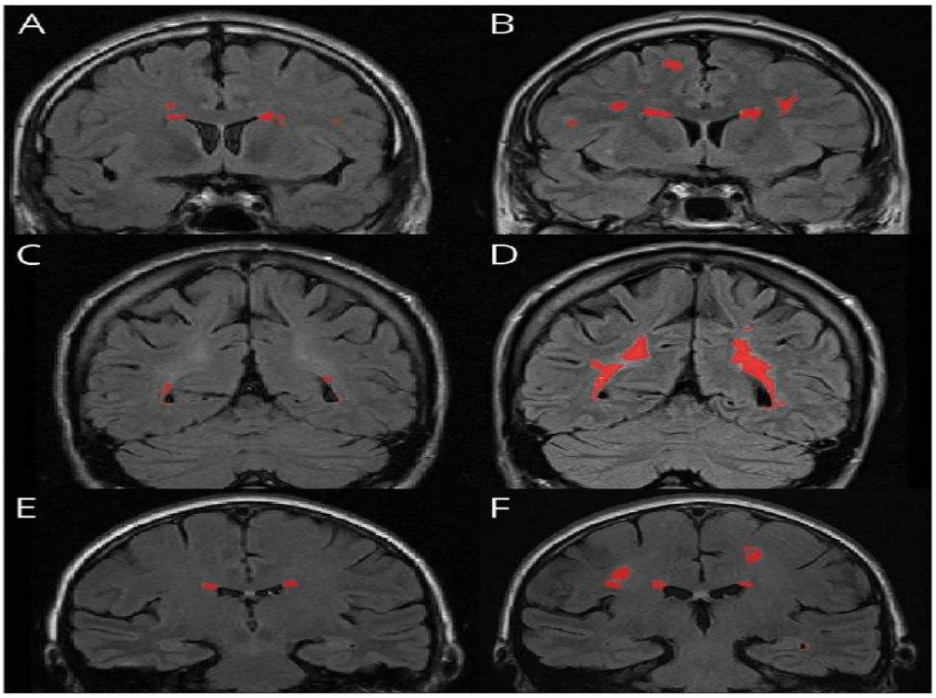


Figure 10: Images showing WMH progression in 3 patients with Fabry disease (Stefaniak et al. 2018)

2.8.11.1 The association between cerebrovascular injury and chronic kidney disease

The relationship between cerebrovascular injury and chronic kidney disease (CKD) has been researched extensively over the last decade. Significant findings show a link between declining renal function, microvascular disease, and cognitive impairment. Vascular calcification and endothelial erythrophagocytosis have significantly been linked to cerebral microvascular disease (Fisher 2020). The in vitro and in vivo studies with cell culture also demonstrate the disruption of the brain endothelial barrier by CKD serum of which the components include endotoxin, urea, indoxyl sulphate, p-cresyl sulphate, and Trimethylamine N-oxide (TMAO). The kidney and the brain are responsible for vasoregulation of the microvasculature in each organ and therefore, are at a higher risk at a vascular level to complicate organ involvement, hypertension, and diabetes mellitus. In patients with FD, the severity of chronic white matter hyperintensities (CWMH)

increased in the presence of a lower eGFR resulting in increased episodes of stroke. Fewer strokes were documented in patients with a more stable eGFR (Tapia and Kimonis 2020).

2.8.11.2 The Investigative Outcomes between Fabry Disease and Parkinson's Disease

Parkinson's disease (PD) is a chronic, progressive, neurodegenerative disease that is initiated by the gradual degeneration of the dopamine neurons located in the substantia nigra of the midbrain (MacMahon Copas *et al.* 2021). Dopamine is a neurotransmitter that assists in coordinating and regulating muscle activity. Primary motor symptoms and the appearance of alpha-synuclein positive cytoplasmic inclusions, termed Lewy bodies, in surviving neurons are the result of the loss of dopamine neurons (Cerri, Mus and Blandini 2019). Primary motor symptoms include bradykinesia, rigidity, resting tremor, and gait disturbances while non-motor symptoms include autonomic dysfunctions, cognitive abnormalities, and psychiatric symptoms. Sleep disorders manifest and include a high incidence of insomnia and REM behaviour disorder (Cerri, Mus and Blandini 2019). Recent studies have demonstrated evidence supporting a correlation between PD and Fabry disease (Gago *et al.* 2020). Alpha Gal-A enzyme activity was significantly lower in the temporal cortex of patients with advanced PD and the substantia nigra of sporadic PD patients (Paciotti *et al.* 2020). This consequently increases the risk of PD in FD patients (Belarbi *et al.* 2020). Parkinson's disease has been diagnosed in a significant number (8.3%) of Fabry patients over the age of 60 years, where all the cases revealed reduced alpha gal-A activity (Blumenreich *et al.* 2020).

2.8.12 The Correlation between Cardiovascular Manifestations and Fabry Disease

Cardiovascular (CV) disease is the primary cause of mortality and morbidity in patients with FD exceeding CKD due to changes in developments in FD therapy (Baig *et al.* 2019). Concentric left ventricular hypertrophy (LVH) is unexplained by abnormal cardiac loading conditions and is a frequent manifestation in FD patients (Hagege *et al.* 2019). Myocardial inflammation and myocardial fibrosis eventually progress to heart failure, restrictive cardiomyopathy, arrhythmias, and death (Perry *et al.* 2019). Cardiac involvement is prevalent in over 60% of both males and females with the classic form of FD. Males are affected around the second decade of life, reducing life expectancy by 15 to 20 years.

Early detection of cardiac abnormalities and LVH in diagnosing FD is important before the onset of renal or neurological damage (Savary *et al.* 2017). Myocardial cells are dependent on mitochondrial function. As a result of Gb3 accumulation in the mitochondrial cells, energy metabolism is inhibited, resulting in cardiomyopathy (Tuttolomondo *et al.* 2021). Histological analysis of hearts affected by FD indicates accumulation of GL-3 in the cardiac tissue cells, cardiomyocytes, valvular fibroblasts, the heart conduction system, and the endothelial cells of the heart (Hagege et al. 2019). A decrease in mitral annulus velocity occurs before LVH and is useful for early detection using tissue Doppler imaging (Kubo 2017). Dyspnoea and exercise intolerance is an indication of diastolic dysfunction and also can be an early indication of FD before the development of LVH. Fabry registries have reported the manifestation of LVH in 53% of men and ≥33% of women after the third decade of life. In 60% of these patients, symptoms that including heart failure with preserved ejection fraction, chest pain, and arrhythmias were reported (Pieroni *et al.* 2021b). Left undetected, diastolic dysfunction progressively leads to left atrial enlargement contributing to atrial arrhythmias and atrial fibrillation (Akhtar and Elliott 2018), leading to the insertion of pacemakers and implantable cardioverter defibrillators (ICD) and commencement of anticoagulation therapy (Hagege *et al.* 2019). Electrocardiogram changes display the high voltage of R waves revealing signs of LVH and right ventricular hypertrophy (RVH) (Selthofer-Relatic 2018). Observations on echocardiography due to accumulation of glycosphingolipids in the myocardial cells and conduction system cells include abnormal Q waves and ST-T changes, shortened PR intervals, sick sinus changes, or arteriovenous block (Kubo 2017). Although shortened PR intervals were found useful in detecting cardiac involvement in FD, PR intervals of <120ms were deemed irrelevant in patients with untreated FD in all stages of cardiomyopathy (Kubo 2017). The severity and incidence of cardiac manifestations are known to increase with age (Serra and Marziliano 2019).

2.8.13 Renal Association in Fabry Disease

Progressive nephropathy is a key complications of FD after cardiovascular disease, leading eventually to end-stage renal disease (ESRD) if left untreated (Levstek, Vujkovac and Trebusak Podkrajsek 2020). The progression from the initiation of renal manifestations to ESRD was reported to be on average over four years in patients with FD (Bernardes, Foresto and Kirsztajn 2020). Manifestation of Fabry nephropathy is

prevalent in the classical phenotype, occurring from the third to the fifth decades of life. Disease severity is extensive in males and females. The rate of progression of chronic kidney disease (CKD) has been often been misdiagnosed as diabetic nephropathy due to the close resemblance (Del Pino *et al.* 2018). Reports from the Fabry Outcome Survey (FOS) revealed a baseline prevalence of nephropathy in 59% of men and 38% of women with FD (Levstek, Vujkovac and Trebusak Podkrajsek 2020). In various studies conducted worldwide, a prevalence of 0.17%- 3.5% of the respective dialysis populations was diagnosed with FD, indicating that FD should be considered as an underlying cause of unexplained ESRD (Del Pino *et al.* 2018).

2.8.13.1 Pathophysiology of Fabry Nephropathy

Early and progressive kidney damage can be detected by microalbuminuria and proteinuria as early as the beginning of the second decade of life. The decline in renal function is in correlation to proteinuria and progresses more rapidly when the estimated glomerular filtration rate (eGFR) is <60ml/min/1.73m^2 (Waldek and Feriozzi 2014). In untreated males with FD, three clinical phases have been described, viz., early manifestation in childhood and adolescence characterized by hyperfiltration being the first phase, renal involvement with proteinuria, lipiduria and Malta crosses crystals in the urine sediment and the commencement of other renal dysfunctions is the second clinical phase, and the third phase involving severe renal disease and involvement of vascular, cardiac, and cerebral systems (Perretta, Antongiovanni and Jaurretche 2018). Although the pathogenesis of Fabry nephropathy is not completely understood, it has been observed that the glomeruli, including the podocytes, endothelial and mesangial cells are characteristically the first to be affected by the accumulation of Gb3 in the kidneys (Waldek and Feriozzi 2014). Accumulation of Gb3 stimulates an inflammatory process similar to hyperglycemia. Inflammatory cytokines are released due to Gb3 through the CD74 receptor pathways, reducing podocyte function (Olivera-González, Josa-Laorden and Torralba-Cabeza 2018). This leads to gradual decline of renal function including glomerulosclerosis which in due course progresses to ESRD, where hemodialysis or transplantation becomes a necessity in the fourth to fifth decade of life.

2.8.13.2 Histology of Fabry Nephropathy

Glomerulosclerosis and fibrosis of the interstitial tubules are the primary histological characteristics of FD indicating progression of renal deterioration. This leads to the requirement for chronic renal replacement therapy (CRRT) (Mena Rodríguez *et al.* 2018). Histological examinations of patients with FD have exhibited vacuolization of podocytes and epithelial cells, expansion of mesangial cells, and progressive segmental and global glomerulosclerosis (Waldek and Feriozzi 2014). Focal segmental glomerulosclerosis (FSGS) is the result of podocyte deterioration and injury to the Bowman's capsule resulting in extrication from the glomerular basement membrane (GBM) following podocyte hypertrophy (Kim *et al.* 2021). Accumulation of Gb3 in the smooth muscle cells of the renal arteries and arterioles progresses to degeneration and eventual death of the smooth muscle cells (Gaballa *et al.* 2020). Investigations with the use of the electron microscope revealed myelin-like, lamellar bodies resembling 'onion skin' known as zebra bodies, and have presented in the renal tubules due to accumulation of Gb3 in the distal tubules and collecting ducts of the affected kidney (Hongo *et al.* 2020).

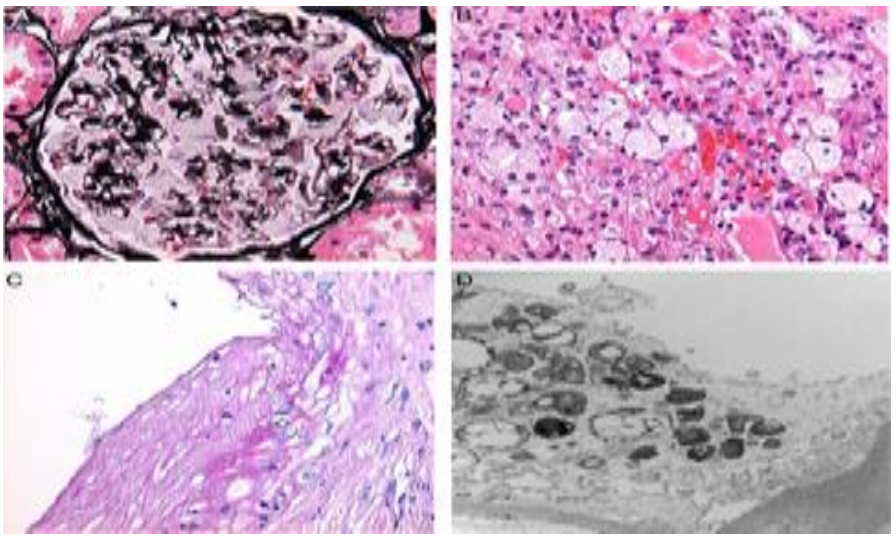


Figure 11: Vacuolation and deposition of Gb3. (A) Vacuolation of podocytes. (B) Tubular and interstitial deposits. (C) Deposits in blood vessels. (D) Zebra bodies in the cytoplasm of podocytes (Mena Rodríguez et al. 2018)

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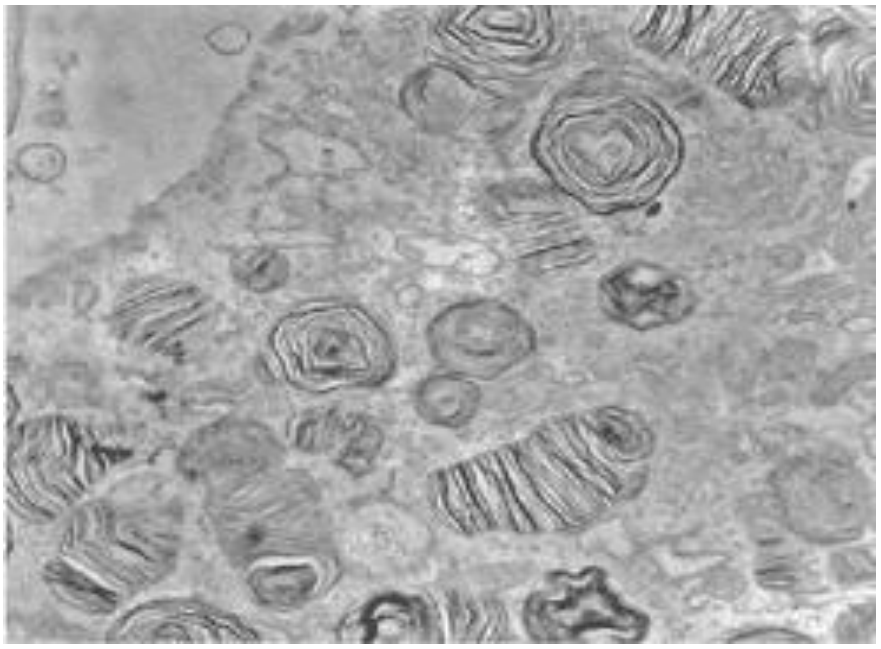

Figure 12: Laminated intra-cytoplasmic inclusions (zebra bodies) and myelin figures within podocytes (Woywodt et al. 2007).

2.9 Renal Biomarkers

Biomarkers are measurable analytes that indicate some biological states or processes that are responsible for the clinical indicators of diseases and can be assessed in various biological samples including blood, urine, and cerebrospinal fluid. The efficacy of enzyme replacement therapy can also be established using biomarkers (Simonetta *et al.* 2020). Fabry nephropathy can present in childhood, however, the presentation is heterogeneous and non-specific, causing a delay in diagnosis by approximately fifteen years (Riccio *et al.* 2019). Currently, the only markers of renal dysfunction in FD are albuminuria and a reduction of glomerular filtration rate (GFR), which in turn act as indicators of renal disease progression. Diagnosis of early nephropathy can be achieved by renal biopsies which can reveal the accumulation of lipids in podocytes and vascular cells as well as the resultant adaptive changes in the tissue (Rossi *et al.* 2021a). Measurement of Gb3 in plasma is the less invasive alternative to renal biopsies, however, it has shown to be unreliable as a biomarker of disease activity.

2.9.1 Urine Microscopy

Urinary mulberry bodies are specific to FD and are evident in the urinary sediment of patients with Fabry disease. It is a useful biomarker in diagnosing the disease. Mulberry bodies are whirl-shaped fat globules. The mulberry cells are distal tubular epithelial cells that are keratinized, exfoliated, and vacuolated due to the accumulation of globotriaosylceramide (Gb3) (Aoyama *et al.* 2020). A characteristic feature of the oval fat bodies is the Maltese cross characterised by a lamellarised manifestation with protrusions (Levstek, Vujkovac and Trebusak Podkrajsek 2020). Follow-up on newborn screening showed the presence of mulberry cells in urinary sediment in children as young as five years old (Sawada *et al.* 2020). Mulberry bodies have also proved to be a useful biomarker in determining the efficacy of enzyme replacement therapy (ERT), where the number of mulberry cells has decreased with the initiation of ERT, however, there have been no studies to confirm the number of mulberry bodies and the progression of renal failure. Although the detection of mulberry bodies is a non-invasive alternative to renal biopsies in diagnosing FD, it is not used frequently. The equipment is highly specialized and adequately trained technicians are required to eliminate incorrect reporting of results (Aoyama *et al.* 2020).

The presence of CD77 and Gb3 positive cells in urine sediment of patients with FD has shown to have a 97% sensitivity level and 100% specificity to FD. The deficiency of or absence of the alpha Gal-A enzyme leads to the accumulation of Gb3 which is identical to the CD77 membrane antigen (Riccio *et al.* 2019). Urinary levels of CD77/ Gb3 have been detected in patients with no clinical nephropathy.

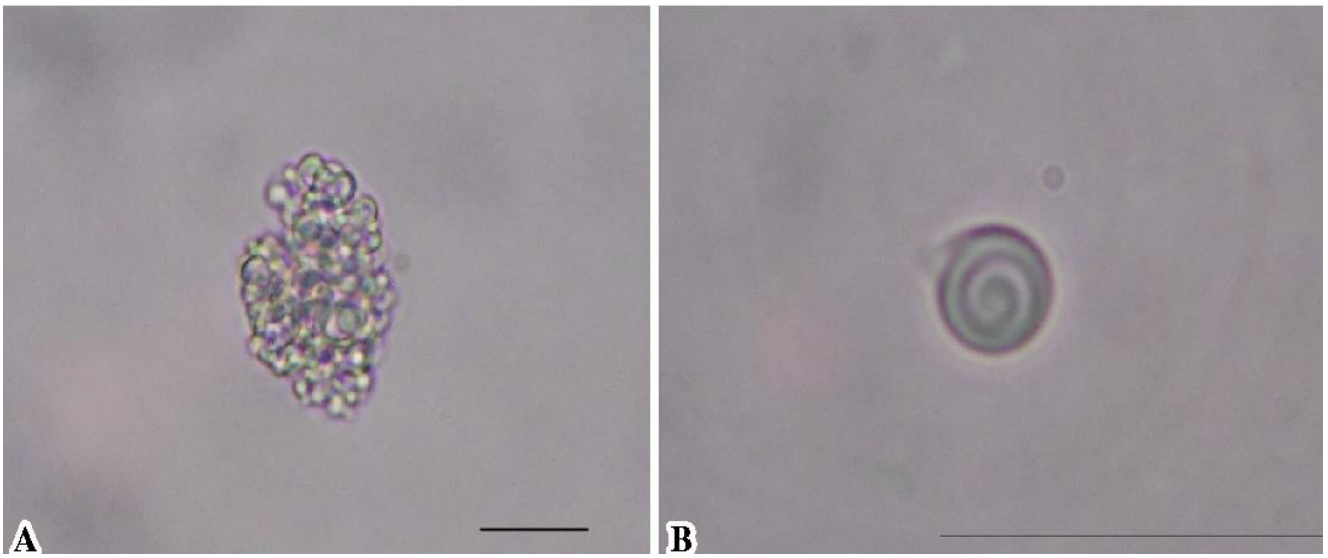

Figure 13: Mulberry cells in the urine sediment (Shimohata et al. 2016).

2.9.2 Proteinuria and Albuminuria

Proteinuria/ albuminuria is evident in patients with the classic phenotype of FD and appears in the second and third decades of life. Although it is most frequently used as a biomarker, its sensitivity to diagnose FD nephropathy is debatable (Riccio *et al.* 2019). Biopsies of FD patients with normal albuminuria levels have revealed kidney damage with advanced lesions. Proteinuria has shown to be more reliable in monitoring the progression of renal function decline, with the level of proteinuria being inversely proportional to the decline in renal function over time (Levstek, Vujkovac and Trebusak Podkrajsek 2020). Interstitial inflammation and fibrosis are stimulated by proteinuria. Partial epithelial-mesenchymal trans-differentiation of tubular cells occurs due to proteinuria inducing cell-cycle arrest and promoting the release of fibrogenic cytokines (Levstek, Vujkovac and Trebusak Podkrajsek 2020).

2.9.3 Hyperfiltration

Glomerular hyperfiltration (GHF) is the age-corrected eGFR > 130 mL/min/1.73/m^2 and can be used as a primary biomarker for Fabry nephropathy (Vardarli *et al.* 2020). Manifestation of GHF is frequent in young patients with FD (Levstek, Vujkovac and Trebusak Podkrajsek 2020). Although it has been employed as a method for early diagnosis, the factors involved in its pathophysiology are multi-factorial and not well

understood. Nitric oxide, prostaglandins, renin-angiotensin-aldosterone-system (RAAS), atrial natriuretic peptide, and reactive oxygen species are some of the known humoral factors involved in the physiopathology of GHF (Perretta, Antongiovanni and Jaurretche 2020). A successful correlation has also been established between GHF and dementia in patients with no FD, where vascular dementia occurs due to endothelial dysfunction causing the deterioration of cerebral blood flow (Kang *et al.* 2020).

2.9.4 Cystatin- C

Cystatin-C (CsC) is a protein belonging to the category of cysteine protease inhibitors and is produced by nucleated cells. The glomerulus freely filters the CsC is then reabsorbed and catabolized by the tubular epithelial cells which prevent it from re-entering the bloodstream or the urine (Torralba-Cabeza *et al.* 2011). Cystatin-C has shown to be a more precise marker than serum creatinine in the early detection of renal function deterioration and indicates the efficacy of ERT (Levstek, Vujkovac and Trebusak Podkrajsek 2020). However, it is costly, laborious, and less available in comparison to creatinine, and therefore, is not frequently utilized as a biomarker tool (Levstek, Vujkovac and Trebusak Podkrajsek 2020).

2.9.5 Urinary Gb3 and Globotriaosylsphingosine

Globotriaosylsphingosine (lyso-Gb3) occurs when accumulated globotriaosylceramide (Gb3) is converted by an acid ceramidase within tissues. The concentration of Gb3 levels decreases after two weeks of commencement of ERT treatment (Jaurretche, Perez and Venera 2019). Measurement is possible with biological fluids and histological samples to detect lyso-Gb3 of affected organs (Simonetta *et al.* 2020). The presence of urinary Gb3 is confirmed by genetic analysis, however, affected females have unaltered urinary Gb3 and its use as a diagnostic tool proved to be limiting. Correlation between urinary Gb3 and eGFR has yet to be established (Sorriento and Iaccarino 2021). Lyso-Gb3 was documented to have a greater sensitivity than Gb3 and is used as an indicator in females with FD. It is also preferred to Gb3 indicators for classic and later-onset phenotypes (Jaurretche, Perez and Venera 2019).

2.9.6 Bikunin

Bikunin occurs in plasma and tissues. It is a serine protease inhibitor that is excreted in the urine and is involved with inflammatory processes. Bikunin is also referred to as a urinary trypsin inhibitor (UTI) (Lepedda *et al.* 2013). Patients with FD nephropathy display a higher level of urinary bikunin in comparison to the general public (Riccio *et al.* 2019). Further studies are required to further understand the mechanism of bikunin elevation in urine as there is no association between serum creatinine and urinary bikunin levels that has been established (Levstek, Vujkovac and Trebusak Podkrajsek 2020).

2.9.7 Tubular and Glomerular Proteins

Abnormal tubular and glomerular protein urinary excretion is the result of impaired tubular and glomerular function in patients with Fabry nephropathy. Kidney involvement and progression of Fabry nephropathy can be measured using the tubular and glomerular protein biomarker (Levstek, Vujkovac and Trebusak Podkrajsek 2020). Patients without signs of deteriorating kidney function still displayed elevated levels of glomerular dysfunction, involving the transferrin and type IV collagen, and tubular dysfunction involving α1- microglobulin, N- acetyl- β glucosaminidase, and alanine aminopeptidase (Levstek, Vujkovac and Trebusak Podkrajsek 2020).

2.9.8 Proteomic

Proteomic analysis in FD is the identification of novel Gb3 isoforms which assist in monitoring disease progression or ERT efficacy (Simonetta *et al.* 2020). In view of the fact that urine comprises less protein than serum or plasma, urine proteomics is, to a lesser extent, an uncomplicated form of analysis (Levstek, Vujkovac and Trebusak Podkrajsek 2020). Altered proteins are categorised as up-regulated or down-regulated, however, their association with the course of the disease and organ involvement has yet to be clearly understood (Levstek, Vujkovac and Trebusak Podkrajsek 2020). It has been suggested that excess cellular Gb3 induces oxidative stress and up-regulates the expression of cellular adhesion molecules in vascular endothelial cells (Simoncini *et al.* 2020). Urinary proteomes of Fabry patients further showed an up-regulation of uromodulin, prostaglandin H2 d-isomerase, and prosaposin.

2.9.9 Parapelvic Cysts

The association between parapelvic cysts (PC) and Fabry disease has been established, though the nature of the association is unspecified (Pisani *et al.* 2018). The prevalence of PC in patients with Fabry disease was 29% during routine ultrasound studies, and 43% during specific ultrasound studies (Pisani *et al.* 2018). The pathophysiology of PC has been attributed to the role of glycosphingolipids. Intra-renal levels of two glycosphingolipids, viz., glucosylceramide (GlcCer) and ganglioside are significantly higher in subjects with polycystic kidney disease (PKD) (Pisani *et al.* 2018). Although parapelvic cysts are not regarded as a definite diagnosis for FD, their manifestation should alert nephrologists to consider testing patients for FD.

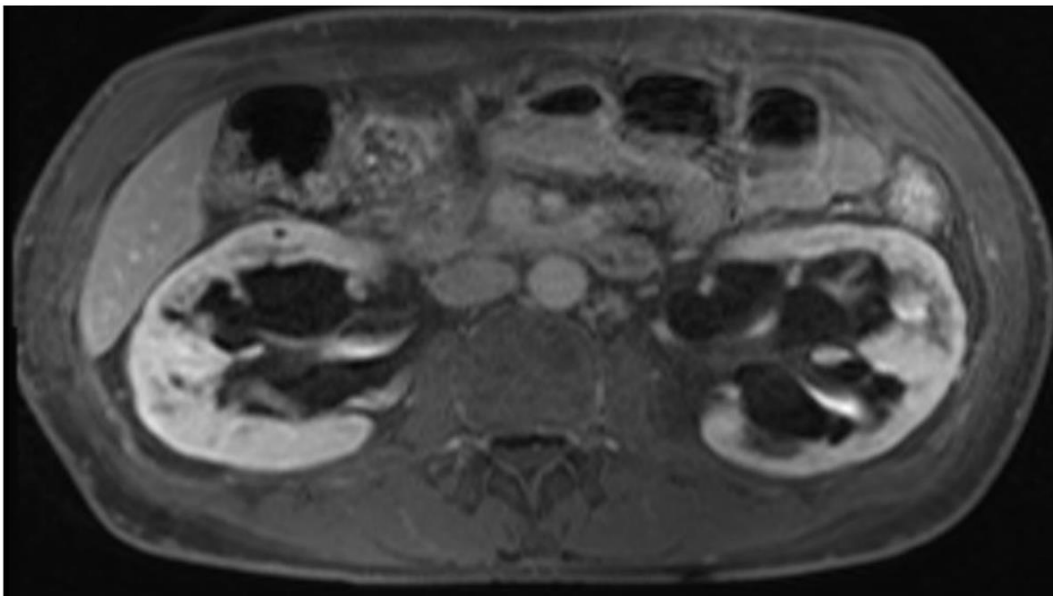

Figure 14: Parapelvic cysts in Fabry disease. MRI scan demonstrating bilateral parapelvic cysts (McCloskey, Brennan and Sayer 2018).

2.10 Scoring Clinical symptoms of Fabry Disease

The Mainz Severity Score Index (MSSI) and the Fabry Disease Severity Scoring System (DS3) are two scoring systems developed and outlined to assess the disease severity and symptoms of Fabry disease. Additionally, the system assists in tracking the course of disease progression in patients being treated with enzyme replacement therapy (ERT). The MSSI and DS3 scoring systems have several primary goals, including assessing disease symptoms and disease load, assessing clinical changes between visits with

individuals with FD, and assessing large populations in cohort studies (Lenders and Brand 2020). The Mainz Severity Score Index was created to assess the severity of Fabry disease clinical symptoms and to track the progress of patients who began enzyme replacement therapy. The signs and symptoms of FD are classified into four categories and weighted based on their contribution to morbidity. The general and renal domains receive a score of 18, and the cardiovascular and neurological domains receive a score of 20. The age of the patients proved to be a concern, with younger patients aged 20-30 having lower MSSI scores than older patients aged 40-50 years (Whybra, Bähner and Baron 2006).

A modification of the MSSI scoring system by the Fabry Outcome Survey (FOS-MSSI) included age and gender adjustment to assess the multisystemic manifestations of the disease. Scoring of clinical symptoms was achieved by adding up individual scores to get a total score, where ≤18 may be categorized as mild, 19-38 would be moderate, and >38 is categorized as severe (Lenders and Brand 2020).

The Fabry Disease Severity Scoring System (DS3) is an alternative scoring system with five domains, viz., peripheral nervous system, renal manifestations, cardiac, central nervous system, and patient-reported outcome.

The detection of disease progression and patient stability is assessed by the recently developed system known as the Fabry Stabilisation Index (FASTEX) (Lenders and Brand 2020). Two sets of scores are required to generate the FASTEX final score. The Raw Score (RS) comprises three domains which are the nervous system, renal, and cardiac domains. The RS is repeated after one year to assess the disease burden. The Weighted Score (WS) is the second score and indicates the severity of organ damage. The WS is established on the domains of the RS.FASTEX scores ≥20% indicate instability and significant clinical deterioration and scores ≤20% indicate improvement in the clinical status of the patient (Simonetta *et al.* 2020).

2.11 Testing for Enzyme Activity: Leukocytes vs Dried Blood Spot

The signs and symptoms of Fabry disease can be non-specific and diagnosis of the disease using clinical manifestations are unreliable. Therefore, confirmation by biochemical or genetic analysis is recommended. Plasma, leukocytes, and fibroblasts, testing is the common biological materials used for the analysis of alpha-Gal-A enzyme activity and are regarded as the “gold standard” for the detection of enzyme levels (Curiati *et al.* 2017). The disadvantage of this method, however, is the invasive nature of sampling, the transportation of the samples, and, the cost of testing (Sozmen *et al.* 2017).

Dried blood spot tests (DBS) are recommended as the first screening due to the non-invasive nature of the sampling, low cost, and transportation is easy. Enzyme stability is acceptable in comparison to leukocyte and fibroblast cultures. The disadvantage of DBS testing has shown higher incidences of false-positive results when compared to other tests (Sozmen *et al.* 2017). Since Fabry disease affects the X chromosome, males can conclusively be diagnosed with the disease if alpha-Gal-A levels are low or undetectable.

2.12 Therapeutic Measures for Fabry Disease

The average life expectancy of an untreated patient with the classic phenotype of FD is approximately 75 years in females and 60 years in males, with sudden cardiac death, renal failure, and, stroke being the most common cause of death. Increased disease progression and serious outcomes are higher in the male FD population than in the female FD population (Ortiz *et al.* 2018a). The primary aim of therapy goals is to reduce pain, delay or prevent the progression of damage to organs (kidneys, heart, and, central nervous system), improve patient quality of life, and increase life expectancy (Lenders and Brand 2021). Treatment options that are available for FD are intended for life-long use and are costly. There are currently two treatment options that include enzyme replacement therapy and chaperone therapy (Lenders and Brand 2021).

2.12.1 Enzyme Replacement Therapy

Enzyme replacement therapy comprises two different forms and has been used since 2003. Agalsidase-alpha is produced in human fibroblasts (human fibrosarcoma cells HT-1080). The recommended dose is 0.2mg/kg body weight and is administered over forty minutes on a bi-weekly basis. The production of Agalsidase-beta is achieved by using Chinese hamster ovary cells and is infused over 240 minutes biweekly using the recommended dose of 1.0mg/kg body weight (van der Veen *et al.* 2020a). The infusion time can be reduced to 90 minutes depending on the patient's level of tolerance (Lenders and Brand 2021). Although patients treated with either form of ERT have exhibited a significant decline in Gb3 accumulation and improvement in neuropathic pain, renal function, and, other major clinical events have been established (Schiffmann *et al.* 2019), treatment with agalsidase-beta has demonstrated a larger decrease in lysoGb3 concentrations when compared to agalsidase-alpha (Arends *et al.* 2018). The half-life of agalsidase-alpha and agalsidase-beta is approximately two hours and is pharmacokinetically similar.

A novel third form of ERT is the pegunigalsidase alfa which is a PEGylated, chemically modified form of the alpha-Gal-A enzyme used in the clinical trial treatment of FD. The alpha-Gal-A enzyme is produced in a tobacco plant cell-based ProCellEX system and further modified chemically (Azevedo *et al.* 2020). Covalently crosslinked monomers with intact 3D structures are produced by chemical modification with homo-bifunctional polyethylene glycol (PEG, 2000 Da) (Schiffmann *et al.* 2019). Under simulated lysosomal-like conditions, pegunigalsidase alpha displayed greater stability when compared to agalsidase-alpha and agalsidase-beta in human plasma at 37^0C (Schiffmann *et al.* 2019). This included a prolonged plasma half-life and biodistribution was significantly greater due to the attachment of additional polyethylene glycol (PEG) moieties to the protein surfaces. An increase in enzymatic activity in the heart and kidney was demonstrated, including reduced clearance by the liver (Kizhner *et al.* 2015). Pegunigalsidase alfa is currently being tested for efficacy in Phase 3 clinical trials and has not been approved by the FDA (Schiffmann *et al.* 2019).

The risk of infusion-based reactions leading to rigors and chills which are prominent more in males than in females have contributed to the challenges of intravenous administration

of ERT. Males with the classic form of FD usually present with anti-drug antibodies within 3-6 months of initiation of ERT with 91% being reported in males treated with agalsidase beta and 20% in males treated with agalsidase alpha (Azevedo *et al.* 2020)The infusion reactions are potentially life-threatening and are mediated by anti-drug antibody (ADA) responses (Felis *et al.* 2020). Inefficient biodistribution due to limited tissue penetration of intravenous ERT by the liver, cardiomyocytes, and podocytes has affected the efficacy of the treatment (Azevedo *et al.* 2020). Further limitations include the inability of intravenous ERT to traverse the blood-brain barrier (Biferi *et al.* 2021).

2.12.2 Chaperone Therapy

Chaperone therapy is a relatively new therapeutic approach to treating FD and is the first oral therapy for treating FD. Chaperone migalastat is administered orally every other day thereby alleviating the inconvenience of bi-weekly intravenous administrations of agalsidase-alpha or agalsidase-beta (Riccio *et al.* 2020). The large volume of distribution allows for traversing into the central nervous system. Mutations of the GLA gene are classified into amenable or non-amenable mutations which aid in the treatment using chaperone migalastat. The mechanism of chaperone therapy is the binding and stabilization of misfolded proteins by small molecules which improve enzymatic activity and reduce substrate accumulation. These therapies are known as pharmacological chaperones and are specific to missense mutations of FD (Caputo *et al.* 2021). The criteria developed for which chaperone migalastat is selected over agalsidase-alpha or agalsidase-beta include patients ≥16 years with a confirmed amenable mutation, an eGFR > 30 mL/min/1.73 m2, medication compliance, and female patients with no intention of becoming pregnant. Hypersensitivity to intravenous administration of ERT and patient preference are also contributing factors to the choice of treatment. A switch from intravenous ERT to chaperone migalastat was shown to be safe and well-tolerated by patients meeting the inclusion criteria (Riccio *et al.* 2020).

2.12.3 Substrate Reduction Therapy

Substrate reduction therapy (SRT) is an oral therapy and is most effective in patients with residual enzyme activity. Substrate production is reduced by glucosylceramide synthetase (GCS) inhibitors to a level compatible with remaining enzyme activity and before accumulation (van der Veen *et al.* 2020a). A combination of SRT and ERT may prove to be more efficacious in patients with minimal or no residual enzyme activity. Significant reduction of Gb3 deposits has been demonstrated in tissues, including the brain and superficial skin capillary endothelium with ibiglustat and Lucerastat has also shown a decrease in Gb3 with stabilization of renal and cardiac manifestations (Azevedo *et al.* 2020).

2.13 Novel Gene Therapies for the Treatment of Fabry Disease

Patients diagnosed with lysosomal storage disorders including mucopolysaccharidosis type 1 and Gaucher's disease were treated previously with bone marrow transplantation, however, this was not attempted in patients with Fabry disease (Kant and Atta 2020). Gene therapy is a novel approach to the treatment of FD. The increasing or introduction of the alpha-Gal-A enzyme activity is the primary objective of gene therapy. Numerous gene therapies are currently under development including ex vivo and in vivo gene therapy and, mRNA gene therapy (Felis *et al.* 2020).

2.13.1 In Vivo Gene Therapy

In vivo therapy involves the infusion of a carrier vector into the Fabry patient and ultimately into the cells including the cells of the liver. Gene editing follows and the missing protein is extracted (Castelli *et al.* 2021). The infusion of the adeno-associated virus (AAV)-mediated gene transfer has been used to improve the levels of enzyme activity. Infusion of the AAV-mediated gene transfer using the liver has produced promising results, showing an increase of the alpha-Gal-A enzyme levels by 300 times in deficient mice. Lyso-GL3 levels were not detected and GL3 levels were reduced by less than 10% after a single dose infusion (Felis *et al.* 2020). In mutations that affect a single organ, cell-specific therapies may be necessary. An increase in transduction in cardiomyocytes has been documented with the use of a novel cardiotropic AAV variant (Felis *et al.* 2020).

2.13.2 Ex Vivo Gene Therapy

The CD34+ cells hematopoietic stem/ progenitor cells (HSPC) are harvested from Fabry patients using leukapheresis (Figure 20). Modification of the cells is achieved through recombinant lentivirus-mediated gene transfer of the alpha-Gal-A gene (Felis *et al.* 2020). The HSPCs are subsequently infused into the autologous patient. Persistent alpha-Gal-A levels have been detected in patients undergoing clinical trials and have consequently discontinued exogenous enzyme intravenous therapy. Ongoing clinical trials have demonstrated variable levels of Gb3 in total plasma and urine, while significant stabilization of cardiac hypertrophy was noted (Khan *et al.* 2021a). In recent studies, patients have been treatment-free but under observation 3 years after the first infusion date (Khan *et al.* 2021b).

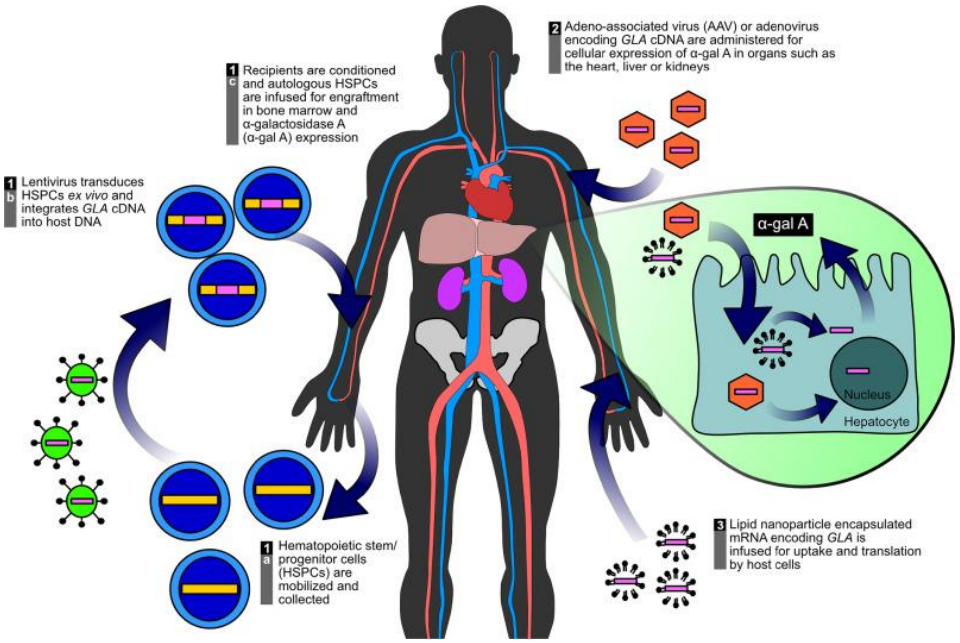

Figure 15: Schematic overview of gene therapy process (Domm et al. 2021)

2.13.3 mRNA Gene Therapy

Messenger RNA (mRNA) is a novel form of gene therapy currently in the clinical study stages for the treatment of FD and other rare monogenic disorders. The alpha-Gal-A mRNA is encapsulated in lipid nanoparticles (LNPs) and administered intravenously. Encoding of the alpha-Gal-A enzyme followed after the administration of the mRNA, stimulating the production of the enzyme (Kok *et al.* 2021). An improvement in Gb3

clearance was demonstrated after a significant increase in alpha-Gal-A levels in the liver, cardiac and kidney tissues (DeRosa *et al.* 2019). The alpha-Gal-A enzyme levels peaked at 6-12 hours post-administration, making the half-life longer than intravenous ERT (Domm *et al.* 2021). A significant advantage of mRNA therapy was demonstrated in Fabry mice where no alpha-Gal- A antibodies were produced after repeated mRNA administrations (Zhu *et al.* 2019).

2.14 Misdiagnosis of Fabry Disease

As awareness about Fabry disease is increasing, the variability of the symptoms still poses a challenge for early diagnosis. The Fabry Registry states that the average age of symptom onset is 10.5 years, yet the average age of diagnosis is 28.5 years (Michael Y Soliman 2016). The Fabry Outcome Survey (FOS) has reported over 25% of misdiagnosed cases (Shah *et al.* 2005). Lin CJ et.al. states that patients who experience stroke at an early age, cardiomyopathy, and end-stage renal disease (ESRD) with unknown aetiology are groups who are at a greater risk of Fabry disease (Lin *et al.* 2018). Previous studies revealed heterozygous females were known to show no symptoms of the disease, however, recent studies indicated that affected females can display multiple symptoms.

Research shows that 33% of males and 37% of females remain undiagnosed with Fabry disease until patients present with end-stage renal disease (Mallett *et al.* 2020). Renal involvement begins with the accumulation of Gb3 in the endothelial, interstitial, epithelial tubular, and glomerular cells. Proteinuria and a decrease in glomerular filtration rate occur in the second to third decades of life. Proteinuria and or albuminuria are the first indications of Fabry disease, although progressive chronic kidney disease in Fabry disease can be compared to diabetic nephropathy with the average decrease in glomerular filtration rates of 12mL/min/yr. (Beirão *et al.* 2016).

Case studies of misdiagnosis show a healthy 49-year-old female who presented with hypertension and proteinuria but displayed no evident signs and symptoms of Fabry disease. There were no cardiac abnormalities. A renal biopsy was performed. Light microscopy revealed that 2 out of 30 glomeruli showed global sclerosis and 3 had fibrous crescent formation diagnosing IgA nephropathy. Immunofluorescence microscopy

revealed lamellar inclusions which are characteristic of Fabry disease (Kakita *et al.* 2010). Fabry disease has also previously been misdiagnosed as Henoch-Schonlein purpura, although it remains unclear if IgA nephropathy is concomitant with Fabry disease or if Fabry disease causes mesangial IgA deposition (Kim *et al.* 2015). A recent study involved a female patient admitted for abnormal urinalysis. She displayed no clinical manifestations of Fabry disease. A renal biopsy was performed and mutant analysis revealed IgM nephropathy and Fabry disease (Wu *et al.* 2019).

Gastrointestinal symptoms in Fabry disease can be non-specific. These symptoms can be the first and only signs of the disease and can be easily misdiagnosed. Glycolipid deposition leads to abnormal smooth muscle activity which causes GI discomfort. Symptoms include abdominal pain, nausea, vomiting, constipation, bloating, and diarrhoea, and can become debilitating. Frequently, these symptoms are misdiagnosed as irritable bowel syndrome, autoimmune diseases, Crohn's disease, or coeliac disease (Politei *et al.* 2016). The frequency of these symptoms in patients diagnosed with Fabry disease remains unclear, however, a high prevalence among children and females has been reported. Statistics from the Fabry Registry indicates that non-specific GI symptoms present in 23% of boys with an average age of 5 years and 11% of girls with an average age of 9 years (Hilz *et al.* 2018). It is therefore important that paediatricians and gastroenterologists have knowledge of Fabry's disease and symptoms and signs to reduce the risk of misdiagnosis.

As with other symptoms of Fabry disease, neurological symptoms are also often misdiagnosed in patients. Neurological symptoms in young children are often mistaken as viral infections, rheumatism, or 'growing pains'(Politei, Durand and Schenone 2016). Ischemic stroke and transient ischemic attacks (TIA) are the main cerebrovascular events of Fabry disease, however, cerebral haemorrhage, micro-bleeding, and cerebral venous thrombosis have also been reported (Brooks and Fragoso 2016). A frequent misdiagnosis of Fabry disease is multiple sclerosis as both conditions present with pain and white matter lesions on magnetic resonance imaging (MRI) which fulfils the McDonald criteria for diagnosing multiple sclerosis. The differentiating characteristics of Fabry disease is the "T2 FLAIR (T2 weighted Fluid Attenuated Inversion Recovery) MRI of the white matter usually produces asymmetric and confluent images, with little involvement of the corpus

callosum and no enhancement of the lesion through gadolinium. There are no lesions in the spinal cord" (Colomba *et al.* 2018). In a study conducted by Colomba et.al. (2018), 86 patients were investigated. These patients were previously diagnosed with possible multiple sclerosis. Four female patients out of the 86 patients were diagnosed with Fabry disease (Colomba *et al.* 2018). With early and correct diagnosis, enzyme replacement therapy is known to stabilize vascular disease progression and reduce the risk of stroke (Brooks and Fragoso 2016).

Left ventricular hypertrophy (LVH), coronary artery disease, hypertension, and aortic and mitral regurgitation are common manifestations of Fabry disease due to progressive accumulation of globotriaosylceramide in the vascular endothelium. The common misdiagnosis for cardiac manifestations is hypertrophic cardiomyopathy (Kunkala *et al.* 2013). Left ventricular hypertrophy is an early cardiac abnormality in Fabry disease and is detected by the use of ECG monitoring. Cardiologists play an important role in the early diagnosis of cardiac manifestations to avoid subsequent renal and neurological damage. The prevalence of Fabry disease in patients with unexplained LVH varied between 1% and 12%. Studies revealed that patients who presented with only cardiac abnormalities were later diagnosed after further investigations, with Fabry disease (Savary *et al.* 2017). In other cases, patients with unexplained cardiac abnormalities were diagnosed with systemic lupus erythematosus due to the similarity of organ involvement (Nandagudi *et al.* 2013).

Early and correct diagnosis of Fabry disease will allow for the initiation of enzyme replacement therapy (ERT). Early commencement of ERT has shown improvement in clinical manifestations of the disease. In trials conducted by El Dib, et.al. (2016), results showed "significant improvement regarding microvascular endothelial deposits of globotriaosylceramide and in pain-related quality of life" (El Dib *et al.* 2016). It has been shown to delay the progression of kidney disease and left ventricular mass reduction (Beirão *et al.* 2016).

CHAPTER 3: METHODS

AIM

This study aimed to investigate the association between Fabry disease (FD), its clinical manifestations, and chronic renal failure in KwaZulu-Natal province. The study design was a prospective, quantitative and descriptive study design method was employed. The study was designed to investigate symptoms of FD in patients with chronic renal failure to determine if there is a correlation between Fabry disease and chronic renal failure.

OBJECTIVES

- The levels of the alpha-galactosidase-A enzyme in the samples were assessed.
- Any association between the clinical symptoms and Fabry's disease was established.
- Any association between chronic renal failure symptoms and signs of Fabry disease by reviewing medical history for undiagnosed causes of chronic renal failure was established.

Due to the small but relevant number of cases of Fabry disease, the study required a large sample size. A cohort of 200 male patients with chronic renal failure was selected from three provincial hospitals, viz., Addington Hospital, Inkosi Albert Luthuli Central Hospital, and St. Aidan’s Hospital. Two patients were confirmed with Fabry disease and were enrolled as positive controls. The patients enrolled for the study were male patients, receiving hemodialysis treatment, patients receiving peritoneal dialysis, and pre-renal patients. Male patients were diagnosed with chronic renal failure, i.e., stage 5 CKD (eGFR < 15ml/min), were receiving HD as outpatients. Participants receiving peritoneal dialysis with stage 5 CKD were considered. Patients who were pre-dialysis candidates with stage 2 CKD (eGFR 60-89ml/min) to stage 4 CKD (eGFR 15-29ml/min) were also enrolled. The research study was explained to all patients who were interested in participating and a consent form was signed before any investigations were performed. Patients were informed that participation was voluntary and they were entitled to withdraw from the study at any point without consequences regarding their treatment. An additional 15 healthy male participants with no renal impairment (eGFR >90ml/min -Stage 1 CKD) were

enrolled as a control group. The total sample size of participants was 215. Demographics of all participants were documented using a questionnaire (Appendix 11).

3.1 Selection Criteria

The following inclusion and exclusion criteria were used to determine if prospective volunteers qualify to participate in the study:

Inclusion criteria:

• All participants who provide consent.
• Male participants over the age of 18 years old up to the age of 75 years of age.
• All recruited patients are required to be chronic haemodialysis, peritoneal dialysis, or pre-renal patients.
• All recruited patients are diagnosed with renal impairment.
• Control participants are required to have no renal impairment (normal GFR ≥ 60ml/min).
• Control participants are required not to present with clinical manifestations of Fabry disease.

Exclusion criteria:

• Participants under the age of 18 years.
• Participants who do give consent to participate.
• Participants who do not understand the English and isiZulu language.
• Patients who are unwell and ailing and unable to provide consent.
• Control participants who have renal impairment.
• Control participants who display clinical manifestations of Fabry disease.

The Good Clinical Practice course was completed with certification obtained online via the TRREE website (Appendix 19). Ethical approval (IREC) was obtained from the Durban University of Technology before any investigative work was commenced (Appendix 17). Permission was also obtained from the hospital managers of Addington Hospital (Appendix 14), St Aidan's Hospital (Appendix13), and Inkosi Albert Luthuli Central Hospital (Appendix 16). Permission was granted from the Department of Health for the above stated Ethekwini hospitals (Appendix 17). All COVID-19 protocols were adhered to during the enrolment of patients.

Patients from the haemodialysis units of Addington Hospital, St Aidan's Hospital, and Inkosi Albert Luthuli Central Hospital were enrolled from March 2021 during the COVID-19 alert level 1 lockdown. Enrolment and samples were collected from the hospitals as well as the pre-renal and peritoneal dialysis clinics from May 2021 (alert level 2 lockdown). In June 2021, during sample collection, the COVID-19 lockdown moved into a level 4 lockdown and patient attendance decreased significantly. Patient enrolment and blood sampling continued and in July 2021 the lockdown level moved to alert level 3 and in September 2021 lockdown moved to alert level 2. Thereafter, in October 2021 the level was adjusted to level 1 where it remained. Patient attendance, however, was still restricted due to continued COVID-19 regulations.

3.2 Blood Samples

Enrolment and blood sampling of participants

Since heterozygous females for FD can present with clinical manifestations ranging from mild symptoms and asymptomatic to severely affected depending on the severity of their X chromosome inactivity profile, measurement of alpha-Gal A activity can be unreliable for identifying heterozygotes, due to subnormal enzymatic levels (Vigneau *et al.* 2021). Therefore, female patients were excluded from this study based on the enzymatic assay, to avoid false negative results. As a result, in our study, male patients with chronic kidney disease stage 2-5D (n=200) were enrolled in line with the inclusion criteria, and consent was obtained from those willing to participate. A control group consisting of males (n=15) was also recruited after consent was provided. Reference numbers were allocated to protect the identity of the participants and maintain patient confidentiality. A questionnaire (Appendix 11) employing the Mainz Severity Score Index (MSSI) was provided to allow the participants to identify any of the clinical manifestations of Fabry disease (Giugliani *et al.* 2016). One control participant unknowingly had diminished kidney function with an eGFR of 37ml/min/1.73m^2 indicating stage 3 CKD and had to be excluded from the study. The participant was counselled and advised to consult his doctor for further examination.

3.3 Haemodialysis Patients

A 5ml blood sample using an Ethylenediamine tetra-acetic acid (EDTA) tube were drawn from the antecubital vein of pre-renal patients (CKD 2-5) and peritoneal dialysis patients during their visit to their respective clinics. Blood samples of haemodialysis patients were drawn pre-dialysis from their dialysis access. The sample was taken from the patient's dialysis access after consent was obtained.

3.4 Pre-renal and CAPD Patients

Patients from the pre-renal and CAPD clinics were interviewed and once consent was obtained, a blood sample of 5mls was taken from the antecubital vein using an EDTA tube.

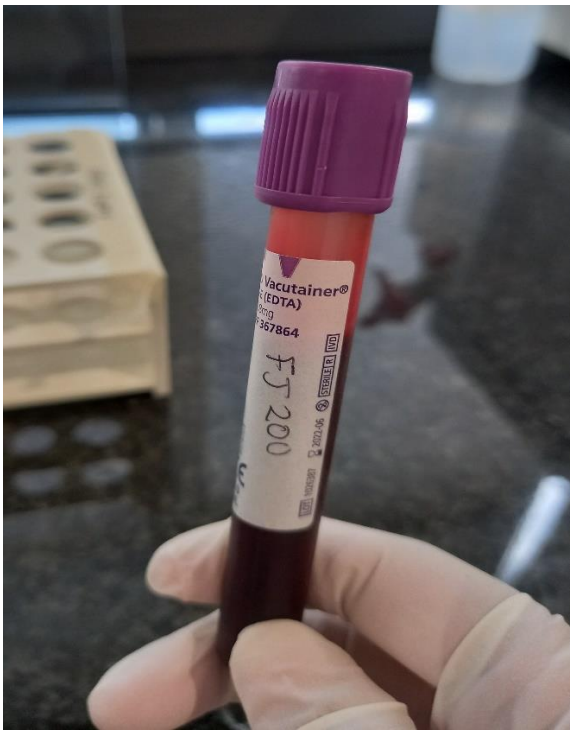

Figure 16: EDTA tubes were used as primary collection tubes (Singh 2022)

3.5 Control Participants

Control participants were interviewed and after consent was obtained, blood samples were taken from the antecubital vein. A 5ml blood sample was taken using a serum separator tube (SST) and transported at room temperature to a pathology laboratory for testing of participants' urea, creatinine, and eGFR levels. A second blood sample of 5mls

was taken from the same site using an EDTA tube which was centrifuged and stored for analysis.

3.6 Transportation and Preparation of Blood Samples

The blood tubes were labelled using a reference number to protect the individual patient's identity. The blood samples were placed in polystyrene tube holders and couriered by the principal investigator in a cooler box lined with ice packs. The blood samples were transferred into 15ml conical bottom centrifuge tubes and centrifuged at 4000rpm for 10 minutes at 5°C to separate the plasma from the blood components. A volume of 1ml of plasma was transferred into microcentrifuge tubes and labelled according to the corresponding patient reference numbers and stored at -80°C. This was done in duplicate. In addition, the buffy coat layer containing the polynuclear cells was transferred into microcentrifuge tubes and stored at -80°C for further future genetic studies. The samples were also labelled according to the corresponding patient reference numbers.

Figure 17: Blood samples were transferred from the primary collection tubes (EDTA) into 15ml centrifuge tubes (Singh 2022)

Figure 18: Samples were centrifuged using the Eppendorf Centrifuge 5810R at 5°C at 4000rpm for 10 minutes (Singh 2022)

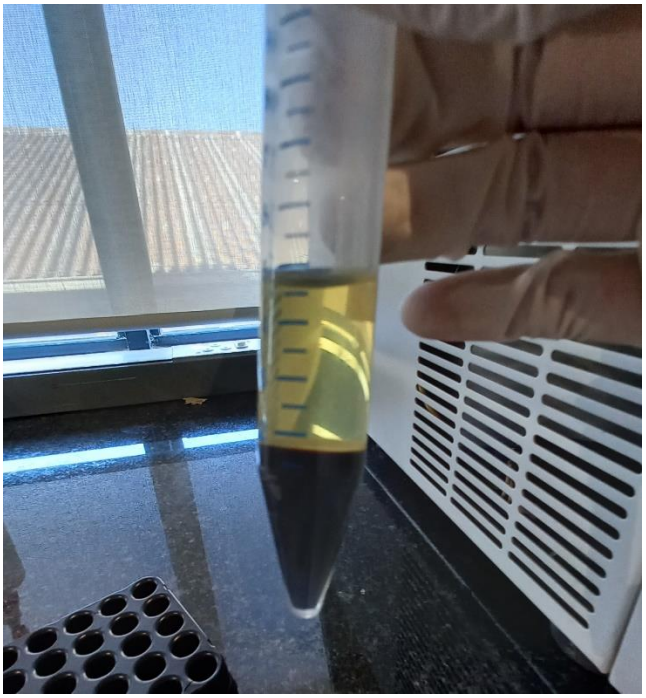

Figure 19: Blood sample after centrifuging for 10 minutes (Singh 2022)

Figure 20: Plasma and buffy coat with red cells were transferred into microcentrifuge tubes respectively and stored at -20°C (Singh 2022)

3.7 Enzyme-Linked Immunosorbent Assay

The ELISA kit used for the test was the Human GLα (Galactosidase Alpha) ELISA kit from Elabscience based in Houston, Texas, United States.

3.8 Principal of the Assay

As per the package insert of the Human GLα (Galactosidase Alpha) ELISA kit, this ELISA kit employed the Sandwich-ELISA principle. was supplied with the 96 wells that were coated with an antibody identifiable to Human GLα by the manufacturer. Samples or Standards were pipetted into the micro-ELISA plate wells and was allowed to mix with the specific antibody. An incubator was used to incubate the microplate for 90 minutes at 37ºC. Subsequently, a biotinylated detection antibody specific for Human GLα and Avidin-Horseradish Peroxidase (HRP) conjugate was pipetted successively into each microplate well and incubated for 1 hour and 30 minutes at 37ºC respectively. The wells were washed, removing the free components. The substrate solution was added to each well ensuring the plate was protected from the light. This was incubated for an additional 15 minutes at 37ºC. The wells containing Human GLα, biotinylated detection antibody, and Avidin-HRP conjugate underwent an immediate colour change and appeared blue in

colour. To terminate the enzyme-substrate reaction, a stop solution was added immediately to each well changing the colour to yellow. Spectrophotometric measurement of the optical density (OD) was performed using a wavelength of 450 nm ± 2 nm. The OD value was relative to the concentration of Human GLα. Measurement of the optical density (OD) was performed spectrophotometrically using a wavelength of 450 nm ± 2 nm.

3.9 Preparation of Blood Samples for Testing

Blood samples tested for the day were brought to room temperature. All samples being tested were arranged according to the plan layout for the ELISA plate. The samples were tested in duplicate.

3.10 Preparation of the Enzyme-Linked Immunosorbent Assay

The reagents used for the Human GLα (Galactosidase Alpha) ELISA kit were prepared following the manufacturer's directions. All the reagents were removed from the fridge and brought to room temperature (18-25℃) before use.

Wash Buffer

A wash buffer of 750mL was prepared by diluting 30mL of concentrated wash buffer with 750mL of distilled water. This was attached to the microplate washer.

Standard working solution

A volume of 1 mL of reference standard and sample diluent was added to the standard working solution. It was allowed to stand for 1-2 min and then mixed thoroughly with a vortex meter at low speed. Serial dilutions were prepared as required. The recommended dilution gradient was as follows: 5000, 2500, 1250, 625, 312.5, 156.25, 78.13, 0 pg/mL.

Preparation of Standards and Blank Solution

To 7 microcentrifuge tubes, 500uL of reference standard and sample diluent was added to each tube. A volume of 500uL of the 5000 pg/mL working solution was pipetted to the first tube and mixed to produce a 2500 pg/mL working solution. Then, 500uL of the solution from the former tube was pipetted into the latter one according to this step until the dilution gradient was achieved. All samples were vortexed prior to and after dilutions.

Preparation and Dilution of Biotinylated Detection Antibody Working Solution

The required amount was calculated before commencing the testing (100μL/well). In the preparation process, slightly more than what was calculated was prepared to avoid a shortage of the solution. The concentrated biotinylated detection Ab was centrifuged at 800×g for 1 minute. The 100× Concentrated Biotinylated Detection Ab to 1× working solution with Biotinylated Detection Ab Diluent was then diluted. The recommended ratio of Concentrated Biotinylated Detection Ab: Biotinylated Detection Ab Diluent is 1:99.

Concentrated HRP Conjugate Working Solution

The required amount was calculated before commencing the testing (100μL/well). In the preparation process, slightly more than what was calculated was prepared to avoid a shortage of the solution. The Concentrated HRP Conjugate was centrifuged at 800×g for 1 minute. The 100× Concentrated HRP Conjugate to 1× working solution with HRP Conjugate Diluent was then diluted. The recommended ratio of Concentrated HRP Conjugate: HRP Conjugate Diluent is 1: 99.

Table 1: Kit components and storage

Item	Specifications	Storage
Micro ELISA Plate (Dismountable)	96T: 8 wells ×12 strips	-20℃, 6 months
Reference Standard	96T: 2 vials	
Concentrated Biotinylated Detection Ab (100×)	96T: 1 vial, 120 μL	
Concentrated HRP Conjugate (100×)	96T: 1 vial, 120 μL	-20℃ (Protect from light), 6 months
Reference Standard & Sample Diluent	1 vial, 20 mL	2-8°C, 6 months
Biotinylated Detection Ab Diluent	1 vial, 14 mL	
HRP Conjugate Diluent	1 vial, 14 mL	
Concentrated Wash Buffer (25×)	1 vial, 30 mL	
Substrate Reagent	1 vial, 10 mL	2-8℃ (Protect from light)
Stop Solution	1 vial, 10 mL	2-8°C
Plate Sealer	5 pieces	

3.11 Assay Procedure

1. The wells for diluted standard, blank, and sample were established using a microplate model. A volume of 100 μL of standard, blank and sample was added to each dilution into

the appropriate wells. All samples and standards were assayed in duplicate. A sealer provided in the kit was used to cover the microplate kit and incubated for 90 min at 37°C.

2. Subsequently, the liquid was emptied from each well without washing. A volume of 100 µL of biotinylated detection Ab working solution was pipetted immediately using a multi-channel pipette into each well. A new sealer was used to cover the plate and incubated for 1 hour at 37°C.

3. After 1 hour, the microplate washer was used to aspirate the solution from each well, and 350 µL of wash buffer was added to each well. It was allowed to soak for 1 min and the solution was aspirated from each well. This wash step was repeated 3 times.

4. A volume of 100 µL of HRP Conjugate working solution was then added to each well. The plate was covered with a new sealer and incubated for a further 30 minutes at 37°C.

5. Thereafter, the solution was aspirated from each well and the wash process was repeated 5 times as conducted in step 3 using the microplate washer.

6. A volume of 90 µL of substrate reagent was then added to each well. A colour change to blue was noted. The plate was covered with a new sealer and incubated for about 15 min at 37°C. The plate was protected from light using a foil cover. The Microplate Reader was prepared and warmed about 15 minutes before OD measurement.

7. A volume of 50 µL of stop solution was added to each well and a colour reaction to yellow was noted.

8. The optical density (OD value) of each well was determined promptly with a micro-plate reader set to 450nm ±2nm.

Figure 21: The BioTek ELx50 microplate washer was used to wash the microplate wells (Singh 2022)

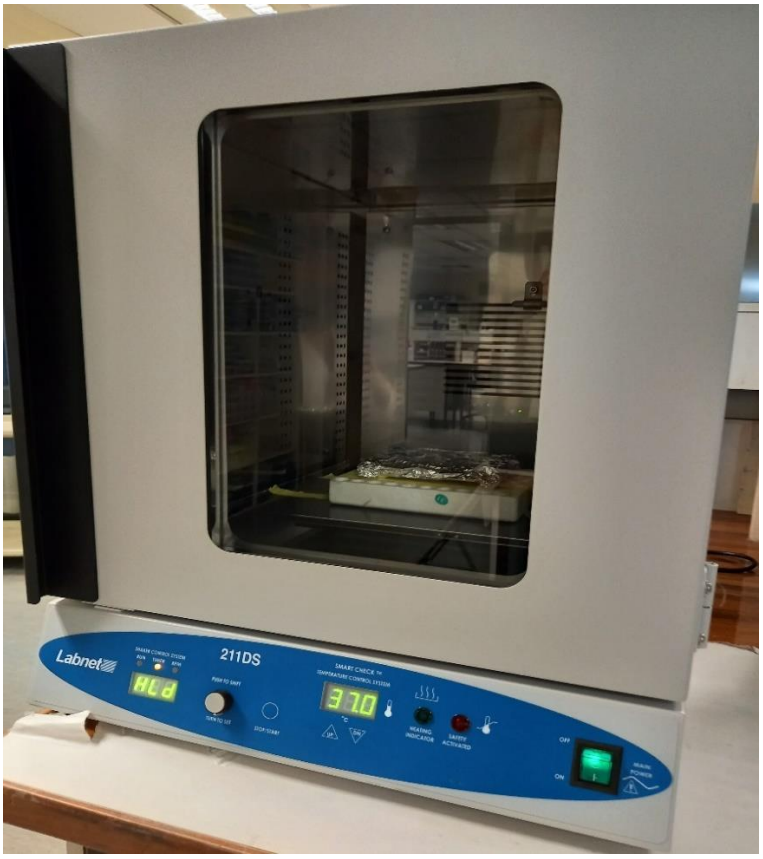

Figure 22: The Labnet 211DS incubator was used to incubate the samples as per manufacturer's instruction

Table 2: Summary of Assay Procedure

PROCEDURE	TIME
1. Standard or sample (100ul) added to the wells.	Incubation for 90 minutes at 37ºC.
2. Liquid is discarded. Biotinylated Detection Ab working solution (100ul) is added to each well.	Incubation for 60 minutes at 37ºC.
3. Liquid is aspirated and the plate is washed 3 times using a microplate washer.	15 minutes.
4. HRP conjugated working solution (100ul) is added.	Incubation for 30 minutes at 37ºC.
5. Liquid is aspirated and the plate is washed 3 times using a microplate washer.	15 minutes.
6. Substrate reagent (90ul) is added. Colour reaction is noted.	Incubation for 15 minutes at 37ºC.
7. Stop solution (50ul) is added. Reaction is terminated and plate is read at 450nm.	

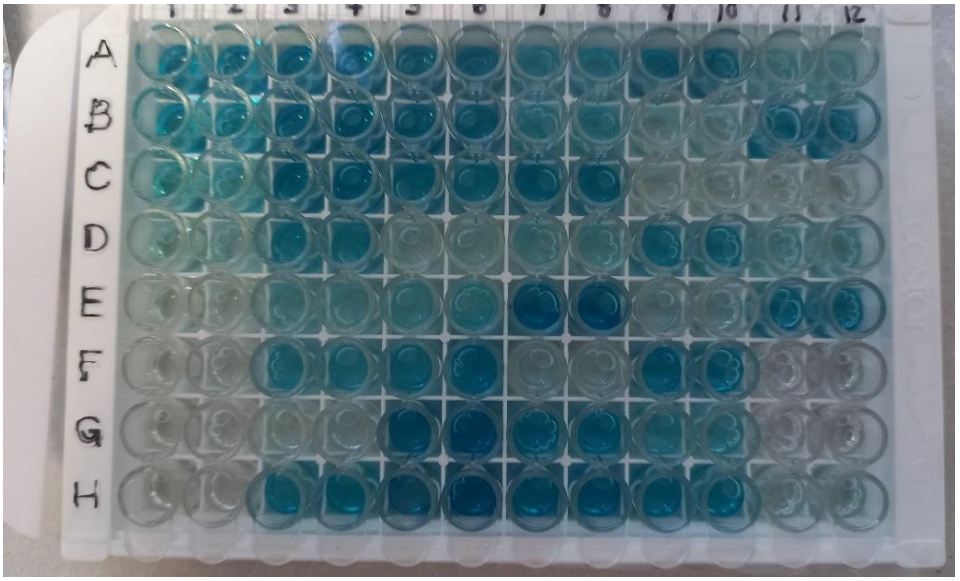

Figure 23: ELISA plate after substrate reagent was added (Singh 2022)

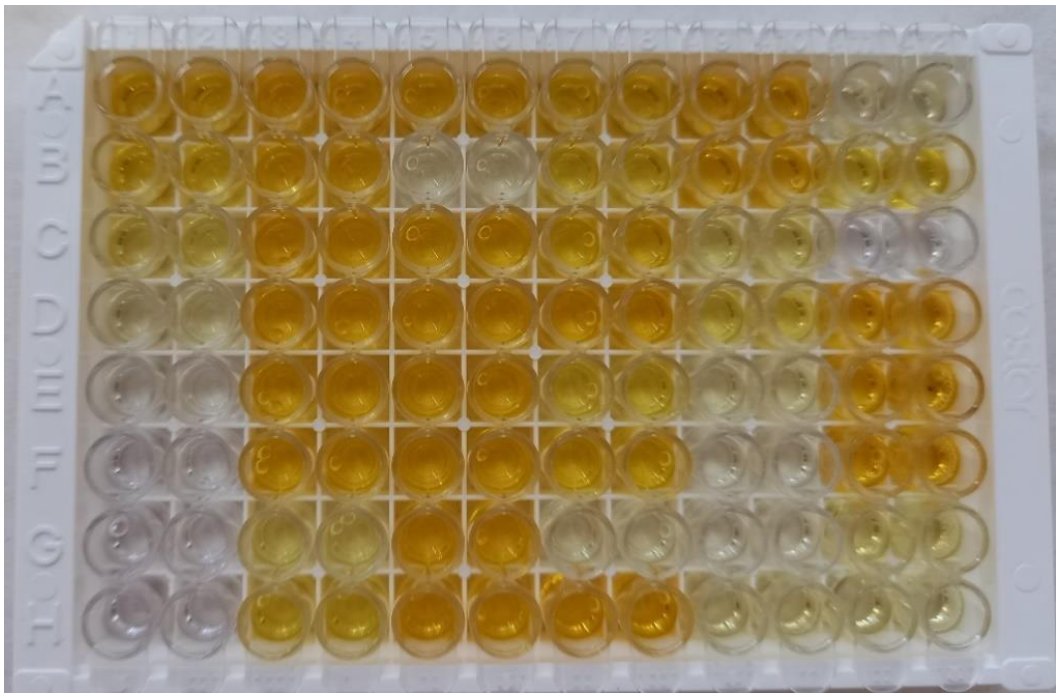

Figure 24: ELISA plate showing colour change using a stop solution to show results for Alpha-Gal A enzyme (Singh 2022)

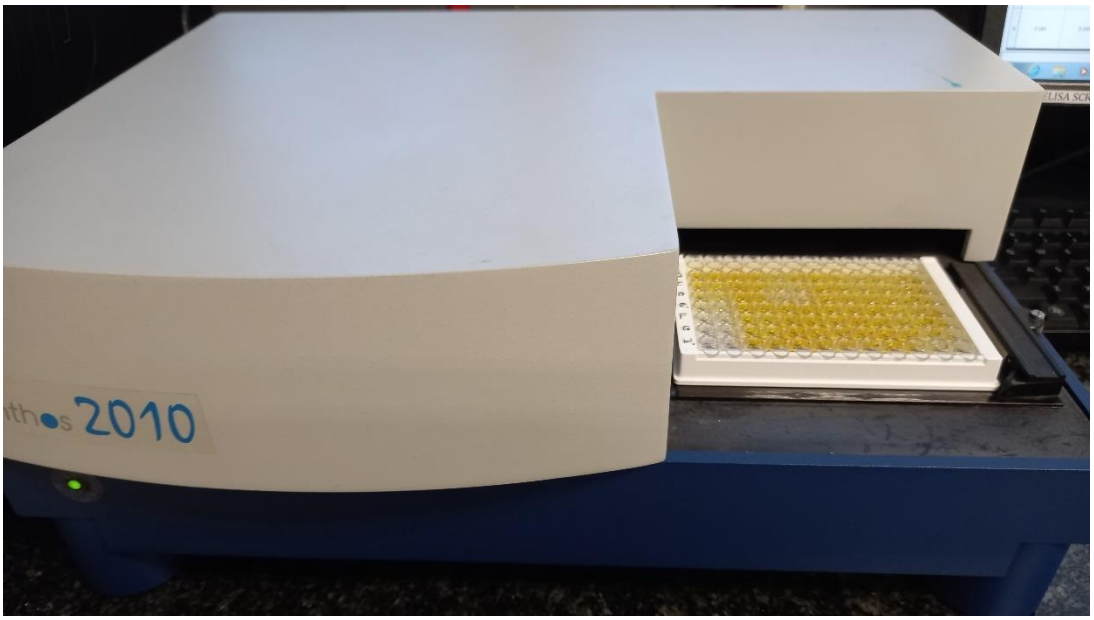

Figure 25: The Anthos 2010 microplate reader was used to read the ELISA plate (Singh 2022)

3.8 Data Analysis

All the data was recorded and saved. Analysis of the data was done accordingly.

3.9 Statistical Analysis

The data collected was organised, cleaned, and analysed using the IBM® SPSS Version 27. Descriptive statistical analysis including the minimum and maximum statistics, the mean and, the standard deviation was used to analyse the data.

3.10 Determining the Cut-off Values of the Alpha-Gal-A Enzyme Concentration Level Values

Using the control group as a benchmark for the cut-off value, we determined the average control value was 756±675. Based on this, 756pg/ml should have been employed as the cut-off value. However, it is established that Fabry disease is unlikely with high levels of the alpha-Gal-A enzyme and we concluded that a value of 500pg/ml was acceptable to utilise as a cut-off value (Appendix 20).

CHAPTER 4: RESULTS

4.1 A sample size of 200 haemodialysis, peritoneal dialysis, and pre-renal patients consented to participate in the study. A pie chart was constructed to depict the ethnic groups of the sample population (Figure 26). In patients with alpha Gal-A enzyme levels <500pg/ml ethnic groups showed no significance (p=0.224).

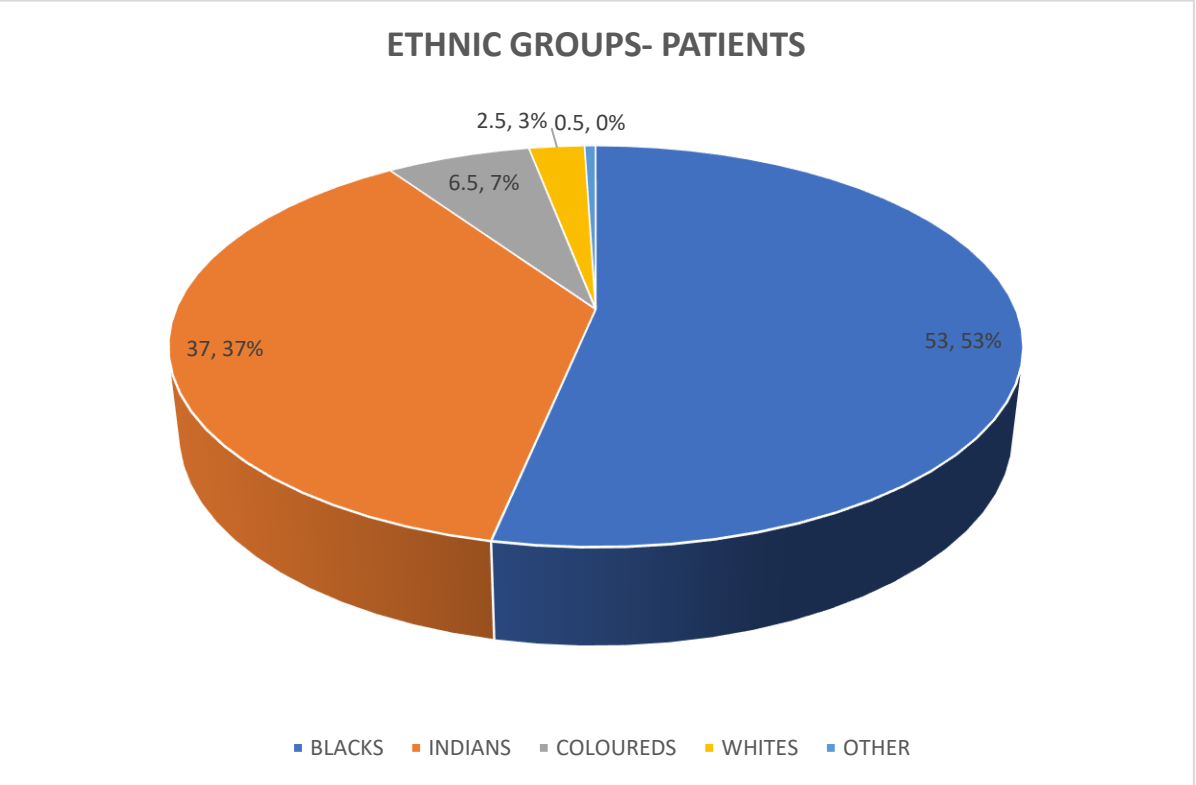

Figure 26: Graphical representation of the ethnic groups of the patient population

4.2 A control group of 14 healthy males was enrolled. A pie chart was constructed to depict the ethnic groups of the sample population (Figure 27).

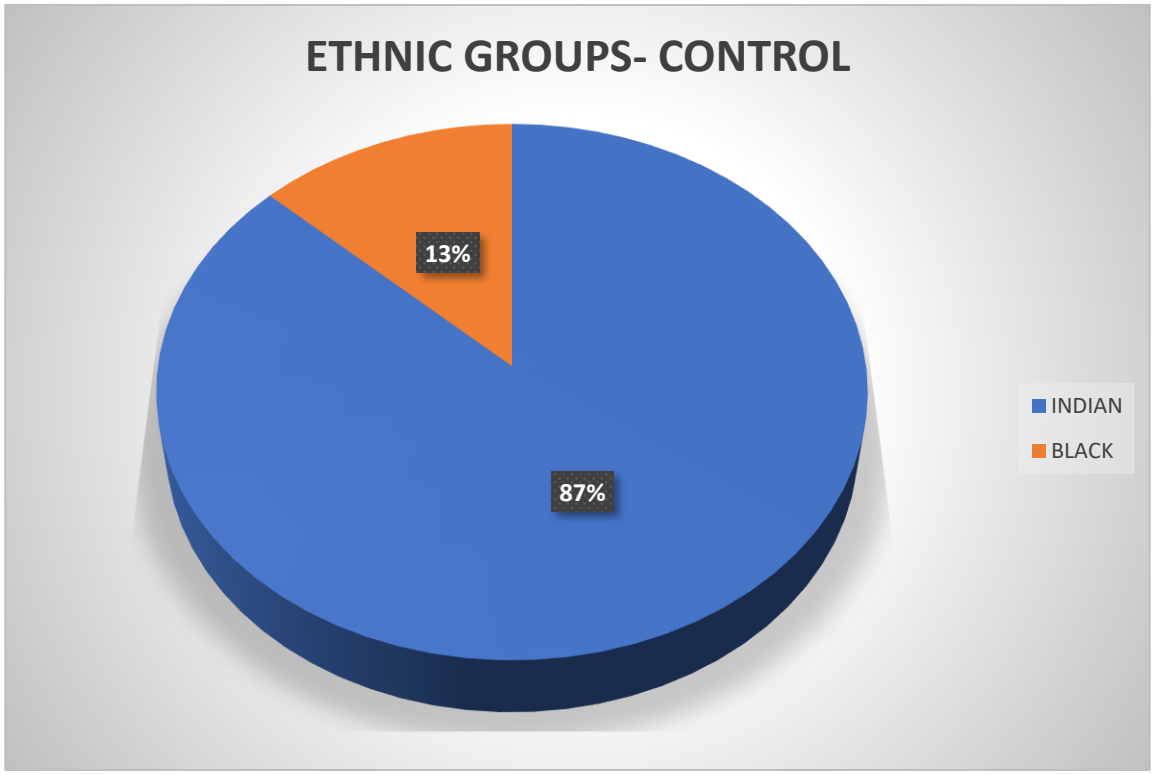

Figure 27: Graphical representation of the ethnic groups of the control group

4.3 The patient group consisted of haemodialysis, peritoneal dialysis and pre-renal patients. A pie chart was constructed to illustrate the percentage of patients in the respective groups.

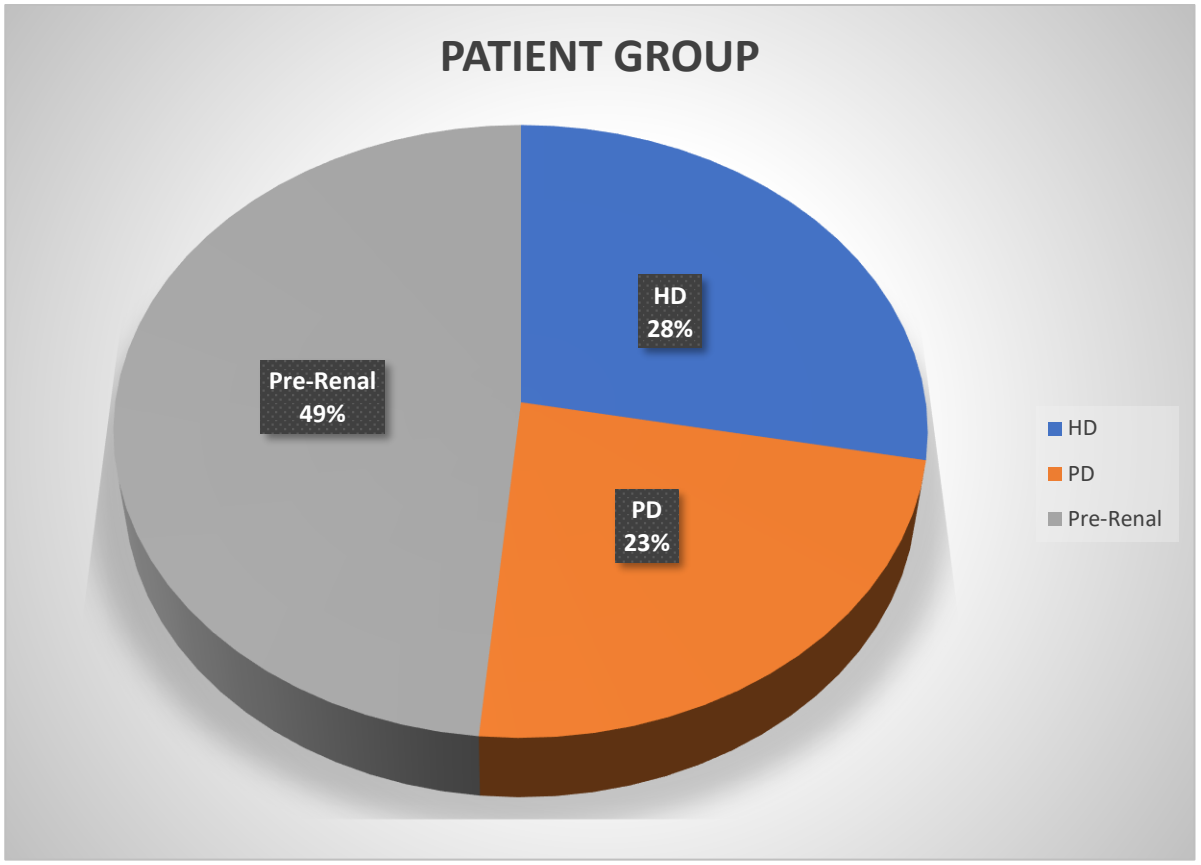

Figure 28: Graphical representation of treatment modalities of the patient group

4.4 Alpha Galactosidase-A enzyme concentration levels of all haemodialysis, peritoneal dialysis, and pre-renal patients (n=200).

The levels of alpha galactosidase-A enzyme were assessed. A graphical representation of the alpha-Gal A concentration levels was constructed using a scatter plot graph (Figure 29). The ELISA assay was unable to determine alpha-Gal levels >5000pg/ml.

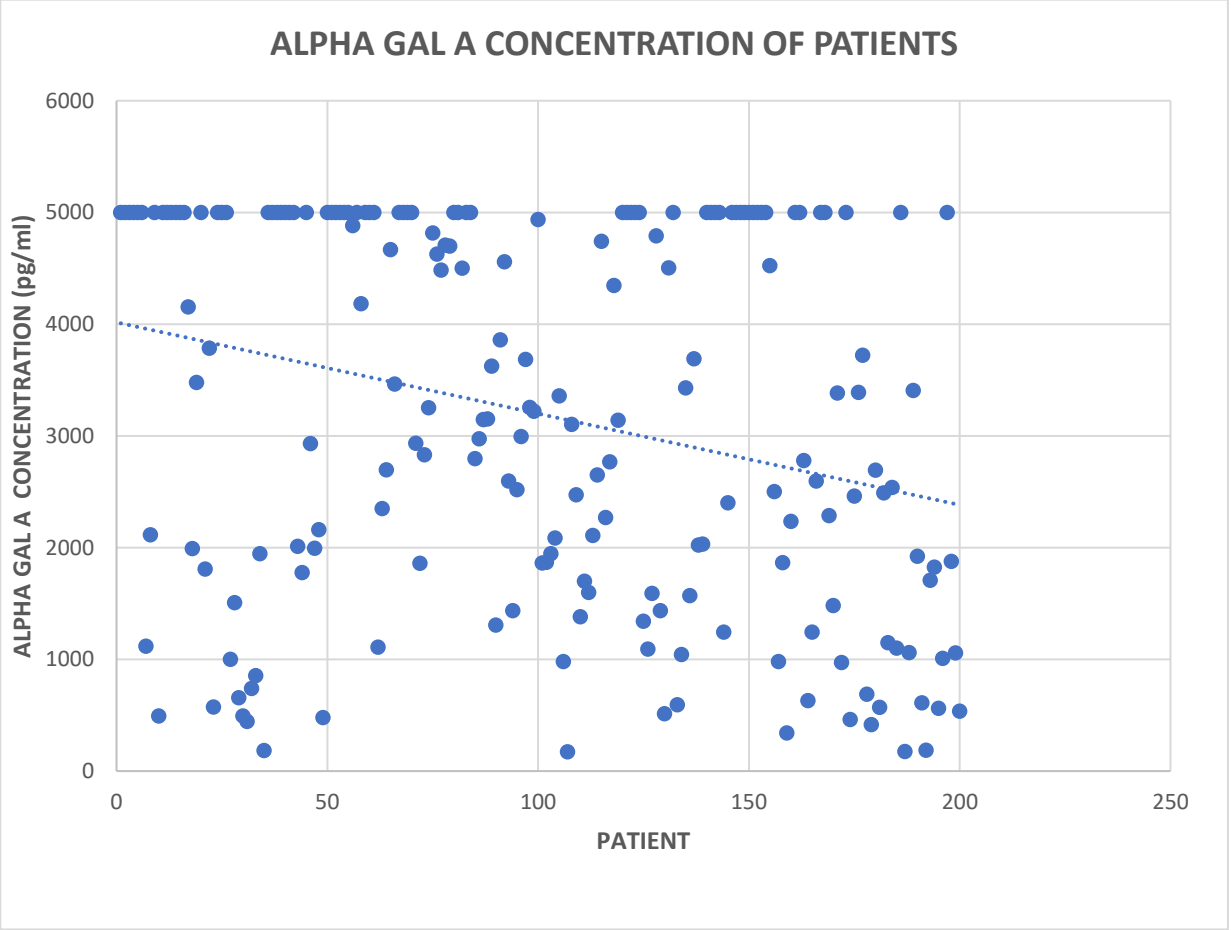

Figure 29: Alpha Gal-A enzyme concentration levels (y-axis), patient (x-axis)

4.5 Alpha Galactosidase-A enzyme concentration levels of control participants (n=15).

The levels of alpha galactosidase-A enzyme for control participants. A scatter plot was constructed to represent the concentration levels of alpha Gal-A enzyme levels (Figure 30). Patient 13 had to be excluded from the study due to his eGFR revealing Stage 3 CKD.

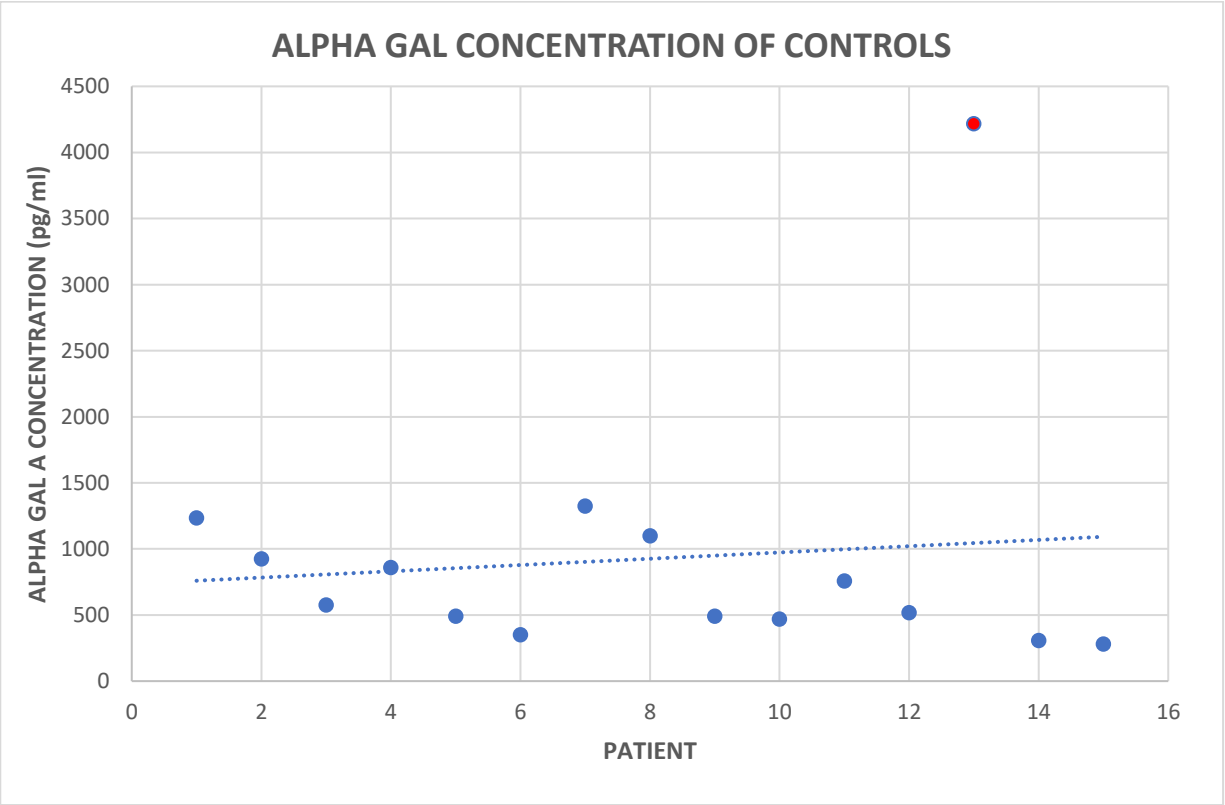

Figure 30: Alpha Gal-A enzyme concentration levels (y-axis), control (x-axis)

4.6 The association between eGFR and the alpha Gal-A enzyme levels of the patient group (n=200) was assessed with the use of scatter plots (Figure 31). The results were random and did not follow any specific pattern.

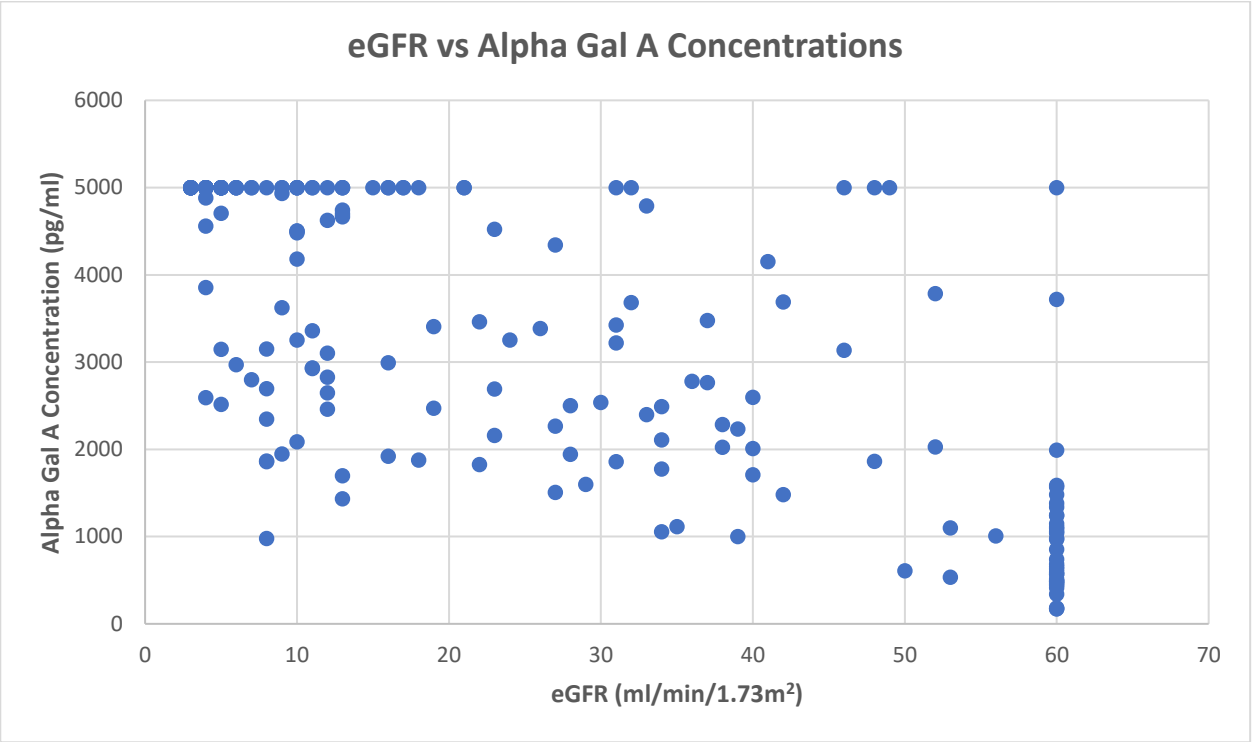

Figure 31: Alpha Gal-A enzyme concentration levels (y-axis), eGFR (x-axis)

4.6.1 The association between eGFR and alpha Gal-A enzyme was further assessed by grouping the stages of chronic kidney disease and the enzyme levels. The different stages of CKD against the alpha-Gal concentration levels demonstrated the increase in alpha-Gal levels as the as kidney function declined. Figure 32 shows patients with CKD stage 2.

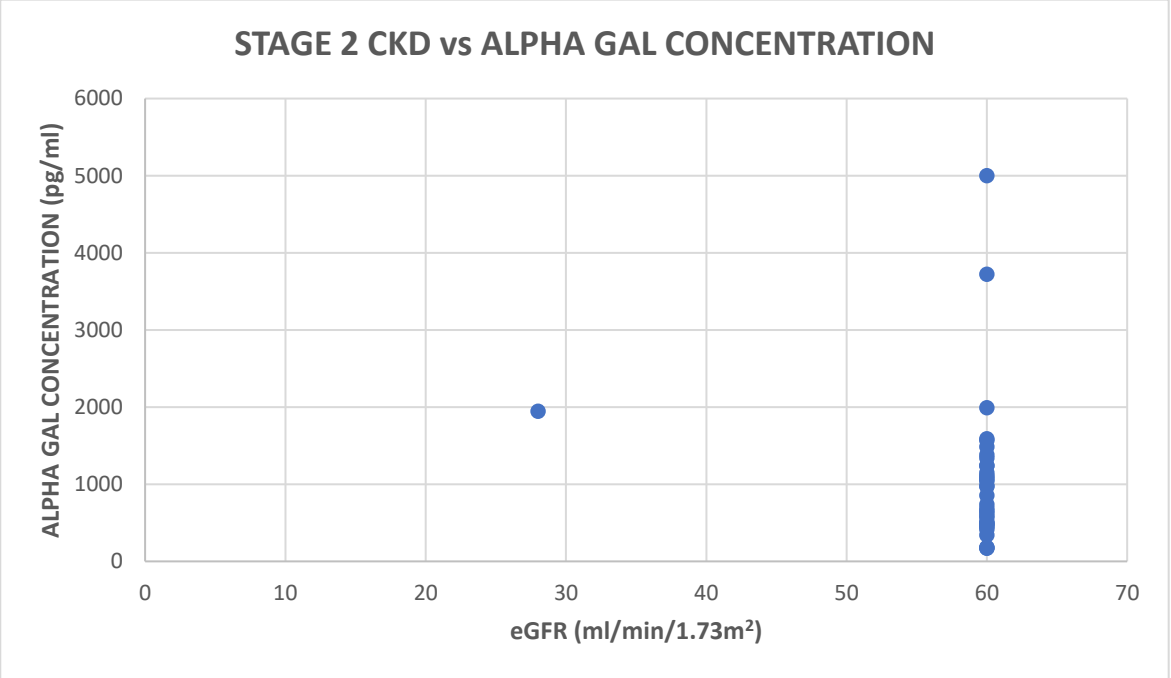

Figure 32: Stage 2 CKD, eGFR (X-axis), Alpha Gal-A enzyme concentration levels (Y-axis)

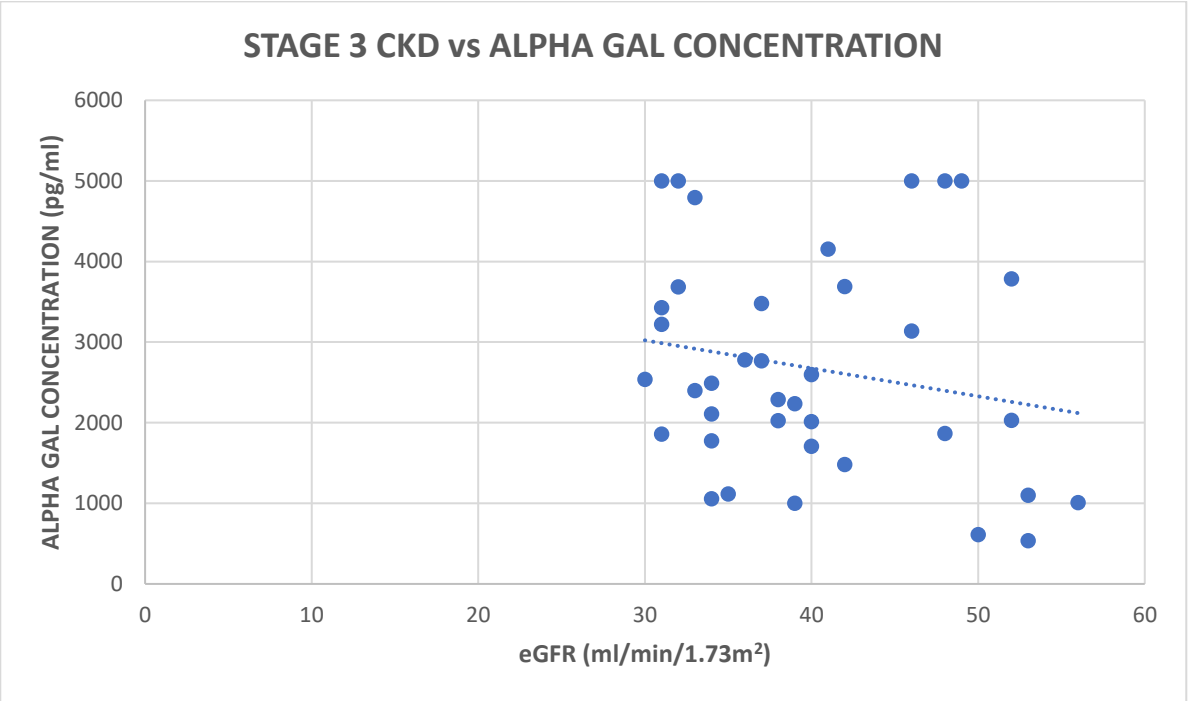

Figure 33: Stage 3 CKD, eGFR (X-axis), Alpha Gal-A enzyme concentration levels (Y-axis)

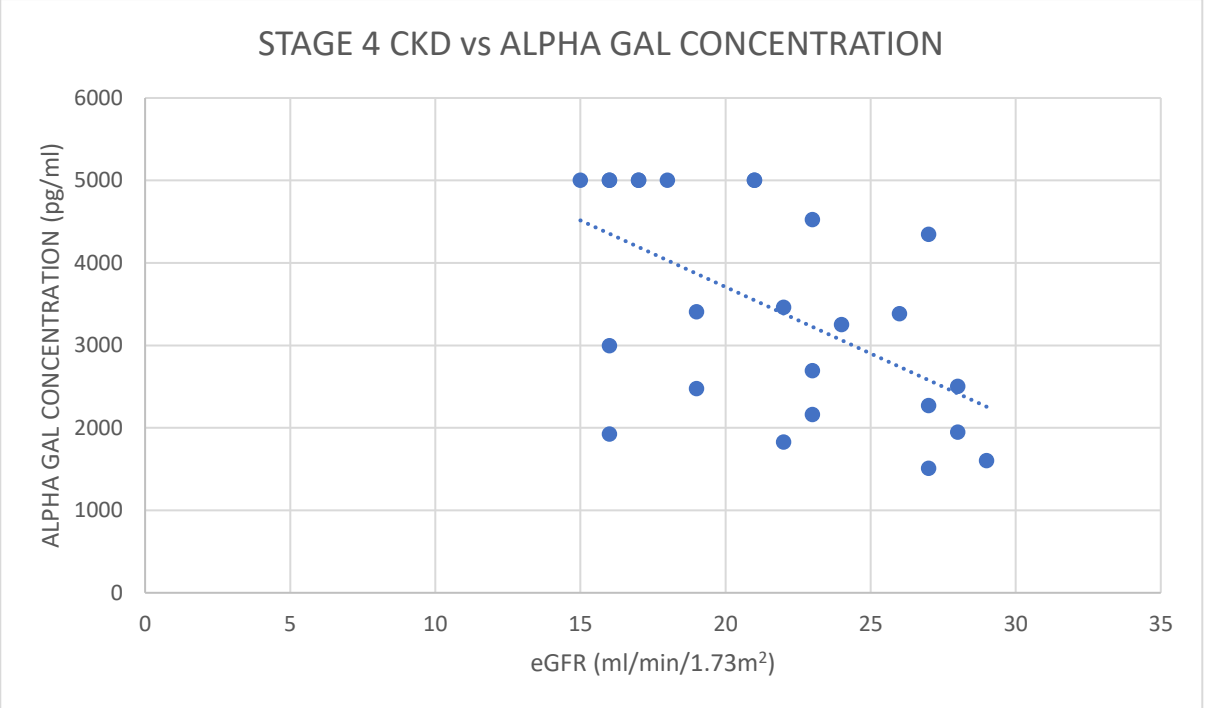

Figure 34: Stage 4 CKD, eGFR (X-axis), Alpha Gal-A enzyme concentration levels (Y-axis)

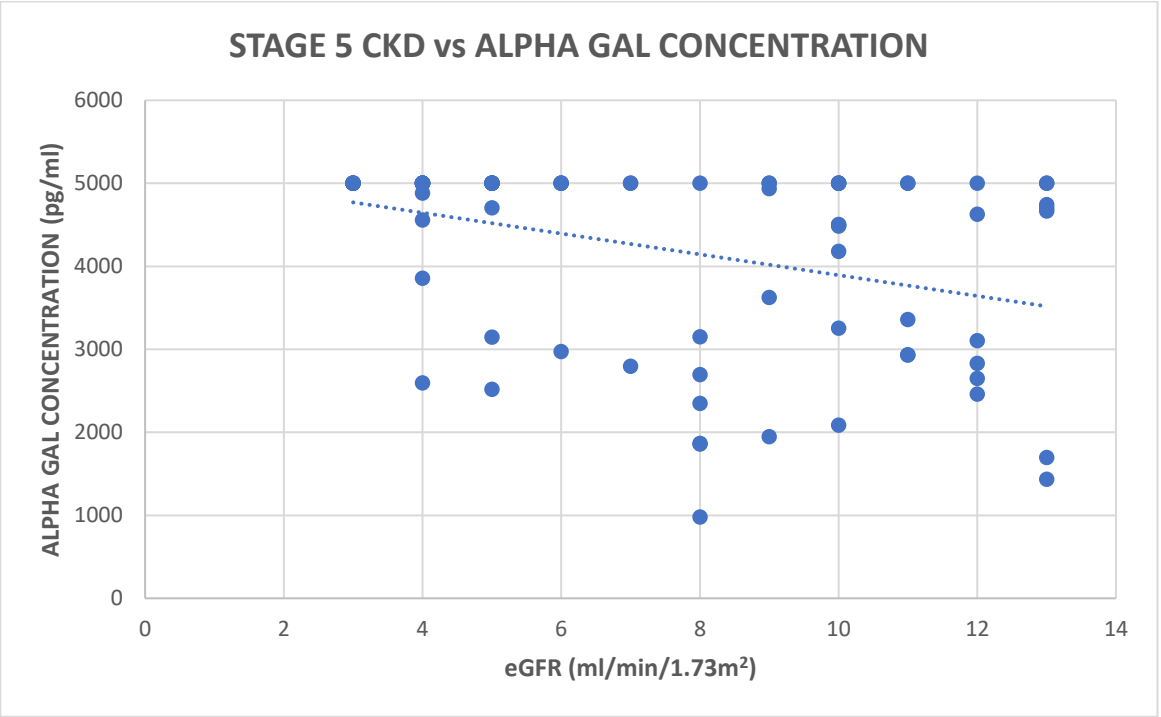

Figure 35: Stage 5 CKD, eGFR (X-axis), Alpha Gal-A enzyme concentration levels (Y-axis)

4.7 A bar graph (Figure 36) displaying the standard mean of error was constructed to demonstrate the average alpha-Gal-A concentration levels of the CKD stage 2-5 of the patient group.

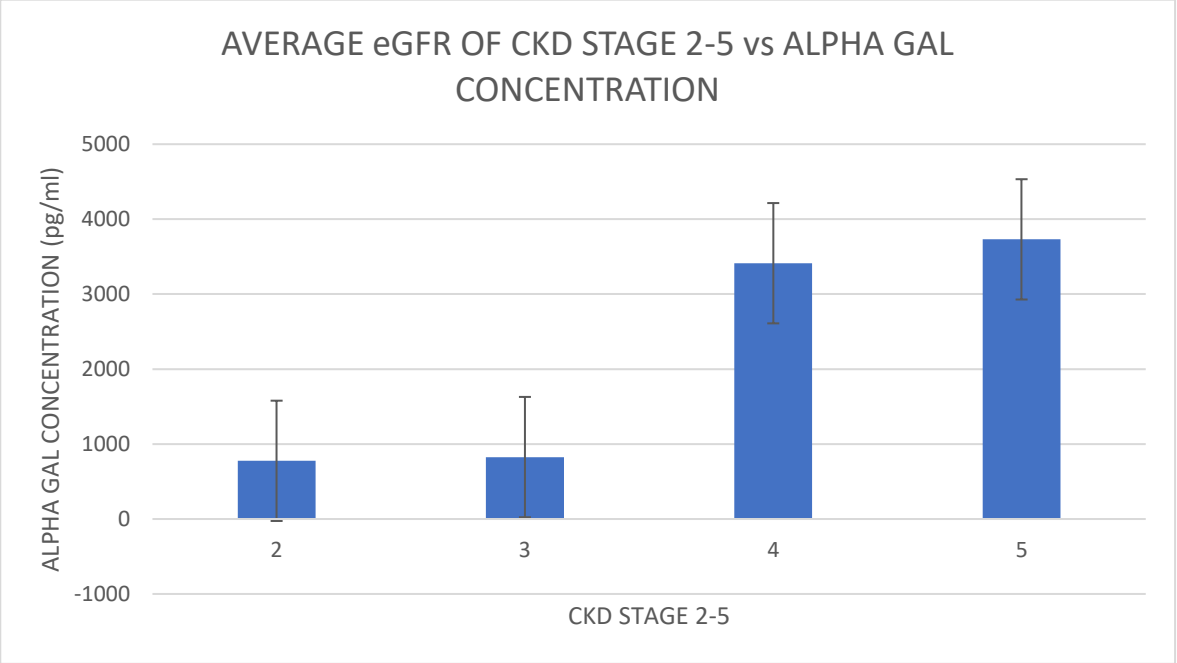

Figure 36: Bar graph showing standard mean of error depicting average alpha Gal-A levels of CKD stage 2-5

4.8 The alpha Gal-A enzyme concentration levels <500pg/ml were assessed using a scatter plot graph (Figure 37). A total of 11 patients exhibited concentration levels of <500pg/ml (n=11).

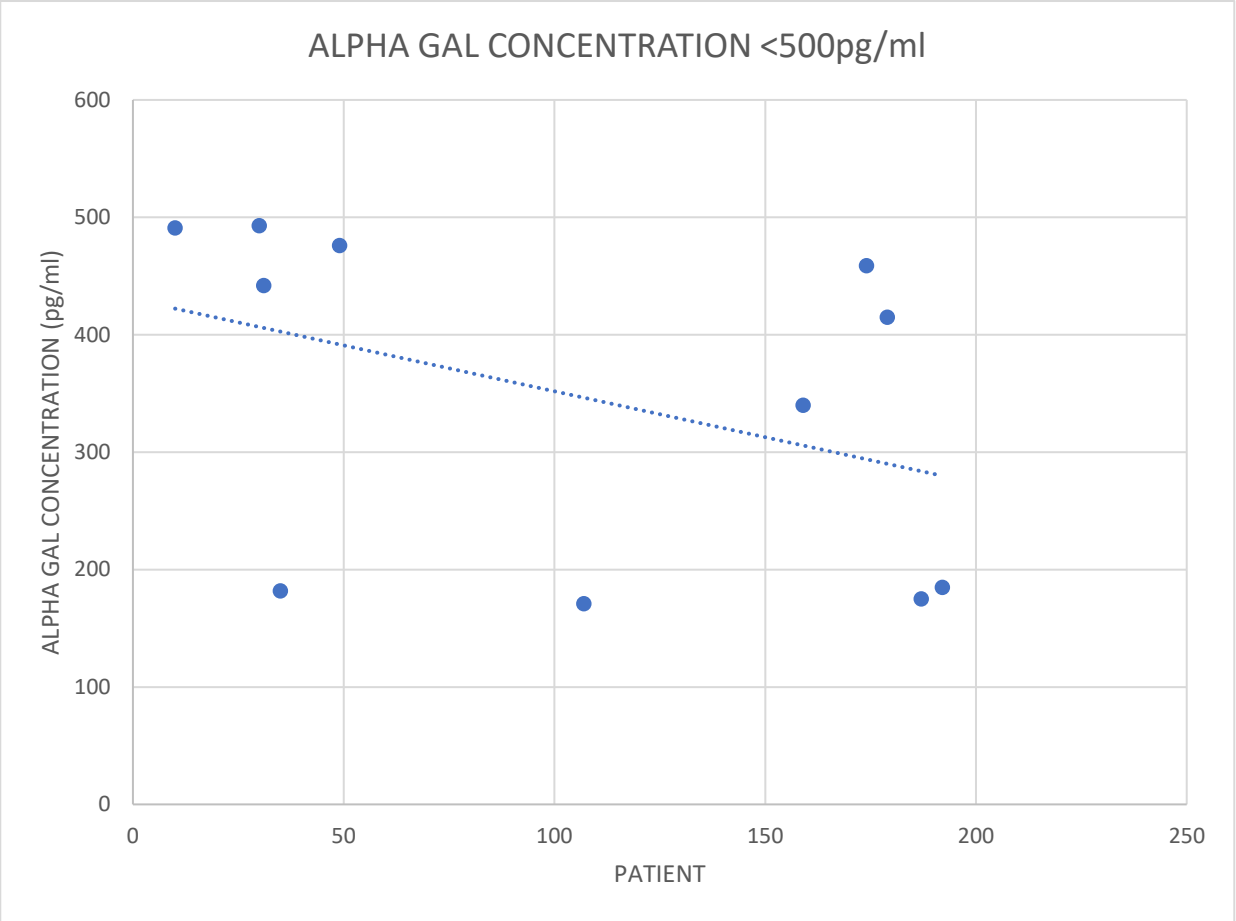

Figure 37: Alpha Gal-A enzyme concentration levels <500pg/ml (y axis), patient (x axis)

4.9 The eGFR of the patients with alpha Gal-A concentration levels of <500pg/ml were assessed (Figure 38). Patients presenting low levels of alpha-Gal- A concentration levels (n=11) had an eGFR >60ml/min/1.73m^2. The univariate analysis of eGFR demonstrated a significance with p=<0.001 while the multivariate analysis showed no significance with *p*=0.089.

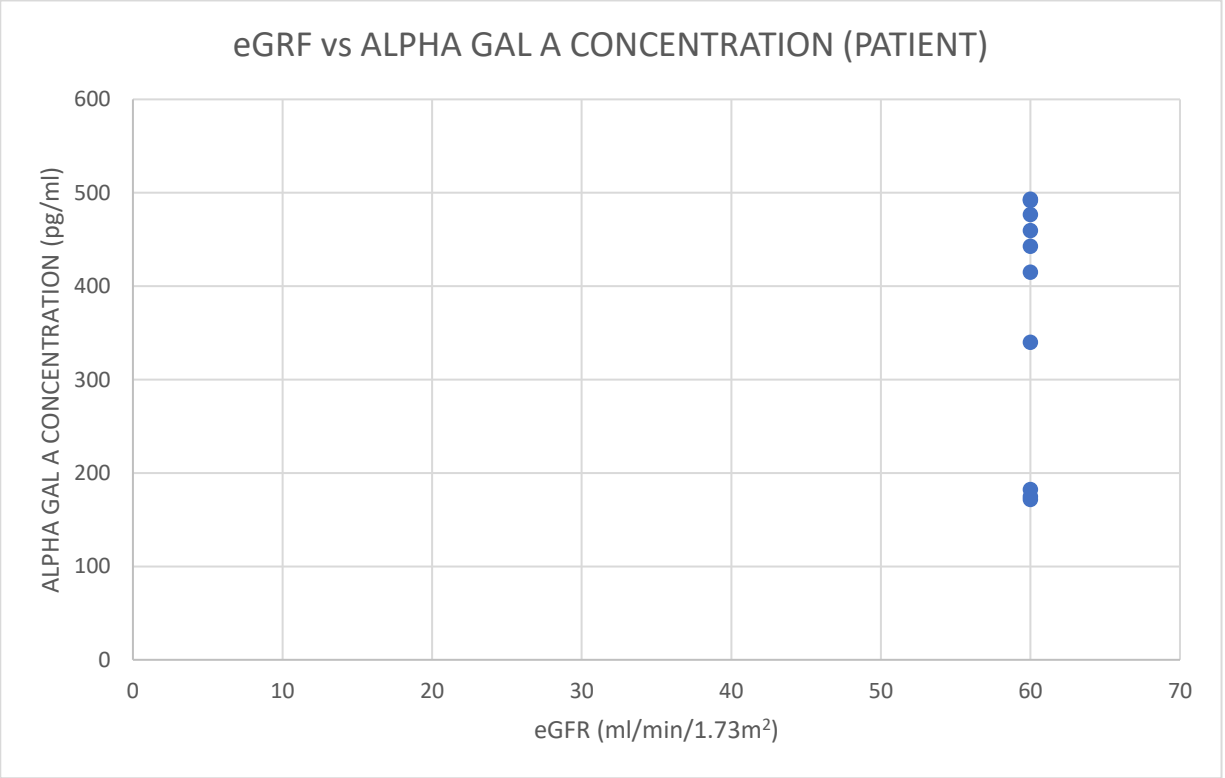

Figure 38: Alpha Gal-A enzyme concentration levels <500pg/ml (y axis), eGFR (x axis)

4.10 The participants of the control group displaying low levels of the alpha Gal-A enzyme of <500pg/ml (n=6) were represented by the construction of a scatter plot graph (Figure 39).

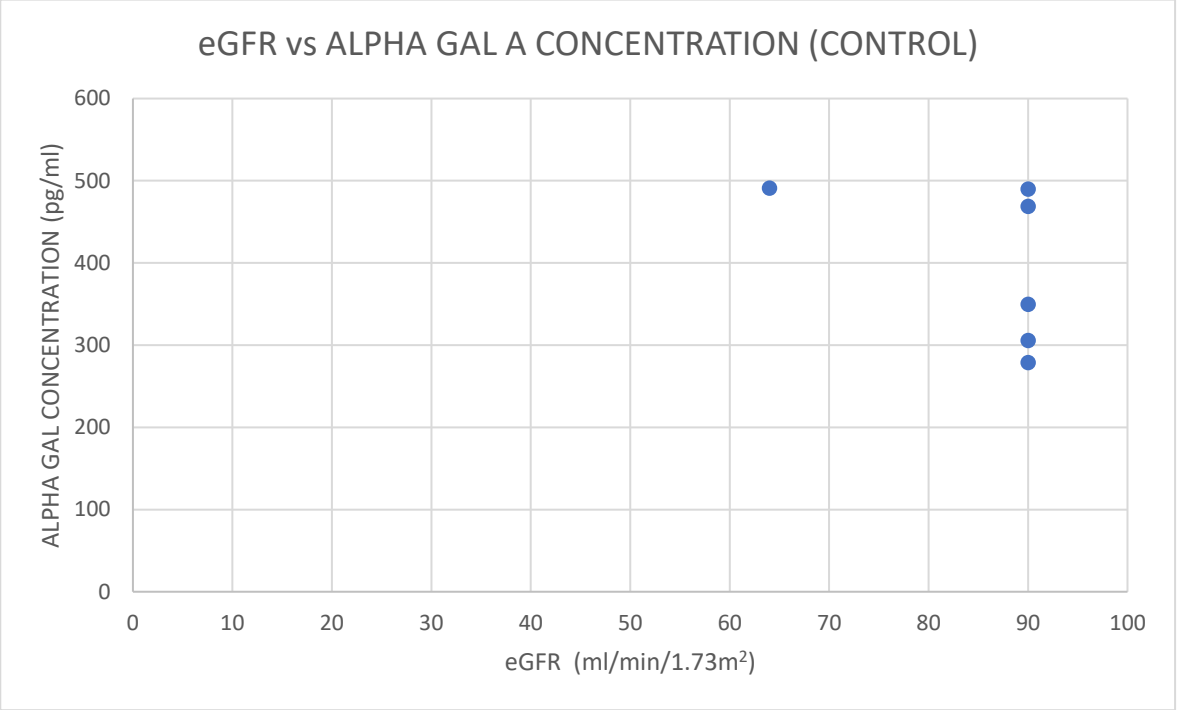

Figure 39: Control group eGFR (X Axis), Alpha-Gal -A enzyme concentration levels (Y Axis)

4.11 The age of the patients with alpha Gal-A enzyme concentration levels of <500pg/ml were assessed. The mean age of all participants with levels <500pg/ml was 30.5 years. There was a significance using the univariate analysis between the age of the patients and the low concentration levels (p=0.007). The multivariate analysis without eGFR as a variable also demonstrated a significance where p=0.044. The multivariate analysis with eGFR as a variable showed no significance where p=0.177.

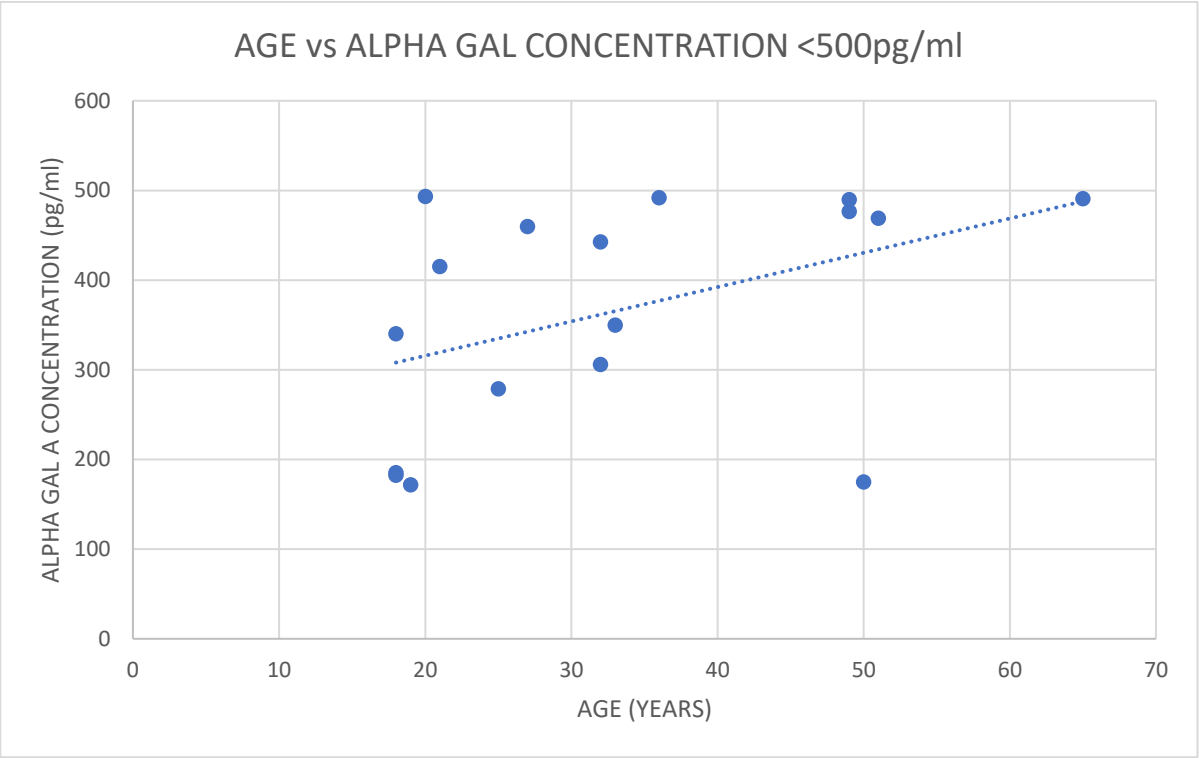

Figure 40: Alpha Gal-A enzyme concentration levels <500pg/ml (y axis), age (x axis)

4.12 The Mainz Severity Score Index (MSSI) was used to identify clinical manifestations of Fabry disease. A graphical representation using a scatter plot was constructed to find a correlation between the score of the patients and the alpha Gal-A concentration level (Figure 41).

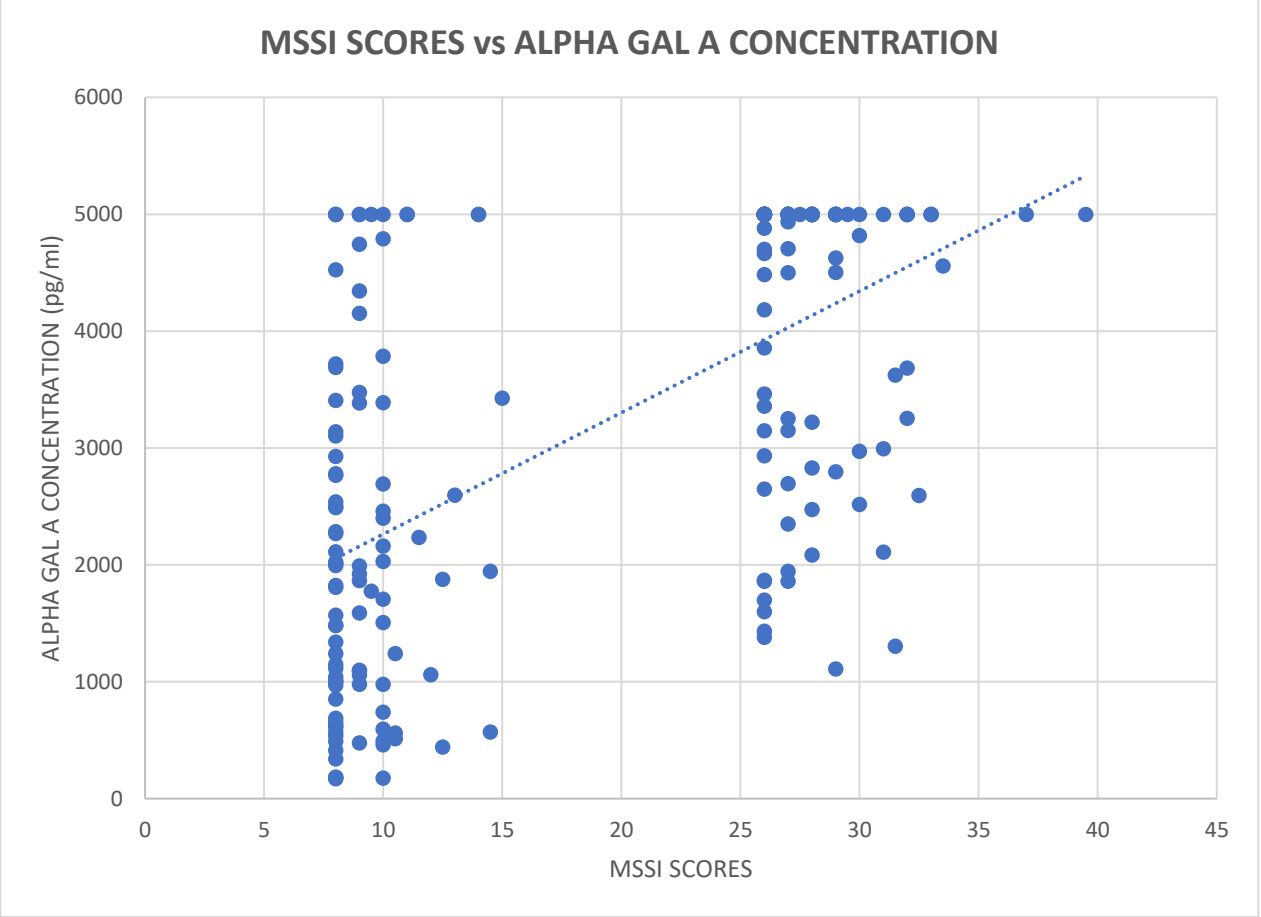

Figure 41: MSSI scores vs Alpha-Gal concentration

4.13 A graphical representation using a scatter plot was constructed to find a correlation between the score of the patients and the alpha Gal-A concentration levels <500pg/ml (Figure 42). There was a negative significance (p=0.001) using the univariate analysis between the MSSI scores and alpha Gal-A levels <500pg/ml. The multivariate analysis with eGFR as a variable showed no significance with MSSI scores (p=0.651), while multivariate analysis excluding eGFR as a variable showed a significance with MSSI scores where p=0.027.

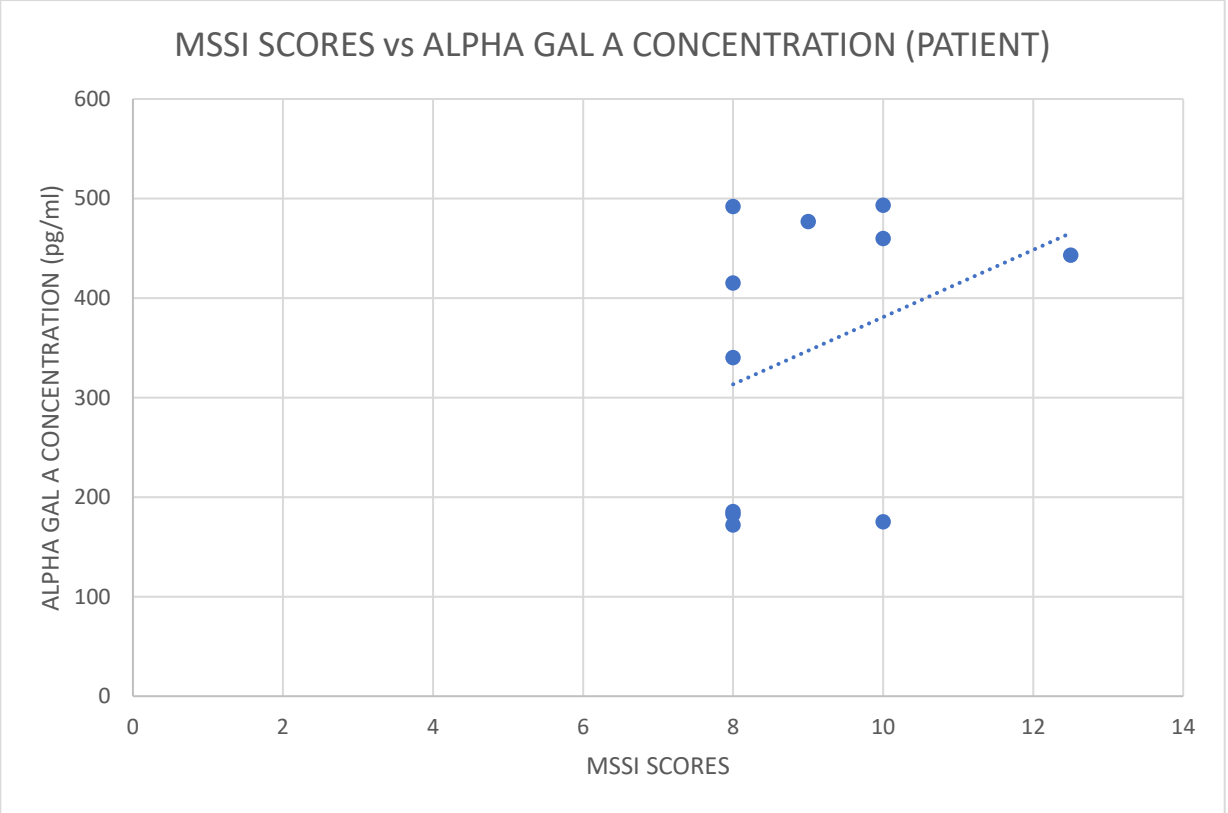

Figure 42: Alpha Gal-A enzyme concentration levels <500pg/ml (y-axis), MSSI score (x- axis)- patient

4.14 A scatter plot graph was constructed to illustrate the association between the MSSI scores of the control group and the alpha-Gal-A concentration levels (Figure 43). Participants in the control group did not report any clinical manifestations.

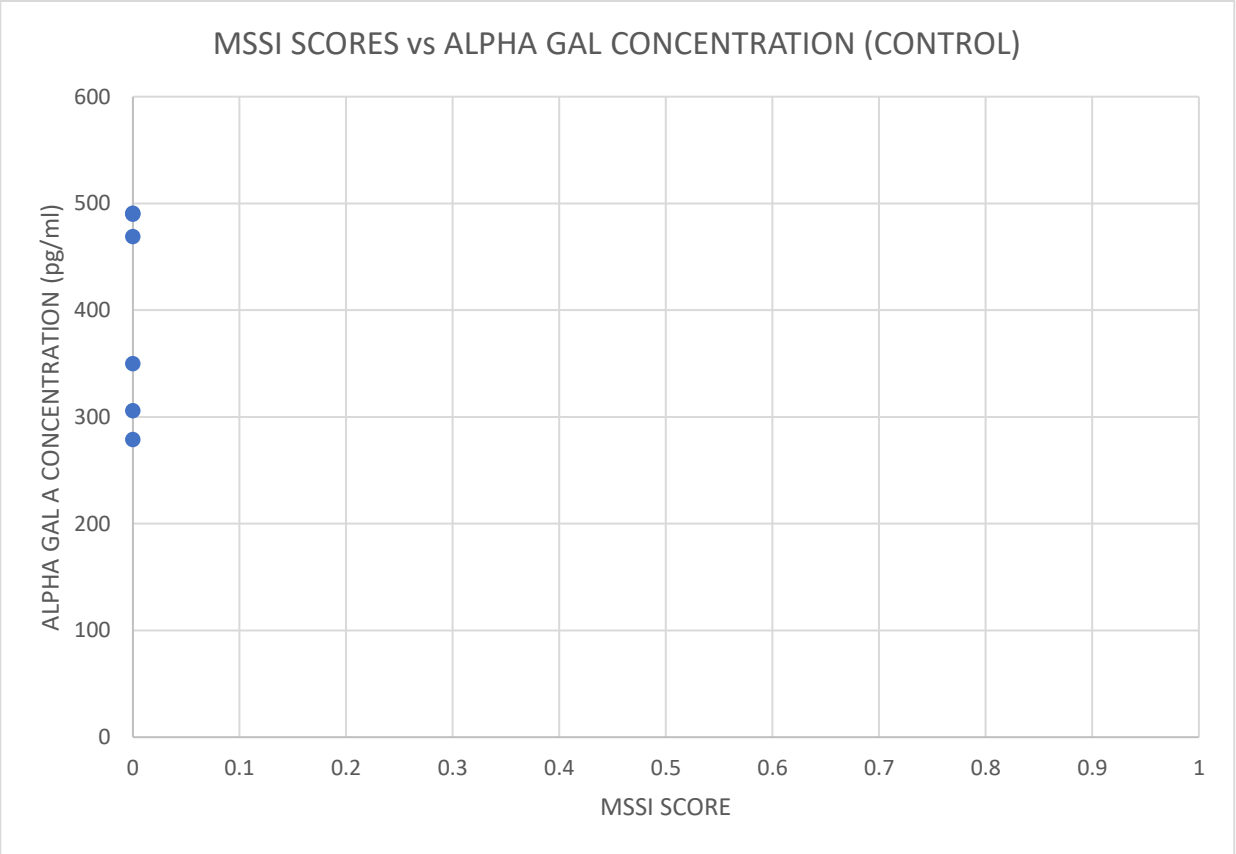

Figure 43: MSSI score vs alpha-Gal concentration -Control group

Table 3: Descriptive statistics of demographics and clinical manifestations of patient and control group

DESCRIPTIVE STATISTICS

				MEAN		
Variable	N Statistic	Minimum Statistic	Maximum Statistic	Statistic	Std. Error	Std.Deviation Statistic
Age	215	18.00	75.00	42.1860	.97349	14.27411
Race	215	1.00	5.00	1.6140	0.5085	.74557
Gender	215	1.00	1.00	1.0000	.00000	.00000
Diabetic	215	.00	1.00	.2326	.2888	.42345
Hypertensive	215	.00	1.00	.7814	.2825	.41426
Duration on HD	215	.00	47.00	5.9921	.44247	6.48794
Cause of CKD	215	.00	22.00	7.0558	.35759	5.24330
Eye	215	.00	.00	.0000	.00000	.00000
Vertigo	215	.00	1.00	.0279	.01126	.016509
Ringing in ear	215	.00	1.00	.1023	.02072	.030378
Heat/cold intolerance	215	.00	1.00	.2744	.03050	.44726
Anhidrosis	215	.00	1.00	.1163	.02191	.32131
Abdominal cramping	215	.00	1.00	.0884	.01940	.28450
Diarrhoea	215	.00	1.00	.0047	.00465	.06820
Constipation	215	.00	1.00	.01163	.02191	.32131
Kidney	215	.00	1.00	.9302	.01741	.25535
Dialysis	215	.00	1.00	.4791	.03415	.50073
Pacemaker	215	.00	1.00	.0047	.00465	.06820
Angina	215	.00	1.00	.0558	.01569	.23010
Skin	215	.00	1.00	.0791	.01845	.27048
Migraines	215	.00	1.00	.0558	.01569	.23010
Depression	215	.00	1.00	.0884	.01940	.28450
MSSI Score	215	.00	39.50	17.6488	.71962	10.55177
Alpha Gal Levels	215	171.97	5000.00	3037.1816	119.46862	1751.75379
Loss of hearing	8	23.00	23.00	23.0000	.00000	.00000
Initial GFR	198	3.00	129.00	31.8889	1.98561	27.94007
Alpha Gal <250pg/ml	215	.00	1.00	.0186	.00924	.13544

Alpha Gal <500pg/ml	*215*	*.00*	*1.00*	*.0698*	*.01741*	*.25535*
Valid N (listwise)	*7*					

Table 4: *Logistic regression analysis for alpha Gal-A concentration <500pg/ml*

UNIVARIATE LOGISTIC REGRESSION ANALYSIS FOR ALPHA GAL CONCENTRATION <500pg/ml

VARIABLE	ODDS RATIO (EXP B)	CONFIDENCE INTERVAL (95%)	P VALUE
Heat/ cold intolerance	0.226	[0.051 - 0.994]	0.049
Angina	0.000	.000	0.999
Skin (angiokeratoma)	0.500	[0.063 - 3.956]	0.511
Ringing in ear	0.609	[0.076 – 4.866]	0.640
Vertigo	0.000	.000	0.999
Hypertension	0.166	[0.067 – 0.409]	<0.001
Age	0.953	[0.920 – 0.987]	0.007
Anhidrosis	1.159	[0.318 – 4.220]	0.823
Diabetic	0.463	[0.132 – 1.627]	0.230
Race	0.650	[0.324 – 1.302]	0.224
Migraine	0.748	[0.092 – 6.074]	0.786
MSSI Score	0.911	[0.863 – 0.961]	0.001
Abdominal cramping	0.000	.000	0.998
Diarrhoea	0.000	.000	1
Constipation	0.000	.000	0.998
Initial eGFR	1.035	[1.019 -1.051]	<0.001

Table 5: Multivariate Regression Analysis for Significant Variables including eGFR

VARIABLE	ODDS RATIO (EXP B)	CONFIDENCE INTERVAL (95%)	*p* VALUE
MSSI Score	**0.982**	**[0.908 – 1.062]**	**0.651**
Initial eGFR	**1.022**	**[0.997 – 1.047]**	**0.089**
Age	**0.976**	**[0.941 – 1.011]**	**0.177**
Heat/ Cold Intolerance	**0.328**	**[0.065 – 1.650]**	**0.176**
Hypertension	**0.519**	**[0.158 – 1.702]**	**0.279**

Table 6: Multivariate Regression Analysis for Significant Variables excluding eGFR

VARIABLE	ODDS RATIO (EXP B)	CONFIDENCE INTERVAL (95%)	*p* VALUE
MSSI Score	**0.936**	**[0.883 – 0.992]**	**0.027**
Age	**0.966**	**[0.934 – 0.999]**	**0.044**
Heat/ Cold Intolerance	**0.366**	**[0.077 – 1.736]**	**0.206**
Hypertension	**0.359**	**[0.124 – 1.033]**	**0.057**

CHAPTER 5: DISCUSSION

To our knowledge, this is the first study in KwaZulu Natal that has investigated the association of Fabry disease, its clinical manifestations, and chronic renal failure in patients attending public hospitals in KwaZulu- Natal. The deficiency or absence of alpha-Galactosidase-A enzyme can confirm the diagnosis of Fabry disease in males. However, confirmatory testing using GLA sequencing is required. The prevalence of Fabry disease in patients with end-stage renal disease is estimated at around 0.12% (Vigneau *et al.* 2021). Levels of enzymatic activity are measured using leukocytes or fibroblasts. This is considered the recommended criterion for testing. However, due to complications in obtaining samples, the dried blood spot method is used as a viable alternative (Varela *et al.* 2020). Levels below 1% or <0.016IU/L are indicative of the classic form of FD and clinical symptoms usually manifest at these levels.

Analysis of Results

The results were analysed using logistic regression analysis and were calculated using the IBM SPSS version 27. A univariate logistic regression analysis (Table 3) was performed to determine the associate between alpha Gal-A enzyme levels <500pg/ml and the individual clinical manifestations. A multivariate logistic regression analysis (Table 4; 5) was performed subsequently to determine the association between alpha Gal-A enzyme levels <500pg/ml and variable that presented with a significant result in the univariate logistic regression analysis. The variables of the patient population and the control group were also analysed using descriptive statistical analysis (Table 2). A *p*-value of <0.05 was used to indicate significance between the variables and the alpha Gal-A enzyme concentration levels <500pg/ml. Scatter plots were constructed to provide a graphical representation of variables in comparison to the alpha Gal-A enzyme concentration levels. The MSSI scores were added and calculated for each participant individually.

Table 7: **Screening for Fabry Disease in Patients with Chronic Kidney Disease**

Study	Sample Size	Population	Gender	Test	Confirmation Test	Positive Cases	Prevalence Per 100 (%)
Turkey-Yeniçerioğlu (2016)	1453	Pre-renal	Male +female	DBS-Alpha Gal enzyme activity	DNA Sanger sequence analysis	3	0.2%
Brazil-Coutinho (2017)	25223	HD	Male +female	DBS- Alpha Gal enzyme activity	GLA sequencing	89	3%
South Africa-Wadee (2018)	1555	HD	Male +female	DBS- Alpha Gal enzyme activity	GLA sequencing	5	0.3%
Canada-Auray-Blais (2019)	397	CKD stage 3-5	Male +female	DBS- Gb3 analysis	GLA sequencing	0	0%
Russia-Moiseev (2019)	5572	HD	Male +female	DBS- Gb3 analysis	GLA sequencing	20	0.36%
Japan-Yoshida (2020)	18199	CKD, cardiac, neurological	Male +female	DBS- Alpha Gal enzyme activity	GLA sequencing	101	0.55%
Saudi Arabia-Alhemyadi (2020)	619	HD	Male +female	DBS- Alpha Gal enzyme activity	DNA Sanger sequence analysis	3	0.48%
Western Australia-Jahan (2020)	526	HD	Male +female	DBS- Alpha Gal enzyme activity	GLA sequencing	0	0%
Western France-Vigneau (2021)	1561	HD + Transplant	Male	DBS- Alpha Gal enzyme activity	GLA sequencing	1	0.12%
Portugal- Da Silva (2021)	72	CKD 5D	Male +female	DBS- Alpha Gal enzyme activity +Gb3	GLA sequencing	4	0.05%
Japan-Nagata (2022)	2122	CKD 1-5 CKD 5D	Males	DBS- Alpha Gal enzyme activity	GLA Sequencing	2 CKD 1-5 1 CKD 5D	0.48% 0.06%
Our Study	200	CKD 2-5D	Males	Alpha Gal enzyme conc.	Nil	4 < 200pg/ml	0.02%

Demographics of Participants

The ethnic groups of the patient population were as follows: blacks (53%), Indians (37%), Coloureds (6.5%), Whites (2.5%), and Philippines (0.5%). The control group consisted of Indians (87%) and Blacks (13%). The race (p=0.224) showed no significance (Table 2). Fabry disease has been known to show no predilection to a specific ethnic group and is described as a pan-ethnic disorder (Bernardes, Foresto and Kirsztajn 2020). In our study, the age of the patients with low alpha Gal-A levels (<500pg/ml), using the univariate analysis showed a significance where p=0.007 (confidence interval [0.920 – 0.987]). The multivariate analysis excluding the initial eGFR values, also showed a significance where p=0.044 and confidence interval [0.934 – 0.999]. Mean age of the patients with CKD stage 2-5 was 27 years. Mean age of the control group with low levels of <500pg/ml was 45 years. In reports by Eng, *et.al.* (2007), the Fabry Registry presented age ranges of enrolled patients as young as a new-born, with the diagnosis being confirmed in the prenatal stages and patients as old as 85 years (Eng *et al.* 2007).

Positive controls

Two known Fabry cases were enrolled as part of the study and tested blindly to observe any significant decrease in the alpha-Gal-A concentration levels. The patients were brothers and both were previously tested for FD. Patient A was transplanted previously with his sister's kidney. However, the transplant was rejected and he commenced haemodialysis 6 months before the study. His alpha-Gal-A enzyme levels were tested using the dried blood spot method. In this study, his eGFR was 11ml/min/1.73m^2. He was receiving enzyme replacement therapy every 2 weeks and his alpha-Gal levels were 3359pg/ml. His MSSI score was 26 and he presented with hearing loss in the left ear, left ventricular hypertrophy, and peripheral neuropathy. Unfortunately, the patient demised before the end of the study. Patient B was in stage 2 CKD with an eGFR of >60 ml/min/1.73m^2 and was regarded as a pre-renal patient. He was receiving enzyme replacement therapy for a few months but it was discontinued due to financial constraints. His initial alpha-Gal-A levels were tested using the dried blood spot method. In this study, his alpha-Gal level was 1990pg/ml and his MSSI score was 9. He presented with constipation and bone degeneration. In this case, the bone degeneration is possibly due to low bone mineral density which is a novel characteristic of FD (Maruyama *et al.* 2020b).

Analysis of the Levels of the Alpha Gal-A Enzyme Concentration

Our assessment of the alpha Gal-A enzyme of the patients revealed that patients with CKD 5D presented with higher levels of alpha Gal-A enzyme, while patients with CKD 2-5 presented with lower levels of the enzyme, increasing the possibility of FD prevalence. A study conducted by Nagata, et.al., (Table 4) concluded that FD occurrence was elevated in male patients with CKD Stages 1–5 than in those with CKD Stage 5D (Nagata *et al.* 2021b) which is comparable with the preliminary results of this study. Unfortunately, no information or studies could be found that explain the catabolic nature of the human alpha Gal-A enzyme to elucidate the higher concentration levels in patients with CKD stage 5D than in patients with CKD stages 2-5. However, earlier studies conducted describe the kinetics of the recombinant human alpha-Gal enzyme replacement therapy infusion which could provide possible insight into the kinetics of the enzyme in the body and tissues. The initial synthesis of the alpha Gal-A in cultured human cells shows that the enzyme is produced as a precursor peptide and is processed in the mature lysosomal subunit. The enzyme, after infusion, is promptly withdrawn from the circulation and taken up by vascular endothelial and parenchymal cells into the lysosomes. The mannose-6-phosphate receptors are responsible for the transportation of the enzyme to the lysosomes. The half-life in plasma is less than five minutes for each glycoform of the enzyme (Siamopoulos 2004). The kinetics or efficacy of the enzyme replacement therapy during haemodialysis has shown no significant change indicating no loss of enzyme activity in the dialysate using even high flux dialysers (Siamopoulos 2004).

Correlation between Clinical Symptoms and Suspected Cases of Fabry Disease

Table 8: Summary of clinical manifestations in patients with Alpha-Gal levels <500pg/ml

PATIENT	DIABETIC	HYPERTENSIVE	EYE	VERTIGO	RINGING	HEAT/COLD INTOLERANCE	ANHIDROSIS	ABDOMINAL CRAMPS	DIARRHOEA	CONSTIPATION	KIDNEY	PACEMAKER	ANGINA	SKIN	MIGRAINE	DEPRESSION	MSSI SCORE
1	x	✓	x	x	x	x	x	x	x	x	✓	x	x	x	x	x	8
2	x	x	x	x	x	x	x	x	x	✓	✓	x	x	x	x	x	10
3	✓	x	x	x	x	✓	✓	x	x	x	✓	x	x	✓	x	x	12.5
4	x	x	x	x	x	x	x	x	x	x	✓	x	x	x	x	x	8
5	x	x	x	x	x	✓	x	x	x	x	✓	x	x	x	x	x	9
6	x	x	x	x	x	x	x	x	x	x	✓	x	x	x	x	x	8
7	x	✓	x	x	x	x	x	x	x	x	✓	x	x	x	x	x	8
8	x	x	x	x	x	x	✓	x	x	x	✓	x	x	x	x	x	10
9	x	✓	x	x	x	x	x	x	x	x	✓	x	x	x	x	x	8
10	x	✓	x	x	x	x	✓	x	x	x	✓	x	x	x	x	x	10
11	x	x	x	x	x	x	x	x	x	x	✓	x	x	x	x	x	8
12	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0
13	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0
14	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0
15	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0
16	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0
17	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	0

X- manifestation absent, ✓- manifestation present

The Mainz Severity Score Index

The Mainz Severity Score Index was included in the questionnaire to identify and evaluate any symptoms of Fabry Disease that participants may present with. Lenders, et.al., concluded in their study that the MSSI was a valid scoring system to evaluate disease burden in affected patients, especially at the time of diagnosis (Lenders and Brand 2020). Scores of the clinical manifestations were allocated as follows: clouding of the eye (1), vertigo (1), tinnitus (1), angiokeratomas (1.5), heat or cold intolerance (1), anhidrosis (2), depression (1), abdominal cramping (2), diarrhoea or constipation (1), kidney disease (8), dialysis (18), angina (2), pacemaker (4), migraines (6) (Tuttolomondo 2013). In our study, scoring of clinical symptoms was achieved by adding up individual scores to get a total score, where ≤18 may be categorized as mild, 19-38 would be moderate, and >38 is categorized as severe. The mean score for patients receiving HD (n=56; 28%) and PD (n=47; 23.5%) was significantly higher (mean=27.75) than the scores of patients in the pre-renal stage of CKD stage 1-5 (n=97; 48.5%) with the mean score of 8. The MSSI scores showed significance (p=0.001) using the univariate logistic regression analysis, however, this is debatable since the scores for patients with alpha-Gal-A levels <500pg/ml were as follows: 54% (n=6) patients had a score of 8, 11% (n=1) patients had a score of

9, 27% (n=3) patients had a score of 10 and, 11% (n=1) patients had a score of 12.5. The multivariate logistic regression analysis was employed to further assess the association between the alpha-Gal-A levels <500pg/ml and MSSI scores. The association with the inclusion of the eGFR shows no significance with p-value of 0.651. When eGFR was excluded from the analysis, a significance was noted (*p*=0.027). The control group with alpha-Gal-A levels <500pg/ml (n=6) disclosed no clinical symptoms of FD and therefore all scores were 0.

Heat or Cold Intolerance

The accumulation of Gb3 affects the thinly myelinated Aδ fibres which are the small nerve fibres. These are predominately responsible for the transmission of mechanical pain sensitivity to pinprick stimuli or unmyelinated C fibres which conduct warm sensation and pain sensitivity to heat (Rajan *et al.* 2021). However, in the early stages of FD, cold perception (Aδ fibres) is primarily affected rather than the warmth perception (C fibres), therefore the Aδ fibres are more susceptible to Gb3 damage (Politei, Durand and Schenone 2016). In our study, only 11% (n=2) of patients with alpha-Gal-A levels <500-g/ml disclosed heat or cold intolerance, however, the univariate analysis determined a significance with a p-value of 0.049. The multivariate analysis with and without eGFR as a variable showed no significance with *p*-value= 0.176 and 0.206 respectively. One patient reported heat intolerance and anhidrosis which typically manifest together with Fabry disease. The second patient reported intolerance to cold. In a study by Bashorum, *et.al*. (2022), one of the most frequently reported symptom was an intolerance to heat or cold (Bashorum *et al.* 2022) which is not in accordance with the results of our study.

Angina and Cardiovascular Manifestations

Left ventricular hypertrophy is reported in 53% of men and 33% of women after the third decade of life, with 60% of patients presenting with symptoms that include heart failure with preserved ejection fraction, chest pain, and arrhythmias. It is therefore recommended that FD should be suspected in patients with the unknown origin of these symptoms (Pieroni *et al.* 2021a). Kodama, *et.al*., conducted studies that concluded that vasospastic angina pectoris is associated with ventricular fibrillation in patients with FD (Kodama *et al.* 2019). In this study, however, no significance was evident (*p*=0.009) between angina and low alpha-Gal-A enzyme levels. Whether this is due to the validity of the information

provided to the patients or the absence of cardiac examinations on patients' records, remains unclear.

Dermatological manifestations

Angiokeratoma corporis diffusum is one of the early manifestations of FD and presents as small reddish to black papules with a diameter of 1mm to 5mm and a smooth or keratotic surface. Recently, however, it has been reported to occur in other lysosomal disorders including fucosidosis, sialidosis, mannosidosis, GM1 gangliosidosis, and Kanzaki disease (Vadher *et al.* 2020). As one of the earlier manifestations of FD, angiokeratoma can be an indicator of diagnosis of the disease (Hsu *et al.* 2019). In this study, there was no significance between angiokeratomas and alpha-Gal levels <500pg/ml (p=0.511). One participant described a rash similar to angiokeratoma on his thighs, however, there were no medical records to confirm this.

Auditory manifestations

Progressive sensorineural hearing loss at high frequencies combined with sudden deafness has been reported more frequently in patients with FD than in the general population (Eyermann *et al.* 2019). Numerous studies have been conducted on the auditory manifestations in FD, and the results have been extensive. Studies conducted by Holy *et.al*., conclude that the incidence of tinnitus and sensorineural hearing loss in males is irrelevant leading symptoms for the screening of FD, and, screening by alpha-galactosidase collection in patients with tinnitus or sensorineural hearing loss is not a rational consideration (Holy *et al.* 2021). In contrast, research by Yazdanfard *et.al*. (2019) demonstrated that patients with FD have a hearing loss of all frequencies and most significantly at high frequencies ranging from 4-8kHz (Yazdanfard *et al.* 2019). In the present study, we found no significance between auditory manifestations (tinnitus p=0.64 and vertigo p=0.999) and alpha-Gal levels <500pg/ml. Only 1 patient reported hearing loss in both ears.

Anhidrosis

Anhidrosis occurs as a result of an accumulation of Gb3 in the sweat glands with sweat gland dysfunction. There have been no studies to date on prospective screening of FD in patients with a conclusive diagnosis of hypohidrosis or anhidrosis. Nagai-Sangawa et.al. conducted a study where the alpha-Gal activity was tested in 17 patients with hypohidrosis. Of the 17 patients, 1 patient was diagnosed with FD (Nagai-Sangawa *et al.* 2022). In our study, 3 patients reported anhidrosis as a symptom, however, there was no significance between symptoms of anhidrosis and alpha-Gal-A levels of <500pg/ml (p=0.823).

Migraine and Cerebrovascular Manifestations

Headaches and migraine in patients with FD are neurological symptoms that occur frequently. Strokes usually occur in patients before a diagnosis is confirmed. It has been reported that stroke in males with FD aged between 25-44 years may occur 12 times more frequently than in the general population (Aksoy Gündoğdu *et al.* 2017). Cerebral magnetic resonance imaging (MRI) often shows multiple white matter lesions (WML), as seen in patients affected by migraine (Sawada *et al.* 2021). It has been reported that people with migraine are at high risk for cerebrovascular disease (Øie *et al.* 2020). However, in this study, there was no significance between migraine or stroke and the alpha-Gal levels <500pg/ml (p= 0.786). Patients who did report headaches attributed it to their hypertensive episodes and stated that it improved after taking their antihypertensive medication.

Gastrointestinal Manifestations

Gastrointestinal (GI) manifestations in patients with FD are underestimated and frequently misdiagnosed as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD). The most common symptoms are abdominal cramps and diarrhoea, followed by constipation, nausea, and vomiting. Data from the Fabry Outcome Survey shows a 51% prevalence of GI symptoms due to abdominal cramps and diarrhoea (Lenders and Brand 2022). In a study conducted by Caputo et.al., 85 patients with IBS symptoms were tested for FD. Only 1 female patient was identified as an FD carrier (Caputo *et al.* 2021). In this study, 3 GI manifestations were examined, abdominal cramping, diarrhoea, and constipation. No significance (abdominal cramping p= 0.998, diarrhoea p=1, constipation

p= 0.998) was evident between the symptoms and alpha-Gal levels <500pg/ml. Only 1 patient reported having symptoms of constipation.

Hypertension

Blood pressure control in patients with FD is essential to minimise disease progression and improve prognosis. Hypertension impacts on disease burden in FD by exacerbating cardiac involvement and by increasing the risk of end-stage renal disease (ESRD) and stroke (Del Pinto and Ferri 2020). Conversely, renal impairment due to FD could also contribute to the development of hypertension and impact blood pressure control. In a study conducted by Dincer *et.al*., the results demonstrated that all blood pressure measurements were lower in the patients with FD than in the control group. They concluded that a decrease in heart rate variability, rather than an increase in blood pressure variability, might be an early indicator of FD (Dincer *et al.* 2022). Another study conducted by Rossi et.al. also demonstrated a lower prevalence of hypertension in patients with ESKD, pre-renal, and patients with transplants (Rossi *et al.* 2021b). In our study, there was a significance (*p* <0.001) between hypertension and patients with alpha-Gal-A levels <500pg/ml using the univariate logistic regression analysis. The multivariate logistic regression analysis with eGFR as a variable, hypertension showed no significance (*p*=0.279). Without eGFR as a variable, *p*-value showed 0.057. In keeping with the statistic that a *p*-value of 0.05 is considered significant, we inferred that hypertension was not significant in our study. In the patient group, 4 patients reported hypertension, however, it is important to note that the patients were in stage 2 CKD, and the cause of hypertension was unknown. Whether the hypertension is due to low levels of alpha-Gal-A enzyme levels or CKD, requires further investigation.

Diabetes

There are minimal studies conducted on the association between diabetes and FD. Diabetes is one of the common causes of CKD globally. It is characterised by albuminuria and a reduced glomerular filtration rate (Triozzi *et al.* 2021). In this study, there was no significance (*p*=0.230) between diabetic patients and alpha-Gal-A levels <500pg/ml. Only 1 patient was reported to be diabetic.

Association between Chronic Kidney Disease and Signs of Fabry Disease

Table 9: Summary of participants and association with chronic kidney disease and alpha-Gal levels <500pg/ml

PATIENT	AGE	KIDNEY COMPLICATIONS	CAUSE OF CKD	CKD	DIALYSIS	MSSI SCORE	ALPHA GAL I	INITIAL eGFR
1	36	6 MONTHS	SLE	2	NO	8	491.93	>60
2	20	7 MONTHS	FSGS	2	NO	10	493.35	>60
3	32	9 YEARS	FSGS	2	NO	12.5	442.88	>60
4	18	8 YEARS	FSGS	2	NO	8	182.52	>60
5	49	2 YEARS	SLE	2	NO	9	476.8	>60
6	19	16 YEARS	FSGS	2	NO	8	171.97	>60
7	18	5 YEARS	FSGS	2	NO	8	340.18	>60
8	27	19 YEARS	FSGS	2	NO	10	459.73	>60
9	21	9 YEARS	SLE	2	NO	8	415.07	>60
10	50	4 YEARS	HIVAN	2	NO	10	175.04	>60
11	18	5 YEARS	MCD	2	NO	8	185.44	>60
12	65	N/A	N/A	N/A	NO	0	491	>60
13	33	N/A	N/A	N/A	NO	0	350	90
14	49	N/A	N/A	N/A	NO	0	490	90
15	51	N/A	N/A	N/A	NO	0	469	90
16	32	N/A	N/A	N/A	NO	0	306	90
17	25	N/A	N/A	N/A	NO	0	279	90

It is estimated that the prevalence of Fabry disease among males undergoing haemodialysis (CKD 5D) is 0 -1.69%, and the prevalence in male patients with chronic kidney disease (CKD) Stages 1–5 is 0.59 -1.8% (Nagata *et al.* 2021a). In this study, patients (n=11) with alpha-Gal-A enzyme levels <500pg/ml were all in stage 2 CKD with eGFR of >60ml/min/1.73m^2. The univariate logistic regression analysis revealed that eGFR in patients with alpha-Gal-A levels <500pg/ml was significant with p<0.001. To verify the significance, a multivariate logistic regression analysis was employed, which revealed no significance (p=0.089) when analysed against variables which showed significance in the univariate analysis. None of the patients with alpha-Gal levels <500pg/ml, had commenced with dialysis. Nagata *et.al.* (2021) (Table 7) concluded in their study that FD prevalence was higher in male patients with CKD stages 1-5 than in those with CKD stage 5D (Nagata *et al.* 2021a). As discussed earlier, in this study, the MSSI scores of the patient group and control group indicated a significance (p=0.001), however, this is questionable. Lower MSSI scores should suggest a lesser possibility of FD. The median MSSI score of the patient group was 8. The control group reported no symptoms and therefore the score was 0. A score of ≤18 is classified as mild FD and the highest score was 12.5 of which only one patient obtained.

In this study, of the 11 participants in the patient group, focal segmental glomerular sclerosis (FSGS) was diagnosed in 6 patients (54%), systemic lupus erythematosus (SLE) was diagnosed in 3 patients (27%), HIV-associated nephropathy (HIVAN) was diagnosed in 1 patient (9%) and minimal change disease (MCD) was diagnosed in 1 patient (9%).

Focal segmental glomerular sclerosis is characterised by segmental scarring involving a part of the glomerulus and affects some of the glomeruli. Patients with FSGS can present with the clinical manifestations of nephrotic syndrome or with haematuria, hypertension, or renal insufficiency (Shabaka, Tato Ribera and Fernández-Juárez 2020). There have been reports of the association between FSGS and FD as far back as 2005 when Svarstad *et.al.* (2005) described the presence of FSGS and vascular changes in a male and female with FD. He concluded that FSGS has the potential role as a marker of progressive renal disease in some Fabry patients (Svarstad *et al.* 2005). Fabry disease and FD share similar pathophysiological characteristics since both cause podocyte damage. Recent studies by Hasbal *et.al.* revealed that alpha-Gal-A enzyme levels in patients with FSGS were lower than in patients on haemodialysis (2.88±1.2 mmol/L/h versus 3.79±1.9 mmol/L/h, p<0.001) (Hasbal *et al.* 2020). In our study, 23 patients (11.5%) were diagnosed with FSGS. In the haemodialysis group (CKD stage 5D), only 1 patient (1.7%) was diagnosed with FSGS with alpha-Gal levels >5000pg/ml. In the pre-renal group (CKD stage 1-5), 22 patients (22%) were diagnosed with FSGS. Only 1 patient (4.5%) (CKD stage 2) from this group had a level of 3271pg/ml. The remaining patients (n=21) (95%) had levels <1600pg/ml. Out of the 21 patients, 6 patients (29%) had alpha-Gal levels below the cut-off of 500pg/ml. This is comparable with studies conducted by Hasbal *et.al*. (Hasbal *et al.* 2020). In a retrospective study of 21 patients with FD conducted by Rusu *et.al*. (2022), patients were classified into 2 groups. The first group was categorised according to the presence of the combined endpoint (50% decrease of estimated glomerular filtration rate (eGFR) from baseline, kidney failure (KF), end-stage kidney disease (ESKD), or death and mortality) and the second group reached the combined endpoint outcome. Investigations revealed the presence of segmental sclerosis in all patients with the combined endpoint and only 33% of patients without the combined endpoint (Rusu *et al.* 2022).

In this study, systemic lupus erythematosus (SLE) was diagnosed in 3 patients (27%) with low alpha-Gal enzyme levels <500pg/ml. All 3 patients had eGFR values >60ml/min/1.73m^2. Overlapping organ involvement is evident in the course of FD and SLE (Kiykim *et al.* 2020). The accumulation of Gb3 causes alterations in the lymphocyte cell membranes enabling an environment suitable for autoimmune disease (Neto *et al.* 2019). The presence of zebra bodies during histological examination is usually associated with FD. However, a study conducted by Manabe et.al. biopsied 5 patients with lupus nephritis during hydroxychloroquine treatment. Zebra body formation and kidney phospholipidosis were evident in the biopsies. None of the patients had clinical manifestations of FD or any family history of FD. No genetic studies were performed to confirm the diagnosis of FD. A comparison of the number and size of the zebra bodies to FD confirmed a likely diagnosis of hydroxychloroquine-associated phospholipidosis (Manabe *et al.* 2021). SLE-associated autoantibodies have been reported in FD. In a study by Kiykim et.al. 76 juvenile SLE patients (mean age 16±3.3 years; range,8 to 23.5 years) were tested for FD by GLA sequencing. There were no positive cases found (Kiykim *et al.* 2020). In our study, the median MSSI score of the 3 patients diagnosed with SLE was 8.3. The median age was 35.3 years. Hearing loss in both ears was reported by one patient with SLE.

In our study, only 1 patient (9%) presented with HIVAN with alpha-Gal levels <200pg/ml. There is little that is known regarding the association between FD and HIV-associated nephropathy (HIVAN). It has been established, however, that sphingolipid accumulation is evident in glomerular diseases such as HIVAN (Hasbal *et al.* 2020). In the present study, the patient was a 50-year-old male who reported hypertension and anhidrosis. His MSSI score was 10 and eGFR >60ml/min/1.73m^2.

Only 1 patient (9%) was diagnosed with minimal change disease in this current study. Minimal-change disease (MCD), otherwise known as lipoid nephrosis or nil disease, is the most common cause of idiopathic nephrotic syndrome in children (Maas, Nijenhuis and van der Vlag 2022). It is characterised by intense proteinuria leading to oedema and intravascular volume depletion. Light microscopic findings reveal normal glomeruli or mild mesangial proliferation with negative immunofluorescence and no immune complex deposition while electron microscopy typically demonstrates diffuse effacement of the epithelial cell foot processes (Meyrier and Niaudet 2018). Nonsense mutation has been

reported in FD with nephrotic syndrome developing secondary to minimal change disease (Fujisawa *et al.* 2019). In the present study, the patient was an 18-year-old male with CKD stage 2 and eGFR >60ml/min/1.73m^2. His MSSI score was 8, however, alpha-Gal levels were <200pg/ml. The patient did not present with any other clinical manifestations of FD.

Limitations and Challenges

There were several challenges and limitations in this study. The patient sample size should have been larger owing to the statistics indicating the low prevalence of FD. The study required only males to be enrolled. The male population in the haemodialysis units from the 3 provincial hospitals could have been larger. The patient numbers at the renal clinics were limited due to the COVID-19 lockdown restrictions, therefore, enrolment and blood sampling was delayed. Visitation as the principal investigator was restricted by the hospital management due to lockdown regulations, delaying the enrolment process. The research was being conducted concurrently at the renal clinic and patients were hesitant to provide blood samples as Covid was a major contributing limiting factor through the entire process of the research study. A larger control group of healthy males would have been more effective for better outcomes, however, the sample size for both groups was approved by the supervisors and statistician.

Limitations of the study included analysing only the alpha-Gal-A enzyme concentration levels. Also, since this was a cross-sectional study, the patients' condition and presentation of clinical symptoms were documented for that day.

CHAPTER 6: CONCLUSION

This study aimed to investigate the association between Fabry disease (FD), its clinical manifestations, and chronic renal failure in KwaZulu-Natal province. A prospective, quantitative and descriptive study design method was employed to investigate symptoms of FD in patients with chronic renal failure to determine if there is an association between Fabry disease and chronic renal failure. This was accomplished by assessing the levels of the alpha-galactosidase-A enzyme in the samples, establishing any association between the clinical symptoms and Fabry's disease, and establishing any association between chronic renal failure symptoms and signs of Fabry disease by reviewing medical history for undiagnosed causes of chronic renal failure.

In this study, FD is suspected in 11 patients and 6 participants from the control group having alpha-Gal levels <500pg/ml. The cause of CKD nephropathy raises interest as conditions such as FSGS have been associated with FD. The low levels of the alpha-Gal enzyme and presentation of the clinical manifestations can be used as preliminary findings; however, they may not be sufficient to confirm the diagnosis. It is recommended that confirmatory tests such as DNA analysis or Gb3 and GL3 analysis should be performed to confirm the diagnosis.

CHAPTER 7: REFERENCES

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APPENDICES

Appendix I

LETTER OF INFORMATION

Dear Sir / Madam

Clinical research are scientific studies conducted to find better ways to prevent, screen for, diagnose or treat certain diseases. These studies follow strict scientific rules to protect patients and help produce reliable results.

Title of the Research Study: An investigation into the association between Fabry disease, its clinical manifestations and chronic renal failure in patients attending public hospitals in KwaZulu-Natal

Principal Investigator/s/researcher: Jillian Singh (BTech: Clinical Technology: Nephrology) studying for MH Clinical Technology

Co-Investigator/s/supervisor/s:
Dr SC Benjamin (D-Tech Clinical Technology)
Senior lecturer at the Department of Biomedical and Clinical Technology, DUT
Tel.: 031-3735411

Professor AGH Assounga (MD.CES.PhD in Nephrology)
Head of Department of Nephrology, Albert Luthuli Hospital
Tel.: 031-3052986

Brief Introduction and Purpose of the Study: There are many causes of kidney disease. One uncommon cause is a disease known as Fabry disease. This is caused when there is a deficiency or absence of an enzyme (alpha-galactosidase A) in the body. This enzyme helps break down a waste substance known as globotriaosylceramide. Because of this deficiency or absence of this enzyme, there is an accumulation of the waste substance and this damages tissues and major organs in the body.

Outline of the Procedures: You are being invited to be a part of this research because we would like to establish if there is an association with your kidney disease and Fabry disease. You will be provided with a consent form prior to any tests are performed. Once consent is obtained, a 5ml blood sample will be taken from you and tested for the abovementioned enzyme. A questionnaire

will also be provided to determine if there is an association with the symptoms and clinical manifestations of the disease. Furthermore, your blood sample will be stored at the Nelson Mandela School of Medicine for 6 months. Thereafter, the blood sample will be discarded. However, if the results are positive, then the blood sample will be stored for future genetic testing. This testing will only be done once we get your consent as well as permission form the ethics committee.

Risks or Discomforts to the Participant: For haemodialysis patients, blood samples will be taken from the dialysis access i.e., the permanent catheter or AV fistula. Peritoneal dialysis patients will have blood drawn from the antecubital vein. This may lead to some discomfort. If the sample is not adequate for testing, a second sample may be required to be taken from you.

Benefits: There will be no direct benefit to you, however, your participation is likely to help us find out more about Fabry disease and help treat it in the early stages.

Reason/s why the Participant May Be Withdrawn from the Study: You may be withdrawn from the study due to non -compliance with regard to divulging information regarding the study. Your participation in this study is voluntary. It is your decision whether or not to participate in this study. If you decide to take part, you will be asked to sign a consent form. After signing the consent form, you are still free to withdraw at any time and without giving reasons. If you withdraw from this study before data collection is completed, your data will be returned to you or destroyed. Your decision to withdraw from the study will not affect your treatment.

Remuneration: There will be no monetary or other remuneration offered to participate in this study.

Costs of the Study: There will be no costs incurred on the participating patient.

Confidentiality: We will not be sharing information about you to anyone outside the research team. The information that I collect from this research project will be kept private. Any information about you will have a number on it instead of your name. Only the research team will know what your number is and information will be kept in a locked cupboard. I will also require to review your medical files for additional information including diagnosis of your kidney disease. Your information will not be shared with anyone outside the research team.

Research-related Injury: If you suffer a physical injury as a direct result of participating in this study, you may obtain medical care in the same manner as you would ordinarily obtain any other medical treatment. In no way does signing this form neither waive your legal rights nor relieve the investigator, sponsor or involved institutions from their legal and professional responsibility.

Persons to Contact in the Event of Any Problems or Queries:

Supervisors: Dr SC Benjamin
Senior Lecturer at the Department of Biomedical and Clinical Technology, DUT
Tel.: 031-3735411

Professor AGH Assounga
Head of Department of Nephrology, Albert Luthuli Hospital

Tel.: 031-3052986

Please contact the researcher Ms Jillian Singh (0827682217), my clinical supervisor Prof AGH Assounga (031 2041325), my supervisor Dr Sherilene Benjamin (031-3735411) or the Institutional Research Ethics Administrator on 031 373 2375. Complaints can be reported to the DVC: Research, Innovation and Engagement Prof S Moyo on 031 373 2576

CONSENT

Statement of Agreement to Participate in the Research Study:

 I hereby confirm that I have been informed by the researcher, Jillian Singh, about the nature, conduct, benefits and risks of this study - Research Ethics Clearance Number: _____,

 I have also received, read and understood the above written information (Participant Letter of Information) regarding the study.

 I am aware that the results of the study, including personal details regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report.

 In view of the requirements of research, I agree that the data collected during this study can be processed in a computerized system by the researcher.

 I am aware that my blood sample will be stored for up to 6 months at the Nelson Mandela School of Medicine.

 I understand that if my results are positive, my blood sample will be stored for further genetic studies only after my consent is given.

 I may, at any stage, without prejudice, withdraw my consent and participation in the study.

 I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.

 I understand that significant new findings developed during the course of this research which may relate to my participation will be made available to me.

_____	_____	_____	_____
Full Name of Participant	**Date**	**Time**	**Signature / Right Thumbprint**

I, Jillian Singh herewith confirm that the above participant has been fully informed about the nature, conduct and risks of the above study.

_____	_____	_____
Full Name of Researcher	**Date**	**Signature**

_____	_____	_____
Full Name of Witness (If applicable)	**Date**	**Signature**

_____	_____	_____
Full Name of Legal applicable)	**Date**	**Signature**

Appendix 2

LETTER OF INFORMATION (CONTROL)

Dear Sir

Clinical research are scientific studies conducted to find better ways to prevent, screen for, diagnose or treat certain diseases. These studies follow strict scientific rules to protect patients and help produce reliable results.

Title of the Research Study: An investigation into the association between Fabry disease, its clinical manifestations and chronic renal failure in patients attending public hospitals in KwaZulu- Natal

Principal Investigator/s/researcher: Jillian Singh (BTech: Clinical Technology: Nephrology) studying for MH Clinical Technology

Co-Investigator/s/supervisor/s:
Dr SC Benjamin (D-Tech Clinical Technology)
Senior lecturer at the Department of Biomedical and Clinical Technology, DUT
Tel.: 031-3735411

Professor AGH Assounga (MD.CES.PhD in Nephrology)
Head of Department of Nephrology, Albert Luthuli Hospital
Tel.: 031-3052986

Brief Introduction and Purpose of the Study: There are many causes of kidney disease. One uncommon cause is a disease known as Fabry disease. This is caused when there is a deficiency or absence of an enzyme (alpha-galactosidase A) in the body. This enzyme helps break down a waste substance known as globotriaosylceramide. Because of this deficiency or absence of this enzyme, there is an accumulation of the waste substance and this damages tissues and major organs in the body.

Outline of the Procedures: You are being invited to be a part of this research as part of a control group because we would like to establish if there is an association with your kidney disease and Fabry disease. You will be provided with a consent form prior to any tests are performed. Once consent is obtained, a 5ml blood sample will be taken from you and tested, firstly, to check that you have no renal impairment. Another 5mls of blood will be taken to test for the abovementioned enzyme. A questionnaire will also be provided for you to answer to establish that you have no symptoms of Fabry disease. Furthermore, your blood sample will be stored at the Nelson Mandela School of Medicine for 6 months. Thereafter, the blood sample will be discarded. However, if any of your results are positive, then you will be informed of the results. This testing will only be done once we get your consent as well as permission form the ethics committee.

Risks or Discomforts to the Participant: Blood samples will be drawn from your antecubital vein. This may lead to some discomfort. If the sample is not adequate for testing, a second sample may be required to be taken from you if you consent.

Benefits: There will be no direct benefit to you, however, your participation is likely to help us find out more about Fabry disease and help treat it in the early stages.

Reason/s why the Participant May Be Withdrawn from the Study: You may be withdrawn from the study due to non -compliance with regard to divulging information regarding the study. Your participation in this study is voluntary. It is your decision whether or not to participate in this study. If you decide to take part, you will be asked to sign a consent form. After signing the consent form, you are still free to withdraw at any time and without giving reasons. If you withdraw from this study before data collection is completed, your data will be returned to you or destroyed. Your decision to withdraw from the study will not be held against you.

Remuneration: There will be no monetary or other remuneration offered to participate in this study.

Costs of the Study: There will be no costs incurred on the recruited participant.

Confidentiality: We will not be sharing information about you to anyone outside the research team. The information that I collect from this research project will be kept private. Any information about you will have a number on it instead of your name. Only the research team will know what your number is and information will be kept in a locked cupboard. Your information will not be shared with anyone outside the research team.

Research-related Injury: If you suffer a physical injury as a direct result of participating in this study, you may obtain medical care in the same manner as you would ordinarily obtain any other medical treatment. In no way does signing this form neither waive your legal rights nor relieve the investigator, sponsor or involved institutions from their legal and professional responsibility.

Persons to Contact in the Event of Any Problems or Queries:

Supervisors: Dr SC Benjamin
Senior lecturer at the Department of Biomedical and Clinical Technology, DUT -
Tel.: 031-3735411

Professor AGH Assounga
Head of Department of Nephrology, Albert Luthuli Hospital
Tel.: 031-3052986

Please contact the researcher Ms Jillian Singh (0827682217), my clinical supervisor Prof AGH Assounga (031 2041325), my supervisor Dr Sherilene Benjamin (031-3735411) or the Institutional Research Ethics Administrator on 031 373 2375. Complaints can be reported to the DVC: Research, Innovation and Engagement Prof S Moyo on 031 373 2576

CONSENT (CONTROL)

Statement of Agreement to Participate in the Research Study:

 I hereby confirm that I have been informed by the researcher, Jillian Singh, about the nature, conduct, benefits and risks of this study - Research Ethics Clearance Number: 143/19 ,

 I have also received, read and understood the above written information (Participant Letter of Information) regarding the study.

 I am aware that the results of the study, including personal details regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report.

 In view of the requirements of research, I agree that the data collected during this study can be processed in a computerized system by the researcher.

 I am aware that my blood sample will be stored for up to 6 months at the Nelson Mandela School of Medicine.

 I understand that if my results are positive, my blood sample will be stored for further genetic studies only after my consent is given.

 I may, at any stage, without prejudice, withdraw my consent and participation in the study.

 I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.

 I understand that significant new findings developed during the course of this research which may relate to my participation will be made available to me.

_____	_____	_____	_____
Full Name of Participant	**Date**	**Time**	**Signature / Right Thumbprint**

I, Jillian Singh, herewith confirm that the above participant has been fully informed about the nature, conduct and risks of the above study.

_____	_____	_____
Full Name of Researcher	**Date**	**Signature**

_____	_____	_____
Full Name of Witness (If applicable)	**Date**	**Signature**

_____	_____	_____
Full Name of Legal Guardian (If applicable)	**Date**	**Signature**

Appendix 3

LETHA LOKWAZI

Isihloko socwaningo lokucwaninga: Ukuphenywa kobudlelwane kanye nokubonakaliswa kwemitholampilo ye-Fabry isifo sokuhluleka okwejwayelekile ukubonakaliswa KwaZulu-Natali

Umphenyi oyinhloko / umphenyi: Jillian Singh (B-Tech: Technology Clinical: Nephrology)

Co-Investigator / s / umphathi / s:
UDkt. Benjamin
Umfundisi omkhulu eMnyangweni Wezolimo Nezobuchwepheshe, i-DUT
Ucingo: 031-3735411

UProfesa AGH Assounga
Inhloko yoMnyango weNeprology, Isibhedlela sase-Albert Luthuli
Ucingo: 031-3052986

Isingeniso esifushane nenhloso yocwaningo: Kunezimbangela eziningi zesifo sezinso. Isizathu esisodwa esingavamile yisifo esaziwa ngokuthi yisifo se-Fabry. Lokhu kubangelwa uma kukhona ukuntuleka noma ukungabi khona kwe-enzyme (i-alpha-galactosidase A) emzimbeni. Le enzyme isisiza ukwephula into edoti ebizwa ngokuthi i-globotriaosylceramide. Ngenxa yalokhu ukuntuleka noma ukungabikho kwalezi zinkimbinkimbi, kukhona ukuqoqwa kwemfucuza futhi lokhu kulimaza izicubu nezinhlaka ezinkulu emzimbeni.

Uhlaka Lwezinqubo: Uyakumenywa ukuthi ube yingxenye yalolu cwaningo ngoba singathanda ukusungula uma kukhona ukuhlangana nesifo sakho sezinso nesifo se-Fabry. Uzohlinzekwa ngefomu lokuvuma ngaphambi kokuhlolwa okukhona. Uma kutholakala imvume, isampula yegazi izothathwa kuwe futhi ihlolwe ngenhla ye-enzyme eshiwo ngenhla. Kuzohlinzekwa inkundla yemibuzo ukuze kunqume ukuthi kukhona yini ubudlelwane nezimpawu kanye nokubonakaliswa kwemitholampilo yesifo.

Izingozi noma Ukuphazamiseka Kwabambe iqhaza: Iziguli ze-hemodialysis, amasampuli egazi azothathwa kusukela ekufinyeleleni kwe-dialysis, isib. I-catheter engapheli noma i-AV fistula. Iziguli ze-peritoneal dialysis zizoba negazi elidonsa emthanjeni we-antecubital. Lokhu kungabangela ukungahambi kahle.

Izinzuzo: Ngeke kube nenzuzo ngqo kuwe, noma kunjalo, ukubamba iqhaza kwakho kungasisiza ukuba sithole kabanzi mayelana nesifo se-Fabry futhi sisize ekutholeni izigaba zakuqala.
Isizathu / ukuthi kungani Umhlanganyeli Angase Akhishwe Esifundweni: Ungasuswa ekutadisheni ngenxa yokungahambisani nokuphathelene nokwazisa ulwazi mayelana nesifundo. Ukuhlanganyela kwakho kulolu cwaningo kungukuzithandela. Kuyisinqumo sakho ukuthi ungahlanganyeli yini kulolu cwaningo. Uma unquma ukuthatha ingxenye, uzocelwa ukuba usayine ifomu lokuvuma. Ngemuva kokusayina ifomu lesivumelwano, usukhululekile ukuhoxisa nganoma yisiphi isikhathi nangaphandle

kokunikeza izizathu. Uma uhoxisa kulolu cwaningo ngaphambi kokuqoqwa kwedatha kuqedile, idatha yakho izobuyiselwa kuwe noma ibhujiswe.

Imali: Ngeke kube khona imali noma enye inkokhelo ehlinzekwa ukuba iqhaza kulolu cwaningo.

Izindleko Zocwaningo: Ngeke kube nezindleko ezithintekayo esigulini esithatha isabelo.

Ukuyimfihlo: Ngeke sabelane ngolwazi ngawe kunoma ubani ngaphandle kwethimba locwaningo. Ulwazi engikuqoqa kulolu phrojekthi lokucwaninga luzogcinwa luyimfihlo. Noma yiluphi ulwazi mayelana nawe luyoba nenombolo kuso esikhundleni segama lakho. Ithimba lezokucwaninga kuphela lizokwazi ukuthi inombolo yakho neyiphi imininingwane izogcinwa ekhabhinini elikhiyiwe.

Ukulimala okuhlobene nocwaningo: Uma uthola ukulimala ngokomzimba njengomphumela oqondile wokubamba iqhaza kulolu cwaningo, ungathola ukunakekelwa kwezokwelapha ngendlela efanayo njengoba uzothola noma yimuphi omunye ukwelashwa. Ngayiphi indlela ukusayina leli fomu akulahli amalungelo akho angokomthetho noma kunciphisa umphenyi, uxhase noma uhileleke izikhungo emithwalweni yabo yomthetho neyomsebenzi.

Abantu Okumele Baxhumane Nesimo Sezinkinga Noma Imibuzo:

Abaphath: AbakwaSc Benjamin
Umfundisi omkhulu eMnyangweni Wezolimo Nezobuchwepheshe, i-DUT
Ucingo: 031-3735411

UProfesa AGH Assounga
Inhloko yoMnyango weNeprology, Isibhedlela sase-Albert Luthuli
Ucingo: 031-3052986

Uyacelwa uxhumane nomcwaningi (0827682217), umphathi wami (031-3735411) noma uMqondisi weziLimi zokuPhenya nge-Institutional ku-031 373 2375. Izikhalazo zingabikwa kuMqondisi: UkuPhepha nokuPhaswa kwePostgraduate, uProf S Moyo ngo- 031 373 2576 noma i-carinn @ dut .ac.za.

Appendix 4

QAPHELA

Isitatimende Sesivumelwane Sokubamba iqhaza Esifundweni Sokucwaninga:

• Ngiyaqinisekisa ukuthi ngitshelwe umcwaningi,

(Jillian Singh), mayelana nemvelo, ukuziphatha, izinzuzo kanye nezingozi zalolu cwaningo - Ukususwa Kwezimiso Zokucwaninga
Inombolo: _,
• Ngiphinde ngithole, ngifunde futhi ngiyiqonde imininingwane ebhaliwe ngenhla (I-Letter Participant of Ulwazi) mayelana nokucwaninga.
• Ngiyazi ukuthi imiphumela yocwaningo, kufaka phakathi imininingwane yomuntu mayelana nobulili bami, ubudala, usuku lokuzalwa, ukuqala kanye nokuxilongwa kuyobekwa ngokungaziwa umbiko wokutadisha.
• Ngokubheka izidingo zocwaningo, ngiyavuma ukuthi idatha eqoqwe phakathi nalolu cwaningo ingacubungulwa ohlelweni lwekhompyutha ngumcwaningi.
• Ngingakwazi, nganoma yisiphi isigaba, ngaphandle kokubandlulula, ngihoxise imvume yami nokuhlanganyela kulolu cwaningo.
• Nginethuba elanele lokubuza imibuzo futhi (ngokuzithandela kwami) ngizibikezele ukuthi ngilungele ukuhlanganyela kulolu cwaningo.
• Ngiyaqonda ukuthi ukutholakala okusha okuphawulekayo kuthuthukiswe phakathi nalolu cwaningo okungenzeka
okuphathelene nokuhlanganyela kwami kuzokwenziwa kimi.

_____ _____ _____
Igama eliphelele lomhla wokubamba iqhaza Usuku Isikhathi Isignesha / Kwesokudla I-Thumbprint

Mina, (Jillian Singh) lapha ukuqinisekisa ukuthi umhlanganyeli ngenhla ugcwele

waziswa ngesimo, ukuziphatha nezingozi zesifundo esingenhla.

_____ _____ _____
Igama eligcwele lomShisholi Usuku Isignesha

_____ _____ _____
Igama eliphelele loFakazi (uma likhona) Usuku Lwesignesha

_____ _____ _____
Igama eligcwele le-Legal Guardian (Uma likhona) Usuku Lwesignesha

APPENDIX 5

14 May 2018

Dr A Aron
Medical Manager
Addington Hospital
Durban
4001

Request for Permission to Conduct Research

Dear Madam

My name is Jillian Singh, a B-Tech: Clinical technology (Nephrology) student at the Durban University of Technology. The research I wish to conduct for my Masters dissertation involves: An investigation into the association between Fabry disease, its clinical manifestations and chronic renal failure in patients attending public hospitals in KwaZulu- Natal.

I am hereby seeking your consent to utilize patient information from the haemodialysis and peritoneal dialysis units at Addington Hospital. This study aims to determine the association of Fabry disease and chronic renal failure. Assessment of symptoms can prove useful for further diagnosing of the disease in the future. It requires the involvement of haemodialysis and peritoneal dialysis patients, collection of data and analysis of blood results. The study requires 5mls of blood to be drawn from the dialysis access of haemodialysis patients' pre-dialysis. Blood will be drawn from the median cubital vein from the patients on peritoneal dialysis.

I have provided you with a copy of my proposal which includes copies of the data collection tools and consent and/ or assent forms to be used in the research process, as well as a copy of the approval letter which I received from the Institutional Research Ethics Committee (IREC).

If you require any further information, please do not hesitate to contact me on my cellphone 0827682217 or email at billatster@gmail.com. Thank you for your time and consideration in this matter.

Yours sincerely,

_____
Jillian Singh (Principal Investigator)
Durban University of Technology

Supervisors:
Dr SC Benjamin
Senior lecturer at the Department of Biomedical and Clinical Technology, DUT
Tel.: 031-3735411

Professor AGH Assounga
Head of Department of Nephrology, Albert Luthuli Hospital
Tel.: 031-3052986

Appendix 6

14 May 2018

Dr Mazizi
Medical Manager
St Aidan'sHospital
Durban
4001

Request for Permission to Conduct Research

Dear Madam

My name is Jillian Singh, a B-Tech: Clinical technology (Nephrology) student at the Durban University of Technology. The research I wish to conduct for my Masters dissertation involves: An investigation into the association between Fabry disease, its clinical manifestations and chronic renal failure in patients attending public hospitals in KwaZulu- Natal.

I am hereby seeking your consent to utilize patient information from the haemodialysis and peritoneal dialysis units at St Aiden's Hospital. This study aims to determine the association of Fabry disease and chronic renal failure. Assessment of symptoms can prove useful for further diagnosing of the disease in the future. It requires the involvement of haemodialysis and peritoneal dialysis patients, collection of data and analysis of blood results. The study requires 5mls of blood to be drawn from the dialysis access of haemodialysis patients' pre-dialysis. Blood will be drawn from the median cubital vein from the patients on peritoneal dialysis.

I have provided you with a copy of my proposal which includes copies of the data collection tools and consent and/ or assent forms to be used in the research process, as well as a copy of the approval letter which I received from the Institutional Research Ethics Committee (IREC).

If you require any further information, please do not hesitate to contact me on my cell phone 0827682217 or email at billatster@gmail.com. Thank you for your time and consideration in this matter.

Yours sincerely,

_____
Jillian Singh (Principal investigator)
Durban University of Technology

Supervisors:
Dr SC Benjamin
Senior lecturer at the Department of Biomedical and Clinical Technology, DUT
Tel.: 031-3735411

Professor AGH Assounga
Head of Department of Nephrology, Albert Luthuli Hospital
Tel.: 031-3052986

Appendix 7

14 May 2018

Dr L Mtshali
Medical Manager
Inkosi Albert Luthuli Central Hospital
Durban
4001

Request for Permission to Conduct Research

Dear Sir

My name is Jillian Singh, a B-Tech: Clinical technology (Nephrology) student at the Durban University of Technology. The research I wish to conduct for my Masters dissertation involves: An investigation into the association between Fabry disease, its clinical manifestations and chronic renal failure in patients attending public hospitals in KwaZulu- Natal.

I am hereby seeking your consent to utilize patient information from the haemodialysis and peritoneal dialysis units at Inkosi Albert Luthuli Central Hospital. This study aims to determine the association of Fabry disease and chronic renal failure. Assessment of symptoms can prove useful for further diagnosing of the disease in the future. It requires the involvement of haemodialysis and peritoneal dialysis patients, collection of data and analysis of blood results. The study requires 5mls of blood to be drawn from the dialysis access of haemodialysis patients' pre-dialysis. Blood will be drawn from the median cubital vein from the patients on peritoneal dialysis.

I have provided you with a copy of my proposal which includes copies of the data collection tools and consent and/ or assent forms to be used in the research process, as well as a copy of the approval letter which I received from the Institutional Research Ethics Committee (IREC).

If you require any further information, please do not hesitate to contact me on my cellphone 0827682217 or email at billatster@gmail.com. Thank you for your time and consideration in this matter.

Yours sincerely,

_____
Jillian Singh (Principal investigator)
Durban University of Technology

Supervisors:
Dr SC Benjamin
Senior lecturer at the Department of Biomedical and Clinical Technology, DUT
Tel.: 031-3735411

Professor AGH Assounga
Head of Department of Nephrology, Albert Luthuli Hospital
Tel.: 031-3052986

Appendix 8

14 May 2018

Ms SW Mbambo
Chief Director
Department of Health Province of KwaZulu- Natal
P/Bag X9124 Pietermaritzburg
3200

Request for Permission to Conduct Research

Dear Mam

My name is Jillian Singh, a B-Tech: Clinical technology (Nephrology) student at the Durban University of Technology. The research I wish to conduct for my Masters dissertation involves: An investigation into the association between Fabry disease, its clinical manifestations and chronic renal failure in patients attending public hospitals in KwaZulu- Natal.

I am hereby seeking your consent to utilize patient information from the haemodialysis and peritoneal dialysis units at Inkosi Albert Luthuli Central Hospital, Addington Hospital and St Aiden's hospital. This study aims to determine the association of Fabry disease and chronic renal failure. Assessment of symptoms can prove useful for further diagnosing of the disease in the future. It requires the involvement of haemodialysis and peritoneal dialysis patients, collection of data and analysis of blood results. The study requires 5mls of blood to be drawn from the dialysis access of haemodialysis patients' pre-dialysis. Blood will be drawn from the median cubital vein from the patients on peritoneal dialysis.

I have provided you with a copy of my proposal which includes copies of the data collection tools and consent and/ or assent forms to be used in the research process, as well as a copy of the approval letter which I received from the Institutional Research Ethics Committee (IREC).

If you require any further information, please do not hesitate to contact me on my cellphone 0827682217 or email at billatster@gmail.com. Thank you for your time and consideration in this matter.

Yours sincerely,

_____
Jillian Singh (Principal investigator)
Durban University of Technology

Supervisors:
Dr SC Benjamin
Senior lecturer at the Department of Biomedical and Clinical Technology, DUT
Tel.: 031-3735411

Professor AGH Assounga
Head of Department of Nephrology, Albert Luthuli Hospital
Tel.: 031-3052986

APPENDIX 9

14 May 2018

Dr N Abbai
Head Clinical Medicine Laboratory
Nelson Mandela School of Medicine
University of KwaZulu-Natal
Durban
4001

Request for Permission to Conduct Research

Dear Mam

My name is Jillian Singh, a B-Tech: Clinical technology (Nephrology) student at the Durban University of Technology. The research I wish to conduct for my Masters dissertation involves: An investigation into the association between Fabry disease, its clinical manifestations and chronic renal failure in patients attending public hospitals in KwaZulu- Natal.

I am hereby seeking your consent to utilize the laboratory for storage and testing the blood specimen at the Nelson R Mandela School of Medicine. This study aims to determine the association of Fabry disease and chronic renal failure. Assessment of symptoms can prove useful for further diagnosing of the disease in the future. It requires the involvement of haemodialysis and peritoneal dialysis patients, collection of data and analysis of blood results. The study requires 5mls of blood to be drawn from the dialysis access of haemodialysis patient's pre-dialysis. Blood will be drawn from the median cubital vein from the patients on peritoneal dialysis.

I have provided you with a copy of my proposal which includes copies of the data collection tools and consent and/ or assent forms to be used in the research process, as well as a copy of the approval letter which I received from the Institutional Research Ethics Committee (IREC).

If you require any further information, please do not hesitate to contact me on my cell phone 0827682217 or email at billatster@gmail.com. Thank you for your time and consideration in this matter.

Yours sincerely,

_____
Jillian Singh (Principal investigator)
Durban University of Technology

Supervisors:
Dr SC Benjamin
Senior lecturer at the Department of Biomedical and Clinical Technology, DUT
Tel.: 031-3735411

Professor AGH Assounga
Head of Department of Nephrology, Albert Luthuli Hospital
Tel.: 031-3052986

Appendix 10

The Director
Research and Postgraduate Support
Durban University of Technology

21 May 2020

Dear Dr Linganiso

RE: REQUEST TO RECRUIT CONTROL GROUP FROM DURBAN UNIVERSITY OF TECHNOLOGY

I am a registered postgraduate student at DUT finalizing my PG2a for the Master of Health Science in Clinical Technology (MH Clinical Technology). My topic for my thesis is**: An investigation into the association between Fabry disease, its clinical manifestations and chronic renal failure in patients attending public hospitals in KwaZulu- Natal.** Presently, my research proposal is being reviewed by IREC. My clinical study requires a control group of 30 healthy males. As per discussions with my clinical supervisor, I was advised to possibly recruit students from the Department of Biomedical and Clinical Technology for the control group. I request permission to utilize students from the department to volunteer to participate in this study. The control group will be selected according to an inclusion and exclusion approved by IREC. The following criteria will be used:

1.1 The inclusion criteria:

- Consent must be provided by all participants.
- All participants must be males.
- All participants must be over 18 years old up to the age of 75 years of age (age matching not required for patient population)
- Control participants that have no known renal impairment (normal GFR ≥ 90ml/min).
- Control participants must have no clinical manifestations of Fabry disease.

1.2 The exclusion criteria:

- Participants under the age of 18 years.
- Participants who do give consent to participate.
- Control participants who have known renal impairment.
- Control participants who display clinical manifestations of Fabry disease.

If permission is granted by the Head of Department:

- Letters of information will be emailed to the potential participants explaining the study and their role as participants (Appendix 1).
- Students who are willing to participate will be emailed consent letters (Appendix 2)
- Once consent is signed, a questionnaire will be given to the participants answer (Appendix 3).
- Scheduled dates and times will be corresponded with the individuals who have accepted and signed consent to be part of the study.
- Blood samples will be taken by myself at a common venue at the campus.
- Protocols will be followed with regard to the recent COVID-19 outbreak. This includes a temperature check of the participant. The principal investigator will wear a mask at all times when in contact with participants. Masks and disinfectant will be provided to the participants.
- The participant will be informed that their participation is voluntary and they may withdraw from the study at any time. Strict confidentiality will be maintained at all times.
- All protocols will be followed with regards to phlebotomy (PPE's, counselling).

- There will be no costs incurred on the participants.
  The blood samples will be kept in a cooler box on ice and transported immediately to the laboratories.
- One sample will be used to test the renal function and the other will be stored at the Nelson Mandela School of Medicine repository until the kidney function tests are done and results are within range.
- The stored samples will then be tested once off for Fabry disease.
- Participants will be informed of all their results upon request.

Although there is no direct benefit to the students with regard to participation, their involvement may give them some insight into the field of research.

I hope my request will be considered as I am being offered a grant by the KwaZulu- Natal Kidney Association (David Hepburn Study Award) to conduct the study and they are awaiting ethics approval to issue the letter.

Thanking you in advance.

Yours sincerely

_____

Jillian Singh (Principal Investigator)

Supervisors:
Dr SC Benjamin
Senior lecturer at the Department of Biomedical and Clinical Technology, DUT
Tel.: 031-3735411

Professor AGH Assounga
Head of Department of Nephrology, Albert Luthuli Hospital
Tel.: 031-3052986

APPENDIX 11

QUESTIONNAIRE

Instructions:

Please answer the questions below as honestly and accurately as possible.
Please tick the most appropriate answer and provide some family history if possible.
Your answers will be kept confidential.

Patient Name: ……. Ref. Number: ……

Age: ……

Race: …….

Gender: …….

Diabetic: …….

Hypertensive: ……..

Peritoneal Dialysis/ Haemodialysis: ……...

eGFR (Initial): …….

Number of years/ months on Dialysis: …….

Nephropathy (Cause of Renal failure): …….

Symptoms:

SYMPTOM	SEVERITY	YES	NO	FAMILY HISTORY OF SYMPTOM
Eye-Clouding of the cornea of the eye without it affecting your vision		(1)	(0)	
Ear- Vertigo	Mild	(1)	(0)	
Ringing in ears	Mild	(1)	(0)	
Skin-Small marks between the belly button and knees	Some	(1.5)	(0)	
Heat or cold intolerance		(1)	(0)	

Inability to sweat (anhidrosis)		(2)	(0)	
SYMPTOM	**SEVERITY**	**YES**	**NO**	**FAMILY HISTORY OF SYMPTOM**
Psychiatric/ Psychosocial-Depression		(1)	(0)	
Gastrointestinal- Abdominal cramping		(2)	(0)	
Diarrhoea/ constipation	Chronic	(1)	(0)	
Kidney- Unexplained kidney disease	Tubular dysfunction/low GFR or creatinine clearance	(8)	(0)	
	Dialysis	(18)	(0)	
Heart-Unexplained heart problems	Angina	(2)	(0)	
	Pacemaker	(4)	(0)	
Brain	Migraines	(6)	(0)	

(<shfc003-fabry-symptom-checklist-06.pdf>)

ADDITIONAL COMMENTS:

APPENDIX 12

UMBUZO

.....

Inombolo yesithenjwa:……..
Ubudala: .....

Umjaho: ....

Ubulili: ....

Isifo sikashukela: .....

PD / HD: ....

Inombolo yeminyaka / izinyanga ku-Dialysis: .....

I-Nephropathy: .....

Izimpawu

I-SYMPTOM	**SEVERITY**	**YEBO**	**CHA**	**UMKHAYA UMLANDO WESIMPENDULO**
I-Eye-Clouding ye-cornea iso ngaphandle kokuthinta umbono wakho		(1)	(0)	
Indlebe- I-Vertigo	Ubumnene	(1)	(0)	
Ukubetha ezindlebeni	Ubumnene	(1)	(0)	
Amakhanda amancane abomvu okubomvu kumbala okwesibhakabhaka phakathi kwenkinobho yesisu namadolo	Abanye	(1.5)	(0)	
Ukubekezelelana okushisa noma okubandayo		(1)	(0)	
Ukungakwazi ukuthuthumela (i-anhidrosis)		(2)	(0)	
Ubuhlungu be-neuropathic- Ubuhlungu obuvuthayo ezandleni / ezinyaweni	Ngezinye izikhathi	(4)	(0)	
I-SYMPTOM	**SEVERITY**	**YEBO**	**CHA**	**UMKHAYA UMLANDO WESIMPENDULO**

Isifo sohudo / ukuqothulwa	Okungapheliyo	(1)	(0)	
Izinso- Isifo sezinso esingaziwa	Ukuhlukunyezwa kwe-Tubular / okuphansi kwe-GFR noma i-creatinine imvume	(8)	(0)	
	I-Dialysis	(18)	(0)	
Izinkinga zenhliziyo ezingenakuchazwa	Angina	(2)	(0)	
	I-Pacemaker	(4)	(0)	
Ubuchopho	I-Migraines	(6)	(0)	

Appendix 13

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

33 ML SULTAN ROAD ,DURBAN,4001
P.O. BOX 547 ,DURBAN ,4000
Tel: 031-314 2261 Fax: 031- 314 2213
Email: prakash.subban@kznhealth.gov.za

ST AIDAN'S REGIONAL HOSPITAL
MEDICAL MANAGER

11 September 2020

Attention: Jillian Billat

PERMISSION TO CONDUCT RESEARCH AT ST AIDAN"S HOSPITAL: "AN INVESTIGATION INTO THE ASSOCIATION BETWEEN FABRY DISEASE, IT'S CLINICAL MANIFESTATIONS AND CHRONIC RENAL DISEASE IN PATIENTS ATTENDING PUBLIC HOSPITALS IN KWAZULUNATAL"

Permission is granted to you by to conduct the research mentioned above at St Aidan's Hospital.

Please adhere to the following:

1. The policies, procedures, protocols and guidelines of the KWAZULUNATAL DEPARTMENT OF HEALTH must be followed.
2. The research can only commence once permission is received in writing from the Provincial Health Research Committee of the KZNPA DOH.
3. The Medical Manager must be informed prior to the research commencing.
4. No resources from St Aidan's Hospital can be used for the research.
5. Feedback on the research must be given to St Aidan's Hospital on completion of the study.

We wish you every success with the research.

Yours Faithfully

DR PS Subban

Appendix 14

KWAZULU-NATAL PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

ADDINGTON HOSPITAL

Erskine Terrace, South Beach, DURBAN 4001
Postal Address: P.O. Box 997, DURBAN 4000
Tel: 031 3272970 Fax: 031 3683300
Email: reshma.boodhai@kznhealth.gov.za
www.kznhealth.com

OFFICE OF THE CHIEF EXECUTIVE OFFICER

Reference: *9/2/3/R*

Date: 27th August 2020

Principal Investigator:
- Mrs J Singh

PERMISSION TO CONDUCT RESEARCH AT ADDINGTON HOSPITAL: "AN INVESTIGATION INTO THE ASSOCIATION BETWEEN FABRY DISEASE, ITS CLINICAL MANIFESTATIONS AND CHRONIC RENAL FAILURE IN PATIENTS ATTENDING PUBLIC HOSPITALS IN KWAZULU-NATAL"

I have pleasure in informing you that permission has been granted to you by Addington Hospital Management to conduct the above research.

Please note the following:

1. Please ensure that you adhere to all the policies, procedures, protocols and guidelines of the Department of Health with regards to this research.
2. This research will only commence once this office has received confirmation from the Provincial Health Research Committee in the KZN Department of Health.
3. Please ensure this office is informed before you commence your research.
4. Addington Hospital will not provide any resources for this research.
5. You will be expected to provide feedback on your findings to Addington Hospital.

DR M NDLANGISA
HOSPITAL MANAGER
ADDINGTON HOSPITAL

Appendix 15

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

Physical Address: 330 Langalibalele Street, Pietermaritzburg
Postal Address: Private Bag X9051
Tel: 033 395 2805/ 3189/ 3123 Fax: 033 394 3782
Email:
www.kznhealth.gov.za

DIRECTORATE:
Health Research & Knowledge Management

NHRD Ref: KZ_202008_097

Dear Mrs J. Singh
(DUT)

Approval of research

1. The research proposal titled **'An investigation into the association between Fabry disease, its clinical manifestations and chronic renal failure in patients attending public hospitals in KwaZulu Natal'** was reviewed by the KwaZulu-Natal Department of Health (KZN-DoH).

   The proposal is hereby **approved** for research to be undertaken at Inkosi Albert Luthuli Central Hospital.

2. You are requested to take note of the following:
   a. *All research conducted in KwaZulu-Natal must comply with government regulations relating to Covid-19. These include but are not limited to: regulations concerning social distancing, the wearing of personal protective equipment, and limitations on meetings and social gatherings.*
   b. *Kindly liaise with the facility manager BEFORE your research begins in order to ensure that conditions in the facility are conducive to the conduct of your research. These include, but are not limited to, an assurance that the numbers of patients attending the facility are sufficient to support your sample size requirements, and that the space and physical infrastructure of the facility can accommodate the research team and any additional equipment required for the research.*
   c. *Please ensure that you provide your letter of ethics re-certification to this unit, when the current approval expires.*
   d. *Provide an interim progress report and final report (electronic and hard copies) when your research is complete to* ***HEALTH RESEARCH AND KNOWLEDGE MANAGEMENT, 10-102, PRIVATE BAG X9051, PIETERMARITZBURG, 3200*** *and e-mail an electronic copy to hrkm@kznhealth.gov.za*
   e. *Please note that the Department of Health shall not be held liable for any injury that occurs as a result of this study.*

For any additional information please contact Mr X. Xaba on 033-395 2805.

Yours Sincerely

Dr E Lutge
Chairperson, Health Research Committee
Date: 16/09/2020

Fighting Disease, Fighting Poverty, Giving Hope

Appendix 16

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

Physical Address: 800 Bellair Road, Mayville, 4058
Postal Address: Private Bag X08, Mayville, 4058
Tel: 0312401059 Fax: 0312401050 Email: ursulanun@ ialch.co.za
www.kznhealth.gov.za

DIRECTORATE:
Office of The Medical Manager
IALCH

Reference: IREC 143/19
Enquiries: Medical Management

19 August 2020

Mrs J Singh
P O Box 65861
Reservoir Hills
4090

Dear Mrs Singh

RE: PERMISSION TO CONDUCT RESEARCH AT IALCH

I have pleasure in informing you that permission has been granted to you by the Medical Manager to conduct research on: **An investigation into the association between Fabry disease, its clinical manifestations and chronic renal failure in patients attending public hospitals in KwaZulu-Natal.**

Kindly take note of the following information before you continue:

1. Please ensure that you adhere to all the policies, procedures, protocols and guidelines of the Department of Health with regards to this research.
2. This research will only commence once this office has received confirmation from the Provincial Health Research Committee in the KZN Department of Health.
3. Kindly ensure that this office is informed before you commence your research.
4. The hospital will not provide any resources for this research.
5. You will be expected to provide feedback once your research is complete to the Medical Manager.

Yours faithfully

.....

pp **Dr L P Mtshali**
Medical Manager

Dr A Harrichandparsad
Clinical Care Manager

Appendix 17

Institutional Research Ethics Committee
Research and Postgraduate Support Directorate
2nd Floor, Berwyn Court
Gate 1, Steve Biko Campus
Durban University of Technology
P O Box 1334, Durban, South Africa, 4001
Tel: 031 373 2375
Email: lavishad@dut.ac.za
http://www.dut.ac.za/research/institutional_research_ethics

18 September 2020

Mrs J Singh
P O Box 65861
Reservoir Hills
4090

Dear Mrs Singh

An investigation into the association between Fabry disease, its clinical manifestations and chronic renal failure in patients attending public hospitals in KwaZulu- Natal

Ethical Clearance number IREC 143/19

The Institutional Research Ethics Committee acknowledges receipt of your gatekeeper permission letters. Please note that FULL APPROVAL is granted to your research proposal. You may proceed with data collection.

Any adverse events [serious or minor] which occur in connection with this study and/or which may alter its ethical consideration must be reported to the IREC according to the IREC Standard Operating Procedures (SOP's).

Please note that any deviations from the approved proposal require the approval of the IREC as outlined in the IREC SOP's.

Yours Sincerely

Prof J K Adam
Chairperson: IREC

Appendix 17

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

DIRECTORATE:
Physical address: 83 King Cetshwayo Highway; Highway House; Mayville 4091
Postal Address: private Bag X 54318, Durban 4000 eThekwini District Office
Tel: 031 240 5308 Fax: 031 240 5555 Email: Ntombenhle.Ngcobo@kznhealth.gov.za
www.kznhealth.gov.za

Enquiries: Mrs. N.P Ngcobo
Date: 28/08/2020

Dear Mrs. J. Singh
Durban University of Technology
Health Sciences

RE: SUPPORT FOR RESEARCH STUDY IN "AN INVESTIGATION INTO THE ASSOCIATION BETWEEN FABRY DISEASE, ITS CLINICAL MANIFESTATIONS AND CHRONIC RENAL FAILURE IN PATIENT'S ATTENDING PUBLIC HOSPITAL IN KWAZULU-NATAL"

I have pleasure in informing you that the District is granting you support to conduct the research study entitled "**An Investigation Into The Association Between Fabry Disease, Its Clinical Manifestations And Chronic Renal Failure In Patient's Attending Public Hospitals in KwaZulu-Natal**" in facilities of Addington Hospital, Inkosi Albert Luthuli Central Hospital and St Aidan's Hospital.

Please note the following:

1. Please ensure you adhere to all the policies, procedures, protocols and guidelines of the department of health with regards to this research.
2. This research will only commence once this office has received confirmation from the provincial health research committee in the KZN department of health.
3. Please ensure this office is informed before you commence your research.
4. The District office/facility will not provide any resources for this research.
5. You will be expected to provide feedback on your findings to the district office/facility.

Thanking you.
Sincerely,

Mrs. N.P. Ngcobo
(P, Monitoring and Evaluation Manager)
EThekwini Health District

Fighting Disease, Fighting Poverty, Giving Hope

Appendix 18

Directorate for Research and Postgraduate Support
Durban University of Technology
Tromso Annexe, Steve Biko Campus
P.O. Box 1334, Durban 4000
Tel.: 031-3732576/7
Fax: 031-3732946
15th July 2020

Mrs Jillian Singh

c/o Department of Clinical and Biomedical Technology
Faculty of Health Sciences
Durban University of Technology

Dear Mrs Singh

PERMISSION TO CONDUCT RESEARCH AT THE DUT

Your email correspondence in respect of the above refers. I am pleased to inform you that the Institutional Research and Innovation Committee (IRIC) has granted **Full Permission** for you to conduct your research “An investigation into the association between Fabry disease, its clinical manifestations and Chronic renal failure in patients attending public hospitals in KwaZulu-Natal” at the Durban University of Technology.

The DUT may impose any other condition it deems appropriate in the circumstances having regard to nature and extent of access to and use of information requested.

We would be grateful if a summary of your key research findings can be submitted to the IRIC on completion of your studies.

Kindest regards.

Yours sincerely

_____
DR LINDA ZIKHONA LINGANISO
DIRECTOR: RESEARCH AND POSTGRADUATE SUPPORT DIRECTORATE

Appendix 19

Zertifikat

Certificat

Certificado

Certificate

Promouvoir les plus hauts standards éthiques dans la protection des participants à la recherche biomédicale
Promoting the highest ethical standards in the protection of biomedical research participants

Certificat de formation - Training Certificate

Ce document atteste que - this document certifies that

Jillian Singh

a complété avec succès - has successfully completed

Good Clinical Practice (GCP-E6(R2) 2016)

du programme de formation TRREE en évaluation éthique de la recherche
of the TRREE training programme in research ethics evaluation

Release Date: 2020/02/27

UCID : iHSUxcs3vI

Professeur Dominique Sprumont
Coordinateur TRREE Coordinator

Appendix 20

Calculating the cut-off Values of Alpha-Gal- A levels for suspected Fabry patients

To calculate a cut-off value for participants with low alpha Gal-A levels and therefore suspected cases of Fabry disease, the mean and standard deviation was calculated for the alpha Gal-A levels of all the control participants. The range was calculated using the mean and standard deviation [756±675] of the enzyme concentration. The calculated range was [81;1431]. It has been established that Fabry disease is caused by the deficiency of the alpha Gal-A enzyme, therefore the upper range was not considered for suspected participants for Fabry disease (Kok *et al.* 2021). The lower range of 81 was in accordance with the lower limit of the ELISA test kit detection range of 78.13pg/ml. There were no patients that presented with levels <81pg/ml. A cut-off value of <200pg/ml was considered, where four patients presented with levels below the cut-off value. However, to ensure that we did not exclude any suspected cases, the cut-off range was increased to 500pg/ml. Participants presenting with levels <500pg/ml (n=17) consisted of both patients (n=11), all of which were pre-renal with CKD stage 2 and control participants (n=6).

Appendix 21

Table displaying clinical manifestations of patient group (n=200). The tick (✓) indicates presence of clinical manifestation and the cross (x) indicates absence of the manifestation. The MSSI score is a total score of each individual present manifestation.

No.	HPT	Vertigo	Ring	Heat/Cold	Anhidrosis	Abdominal	Diarrhoea	Constipation	Kidney	Dialysis	Angina	Skin	Migraine	Depression	MSSI Score
1	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
2	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
3	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
4	✓	X	✓	X	X	X	X	X	✓	✓	X	X	X	✓	28
5	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
6	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
7	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
8	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
9	✓	X	X	✓	X	X	X	X	✓	✓	X	X	X	X	27
10	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
11	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	✓	27
12	✓	X	X	✓	X	X	X	X	✓	✓	X	X	X	X	27
13	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
14	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
15	✓	X	X	✓	X	X	X	X	✓	✓	X	X	✓	X	33
16	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
17	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
18	✓	X	✓	X	✓	X	X	X	✓	✓	X	X	X	X	29
19	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
20	X	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
21	✓	X	X	X	X	X	X	✓	✓	✓	X	X	X	✓	28
22	✓	X	X	X	X	X	X	✓	✓	✓	X	X	X	X	27
23	✓	✓	✓	X	X	X	X	✓	✓	✓	X	X	X	✓	30
24	✓	X	✓	X	X	X	X	X	✓	✓	✓	X	X	X	29
25	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
26	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	✓	27
27	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
28	✓	X	X	X	X	X	X	✓	✓	✓	X	X	X	X	27
29	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
30	✓	X	✓	X	X	X	X	X	✓	✓	X	X	X	X	27
31	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
32	✓	X	X	✓	X	X	X	X	✓	✓	X	X	X	X	27
33	✓	X	X	✓	✓	X	X	X	✓	✓	X	X	X	X	29
34	✓	X	X	✓	X	X	X	✓	✓	✓	✓	✓	X	✓	32.5
35	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
36	✓	X	✓	✓	X	✓	X	X	✓	✓	X	X	X	X	30
37	✓	X	X	✓	X	✓	X	✓	✓	✓	✓	X	X	X	31
38	✓	X	X	X	X	X	X	X	✓	✓	X	X	✓	X	32
39	✓	X	✓	✓	✓	X	X	✓	✓	✓	✓	X	X	X	32
40	X	X	X	X	✓	X	X	X	✓	✓	X	X	X	X	28

41	✓	X	X	X	✓	X	X	X	✓	✓	X	X	X	X	27
42	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	✓	27
43	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
44	✓	X	X	✓	X	X	X	X	✓	✓	X	X	X	X	27
45	✓	X	✓	X	X	X	X	X	✓	✓	✓	X	X	X	28
46	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
47	✓	✓	✓	X	X	X	X	X	✓	✓	X	X	X	X	28
48	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
49	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
50	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
51	✓	X	✓	✓	✓	X	X	✓	✓	✓	X	X	X	X	31
52	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
53	X	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
54	✓	X	X	✓	✓	X	X	✓	✓	✓	✓	X	X	X	32
55	✓	X	X	X	✓	X	X	X	✓	✓	X	X	X	X	28
56	X	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
57	✓	X	✓	X	X	X	X	X	✓	✓	X	X	X	X	28
58	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	27
59	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
60	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	27
61	✓	X	X	X	X	X	X	X	✓	✓	X	X	✓	X	32
62	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	27
63	X	X	X	X	✓	X	X	X	✓	✓	X	X	X	X	28
64	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
65	✓	X	X	✓	X	✓	X	X	✓	✓	X	X	X	X	29
66	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	✓	29
67	✓	X	X	X	✓	✓	X	X	✓	✓	X	X	X	X	30
68	✓	X	X	✓	X	X	X	X	✓	✓	X	X	✓	X	33
69	✓	X	X	✓	X	X	X	X	✓	✓	X	X	X	X	27
70	✓	X	X	X	✓	X	X	X	✓	✓	X	X	X	X	28
71	✓	X	X	X	X	X	X	✓	✓	✓	X	X	X	X	27
72	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
73	✓	X	X	✓	X	✓	X	X	✓	✓	X	X	X	X	29
74	✓	X	X	✓	X	✓	X	X	✓	✓	X	X	X	X	29
75	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
76	✓	X	X	X	X	X	X	X	✓	✓	X	X	✓	X	32
77	✓	X	X	X	X	X	X	✓	✓	✓	X	X	X	✓	28
78	X	X	X	✓	X	✓	X	X	✓	✓	✓	X	X	X	29
79	✓	X	X	✓	X	✓	X	✓	✓	✓	X	X	X	X	30
80	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
81	X	X	X	✓	X	X	X	X	✓	✓	X	X	X	X	27
82	✓	X	✓	✓	✓	X	X	X	✓	✓	X	✓	X	X	31.5
83	✓	X	X	✓	X	✓	X	X	✓	✓	X	✓	X	X	31.5
84	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
85	✓	✓	X	✓	✓	X	X	✓	✓	✓	X	✓	X	✓	33.5
86	✓	X	X	X	X	X	X	X	✓	✓	X	✓	X	X	27.5
87	✓	X	✓	✓	X	X	X	X	✓	✓	X	X	X	X	28

88	X	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
89	✓	X	X	✓	✓	X	X	✓	✓	✓	X	X	✓	✓	37
90	✓	X	X	X	X	✓	X	✓	✓	✓	X	X	X	X	29
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92	✓	X	X	X	X	X	X	✓	✓	✓	X	X	✓	X	31
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94	✓	X	X	X	X	✓	X	✓	✓	✓	✓	✓	✓	X	39.5
95	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
96	X	X	X	✓	✓	✓	X	✓	✓	✓	X	X	X	X	32
97	✓	X	X	✓	✓	X	X	X	✓	✓	X	X	X	X	29
98	✓	X	X	✓	X	X	X	X	✓	✓	X	X	X	X	27
99	✓	X	X	✓	X	X	X	X	✓	✓	✓	X	X	X	29
100	✓	X	X	X	X	X	X	X	✓	✓	X	X	X	X	26
101	✓	X	✓	✓	X	X	X	X	✓	✓	X	X	X	X	28
102	✓	X	X	X	X	X	X	✓	✓	✓	X	X	X	X	27
103	✓	X	X	X	✓	X	X	X	✓	✓	X	✓	X	X	29.5
104	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
105	X	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
106	X	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
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108	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
109	✓	X	X	X	X	X	X	✓	✓	X	X	X	X	X	9
110	✓	X	X	X	X	X	X	✓	✓	X	X	X	X	X	9
111	✓	X	✓	✓	X	X	X	X	✓	X	X	X	X	X	9
112	✓	X	X	X	✓	X	X	X	✓	X	X	X	X	✓	11
113	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
114	✓	X	X	X	X	✓	X	X	✓	X	X	X	X	X	10
115	✓	X	X	✓	X	✓	X	X	✓	X	X	✓	X	✓	14.5
116	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
117	✓	X	X	X	X	X	X	X	✓	X	✓	X	X	X	10
118	X	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
119	X	X	X	X	X	X	X	✓	✓	X	X	X	X	X	10
120	✓	X	X	✓	✓	X	X	X	✓	X	X	✓	X	X	12.5
121	✓	X	X	X	X	✓	X	X	✓	X	X	X	X	X	10
122	X	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
123	X	X	X	✓	X	✓	X	X	✓	X	✓	✓	X	X	14.5
124	X	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
125	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
126	✓	X	X	X	X	X	X	X	✓	X	X	X	X	✓	9
127	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
128	✓	X	X	✓	X	X	X	X	✓	X	X	✓	X	X	9.5
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130	X	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
131	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
132	✓	X	X	X	✓	X	X	X	✓	X	X	X	X	X	10
133	X	X	X	✓	X	X	X	X	✓	X	X	X	X	X	9
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135	X	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
136	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
137	✓	X	X	✓	X	X	X	X	✓	X	X	X	X	X	9
138	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
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140	✓	X	X	✓	X	X	X	X	✓	X	X	X	X	X	9
141	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
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143	✓	X	X	✓	X	X	X	X	✓	X	X	X	X	X	9
144	✓	X	X	✓	X	X	X	X	✓	X	X	X	X	✓	9
145	✓	X	✓	✓	X	X	X	X	✓	X	X	X	X	X	10
146	X	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
147	X	X	X	✓	X	X	X	X	✓	X	X	✓	X	X	10.5
148	X	X	X	X	X	X	X	X	✓	X	X	X	X	X	10
149	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
150	✓	X	X	X	X	X	X	X	✓	X	X	X			15
151	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
152	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
153	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
154	X	X	X	✓	X	X	X	X	✓	X	X	X	X	X	10
155	✓	X	X	X	X	X	X	X	✓	X	X	X	✓	X	14
156	X	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
157	X	X	X	X	✓	X	X	X	✓	X	X	X	X	X	10
158	✓	X	✓	X	X	X	X	✓	✓	X	X	X	X	X	10
159	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
160	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
161	X	X	✓	✓	X	X	X	X	✓	X	X	X	X	X	10
162	✓	✓	X	X	X	X	X	X	✓	X	X	X	X	X	9
163	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
164	✓	X	X	✓	X	X	X	X	✓	X	X		X		11.5
165	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
166	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
167	✓	X	X	✓	X	X	X	X	✓	X	X	✓	X	X	10.5
168	✓	✓	✓	X	X	✓	X	X	✓	X	X	X	X	✓	13
169	✓	X	X	✓	X	✓	X	X	✓	X	X	X	X	X	11
170	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
171	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
172	✓	X	X	✓	X	X	X	X	✓	X	X	X	X	X	9
173	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
174	X	X	X	X	✓	X	X	X	✓	X	X	X	X	X	10
175	X	X	X	X	✓	X	X	X	✓	X	X	X	X	X	10
176	✓	X	X	X	✓	X	X	X	✓	X	X	X	X	X	10
177	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
178	X	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
179	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
180	X	X	✓	✓	X	X	X	X	✓	X	X	X	X	X	10
181	X	X	X	X	X	X	X	X	✓	X	X	X	X	X	8

182	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
183	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
184	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
185	✓	X	X	✓	X	X	X	X	✓	X	X	X	X	X	9
186	✓	X	X	X	X	X	X	X	✓	X	X	✓	X	X	9.5
187	✓	X	X	X	✓	X	X	X	✓	X	X	X	X	X	10
188	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	12
189	X	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
190	✓	X	X	✓	X	X	X	X	✓	X	X	X	X	X	9
191	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
192	X	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
193	✓	X	✓	X	X	X	X	X	✓	X	X	X	X	□	10
194	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
195	✓	X	X	✓	X	X	✓	X	✓	X	X	✓	X	X	10.5
196	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
197	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8
198	X	X	X	✓	X	✓	X	X	✓	X	X	✓	X	X	12.5
199	✓	X	✓	X	X	X	X	X	✓	X	X	X	X	X	9
200	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	8

Appendix 22- Draft copy of Article for Publication

Fabry Disease Screening in Patients Attending Dialysis and Nephrology Clinics in Durban, South Africa

J. Singh, SC Benjamin, AGH Assounga

Abstract

Background: Fabry disease is characterized as a genetic, progressive, lysosomal storage disorder. It is inherited in an X-linked manner in which the mutated gene inhibits the functioning of the alpha-Galactosidase-A enzyme causing a deficiency or absence of the enzyme. This results in the accumulation of glycolipids, particularly globotriaosylceramide (Gb3) in the lysosomes causing progressive damage to tissues and major organs. Fabry nephropathy is progressive and is one of the major organ complications after cardiovascular manifestations caused by Fabry disease. Left untreated, Fabry nephropathy can result in end-stage kidney disease.

To our knowledge, no research has been conducted to determine the association between Fabry disease, its clinical manifestations, and chronic kidney disease in KwaZulu-Natal.

Methods: This study was a prospective, quantitative study. A total of 200 male patients with chronic kidney disease (CKD stage 2-5D) were enrolled in three dialysis clinics at Inkosi Albert Luthuli Central Hospital, Addington Hospital and St Aidan's Hospital in KwaZulu-Natal. A control group of 14 healthy males was also enrolled for this study. The ELISA technique was employed to determine the alpha Gal-A enzyme concentration levels in the plasma. A questionnaire using the MSSI scoring system was presented to the participants to identify clinical manifestations.

Results: A cut-off value for the alpha Gal-A enzyme concentration levels of <500pg/ml was calculated using the standard deviation and mean. A total of 17 participants from the patient group (n=11) and the control group (n=6) displayed alpha-Gal-A enzyme levels <500pg/ml. A *p*-value of <0.05 was considered to be statistically significant. A statistically significance result was exhibited between alpha-Gal levels of <500pg/ml and demographic parameters such as age (p=0.007), where the mean age was 30.5 years. Clinical parameters such as heat or cold intolerance, MSSI scores and hypertension also displayed significance. Heat and cold intolerance displayed a p-value of 0.049, where 2 patients reported the manifestation. MSSI scores displayed a negative association where p=0.001. Low MSSI scores should correlate with high alpha-Gal levels, however, in this study, all the patients displayed low MSSI scores

between 9 and 12.5 with low alpha-Gal levels. Hypertension also presented with a significance of $p<0.001$. A total of 4 patients were diagnosed with hypertension.

Conclusion: Fabry disease is suspected in a total of 17 participants with alpha-Gal levels of <500pg/ml. The cause of CKD nephropathy raises interest as conditions such as FSGS have been associated with FD. The low levels of the alpha-Gal enzyme and presentation of the clinical manifestations can be used as preliminary findings. It is recommended that confirmatory tests such as DNA analysis or Gb3 and GL3 analysis should be performed to confirm the diagnosis.

Keywords: Fabry Disease, Alpha-Gal-A enzyme, ELISA assay

Introduction

Fabry disease (Online Mendelian Inheritance in Man #301500) (FD, OMIM) is classified as a lysosomal storage disorder. Lysosomal storage disorders (LSD) are a group of disorders that are inherited or acquired. The disruption of the primary function of the recycling and disposal centres of the lysosome due to errors with the encoding of different lysosomal proteins, lysosomal enzymes, and lysosomal membrane proteins is the main feature of LSD (Platt *et al.* 2018). Fabry disease is defined as a complex multisystem disease with non-specific signs and symptoms and is the second most frequent disorder of this type after Gaucher's disease (Colpart and Felix 2017). The characteristic feature of Gaucher's disease is the presence of lipid-laden reticuloendothelial cells present in the spleen, liver, and bone marrow contributing to symptoms such as hepatosplenomegaly and pancytopenia (Kok *et al.* 2021). Fabry disease, also known as Alpha- Galactosidase A Deficiency or Anderson- Fabry disease is the deficiency of the alpha galactosidase-A (α- Gal A) enzyme. The monogenic disease is inherited in an X-linked manner. The alpha-galactosidase A (GLA) gene is situated on the Xq22.1 position on the X chromosome, affecting all hemizygous males, their daughters become heterozygous carriers and their sons are non-carriers and remain unaffected (Di Toro, Favalli and Arbustini 2018). An incomplete functioning or deficiency of the alpha-galactosidase A enzyme results in a systemic, intracellular accumulation of complex glycosphingolipids, mainly globotriaosylceramide (Gb3) or the water-soluble deacylated Gb3 known as globotriaosylsphingosine (lyso-Gb3) causing progressive damage to tissues and major organs, including amongst others, the heart, brain, vascular endothelium and kidneys leading to end-stage renal disease (Perretta 2018). Ethnic preference has not been observed in FD. Due to the low incidence rate of the condition, the prevalence rate can only be estimated as ranging

from 1:40 000 men to 1:117 000 live births (Perretta, Antongiovanni and Jaurretche 2018). The prevalence in live births has significantly increased with atypical mutations of the disease included, with statistics varying between 1:2900 to 1:3900 (Giugliani *et al.* 2016).

Early symptoms manifesting during childhood and adolescence include pain in the extremities, angiokeratomas, tinnitus, and anhidrosis. Disease progression ultimately results in left ventricular hypertrophy (LVH), stroke, proteinuria, and renal failure. Complications of the heart, cerebrovascular, or kidneys become pronounced after the age of 30 reducing mortality by 20 years (Germain 2010). Under- or misdiagnosis of the disease during later manifestations is common due to the non-specific nature and the mimicking of symptoms associated with diabetes and hypertension (Terryn *et al.* 2013).

Diagnosis of FD in males requires observing low levels or absence of the alpha-galactosidase-A enzyme activity in leukocytes, plasma, or fibroblasts and increased levels of Gb3 and lyso-Gb3 concentrations in plasma and urine. Pathogenic mutations can be assessed by genetic analysis (Yenicerioglu *et al.* 2017). The absence of residual enzyme activity in males is categorized as a severe classical phenotype, where characteristic FD symptoms manifest and progress into more severe symptoms later in life and affect multiple organs. The non-classical phenotype is milder in males where residual enzyme activity is evident. Patients are less severely affected and only one organ is affected later in life. Heterozygous females can present with normal alpha-galactosidase levels due to skewed X-inactivation (as per Lyons hypothesis whereby the process of the X chromosome is rendered inactive) and therefore are not reliably diagnosed by enzymatic assay. In such cases, molecular analysis is required (Curiati et al. 2017).

The Fabry Outcome Survey (FOS) established in 2001, is an international database designed to enhance the clinical management of patients diagnosed with Fabry disease (Giugliani *et al.* 2016). The data collected on the FOS provides information on the safety and efficacy of enzyme replacement therapy as well as the natural history of Fabry disease. The FOS patient report confirmed that as of January 2019, 3855 patients were enrolled in the FOS from 26 countries, which is a 10% increase from January 2018.

Diagnosing Fabry disease in its early stages has proved to be challenging due to the variability of symptoms, thereby increasing the frequency of organ involvement in the later stages of the

disease. Chronic kidney disease (CKD) remains as one of the main characteristics of Fabry disease. The deposition and accumulation of Gb3 occur first in the glomeruli and progress to various areas of the nephron including the mesangial, and interstitial cells, podocytes, cells of the proximal and distal tubules, and loop of Henle, and the vascular endothelial cells including the smooth muscle cells (Levstek, Vujkovac and Trebusak Podkrajsek 2020). Proteinuria is an early indication in detection of the disease in both males and females and is usually a significant clinical manifestation of renal involvement (Mena Rodríguez *et al.* 2018). The appearance of proteinuria occurs mainly during the second to third decades of life, however, it is also shown to be evident in male and female adolescents as well as boys as young as six years old (Mena Rodríguez *et al.* 2018).

The Kidney Disease Improving Global Outcomes (KDIGO) foundation recommends testing patients with chronic kidney disease when biopsies are not performed and there is no definitive cause of nephropathy (Schiffmann *et al.* 2017). The European Best Practice Guidelines (EBPG) recommend testing males under 50 years of age with chronic kidney disease with no definitive diagnosis (Terryn *et al.* 2013). Although there have been documented cases of Fabry disease in South Africa, the prevalence has not been established.

Treatment for Fabry disease currently comprises two forms of recombinant enzyme replacement therapy (ERT): Agalsidase-alpha produced in human fibroblasts and agalsidase-beta produced in Chinese hamster ovary cells (van der Veen *et al.* 2020a). Both forms of treatment with enzyme replacement require biweekly administration. Oral chaperone therapy is a relatively new form of treatment, however, is effective in patients with specific mutations of the disease (Lenders and Brand 2021). Novel gene therapies are in the clinical trial phase and are producing promising results through in vivo and ex vivo procedures (Domm *et al.* 2021). Although reports have shown that after a short course of enzyme replacement therapy patients showed improvements in pain relief, ability to sweat, and quality of life, there were no improvements to the cerebrovascular and renal damage (Schiffmann *et al.* 2019). In this prospective, quantitative study 200 male patients with chronic renal failure were tested for low levels or absence of the alpha- Galactosidase- A enzyme. Clinical manifestations were assessed and scored using the Fabry Outcome Survey Mainz Severity Score Index (FOS-MSSI). Results displaying low levels or absence of the Alpha-Galactosidase-A enzyme in conjunction with high scores in the assessment of their clinical manifestation will establish an

association between Fabry disease and chronic renal failure. Therefore, this study aims to demonstrate that early testing for Fabry disease is required when the diagnosis for chronic kidney disease is unconfirmed. This can ultimately retard or prevent damage to all major organs.

Materials and Methods

Study Participants

A cohort of 200 male patients with chronic renal failure was selected from three provincial hospitals, viz., Addington Hospital, Inkosi Albert Luthuli Central Hospital, and St. Aidan's Hospital. Two patients were confirmed with Fabry disease and were enrolled as positive controls. The patients enrolled for the study were male patients, receiving hemodialysis, peritoneal dialysis, and pre-renal patients. Male patients were diagnosed with chronic renal failure, i.e., stage 5 CKD (eGFR < 15ml/min) and were receiving HD as outpatients. Participants receiving peritoneal dialysis with stage 5 CKD were also considered. Pre-dialysis candidates with stage 2 CKD (eGFR 60-89ml/min) to stage 4 CKD (eGFR 15-29ml/min) were also enrolled. The research study was explained to all patients who were interested in participating and a consent form was signed before any investigations were performed. Patients were informed that participation was voluntary and they were entitled to withdraw from the study at any point without consequences regarding their treatment. An additional 15 healthy male participants with no renal impairment (eGFR >90ml/min -Stage 1 CKD) were enrolled as a control group. A questionnaire employing the Mainz Severity Score Index (MSSI) was provided to allow the participants to identify any of the clinical manifestations of Fabry disease. The total sample size of participants was 215, however, one control participant unknowingly had diminished kidney function with an eGFR of 37ml/min/1.73m2 indicating stage 3 CKD and had to be excluded from the study. The participant was counselled and advised to consult his doctor for further examination. Ethical approval (IREC) was obtained from the Durban University of Technology before any investigative work was commenced.

Blood Sampling and Storage of Samples

Blood samples of haemodialysis patients were drawn pre-dialysis from their dialysis access. The 5ml blood sample was taken using an EDTA tube from the patient's dialysis access after consent was obtained. Patients from the pre-renal and CAPD clinics were interviewed and once consent was obtained, a blood sample of 5mls was taken from the antecubital vein using an EDTA tube. Control participants were interviewed and after consent was obtained, blood

samples were taken from the antecubital vein. A 5ml blood sample was taken using a serum separator tube (SST) and transported at room temperature to a pathology laboratory for testing of participants' urea, creatinine, and eGFR levels. A second blood sample of 5mls was taken from the same site using an EDTA tube which was centrifuged and stored for analysis.

The blood tubes were labelled using a reference number to protect the individual patient's identity. The blood samples were transferred into 15ml conical bottom centrifuge tubes and centrifuged at 4000rpm for 10 minutes at 5°C to separate the plasma from the blood components. A volume of 1ml of plasma was transferred into microcentrifuge tubes and labelled according to the corresponding patient reference numbers and stored at -80°C. This was done in duplicate. In addition, the buffy coat layer containing the polynuclear cells was transferred into microcentrifuge tubes and stored at -80°C for further future genetic studies. The samples were also labelled according to the corresponding patient reference numbers.

Enzyme-Linked Immunosorbent Assay

The Enzyme-Linked Immunosorbent Assay (ELISA) technique was employed to determine the levels of Alpha-Gal-A enzyme concentration levels in the plasma samples.

Statistical Analysis

The results were analysed using logistic regression analysis and were calculated using the IBM SPSS version 27. A univariate logistic regression analysis was performed to determine the associate between alpha Gal-A enzyme levels <500pg/ml and the individual clinical manifestations. A multivariate logistic regression analysis was performed subsequently to determine the association between alpha Gal-A enzyme levels <500pg/ml and variable that presented with a significant result in the univariate logistic regression analysis. The variables of the patient population and the control group were also analysed using descriptive statistical analysis. A p-value of <0.05 was used to indicate significance between the variables and the alpha Gal-A enzyme concentration levels <500pg/ml. Scatter plots were constructed to provide a graphical representation of variables in comparison to the alpha Gal-A enzyme concentration levels. The MSSI scores were added and calculated for each participant individually.

Results

Using the control group as a benchmark for the cut-off value, we determined the average control value was 756±675. Based on this, 756pg/ml should have been employed as the cut-off value.

However, it is established that Fabry disease is unlikely with high levels of the alpha-Gal-A enzyme and we concluded that a value of 500pg/ml was acceptable to utilise as a cut-off value. In our study, a total of 11 patients exhibited concentration levels of <500pg/ml (n=11). The eGFR of the patients with alpha Gal-A concentration levels of <500pg/ml were also assessed. Patients presenting with low levels of alpha-Gal- A concentration levels (n=11) had an eGFR >60ml/min/1.73m2. The univariate analysis of eGFR demonstrated a significance with p<0.001 while the multivariate analysis showed no significance with *p*=0.089. The mean age of all participants with levels <500pg/ml was 30.5 years. There was a significance using the univariate analysis between the age of the patients and the low concentration levels (*p*=0.007). The multivariate analysis without eGFR as a variable also demonstrated a significance where *p*=0.044. The multivariate analysis with eGFR as a variable showed no significance where *p*=0.177. There was a negative significance (*p*=0.001) using the univariate analysis between the MSSI scores and alpha Gal-A levels <500pg/ml. The multivariate analysis with eGFR as a variable showed no significance with MSSI scores (*p*=0.651), while multivariate analysis excluding eGFR as a variable showed a significance with MSSI scores where *p*=0.027.

Discussion

The deficiency or absence of alpha-Galactosidase-A enzyme can confirm the diagnosis of Fabry disease in males. However, confirmatory testing using GLA sequencing is required. The prevalence of Fabry disease in patients with end-stage renal disease is estimated at around 0.12% (Vigneau *et al.* 2021a). It is estimated that the prevalence of Fabry disease among males undergoing haemodialysis (CKD 5D) is 0 -1.69%, and the prevalence in male patients with chronic kidney disease (CKD) Stages 1–5 is 0.59 -1.8% (Nagata *et al.* 2021a). In this study, patients (n=11) with alpha-Gal-A enzyme levels <500pg/ml were all in stage 2 CKD with eGFR of >60ml/min/1.73m2. The univariate logistic regression analysis revealed that eGFR in patients with alpha-Gal-A levels <500pg/ml was significant with *p*<0.001. To verify the significance, a multivariate logistic regression analysis was employed, which revealed no significance (*p*=0.089) when analysed against variables which showed significance in the univariate analysis. None of the patients with alpha-Gal levels <500pg/ml, had commenced with dialysis. Nagata *et.al*. (2021) concluded in their study that FD prevalence was higher in male patients with CKD stages 1-5 than in those with CKD stage 5D (Nagata *et al.* 2021b). In this study, the MSSI scores of the patient group and control group indicated a significance (*p*=0.001), however, this is questionable. Lower MSSI scores should suggest a lesser possibility of FD. The median MSSI score of the patient group was 8. The control group reported no symptoms and therefore

the score was 0. A score of ≤18 is classified as mild FD and the highest score was 12.5 of which only one patient obtained.

In this study, of the 11 participants in the patient group, focal segmental glomerular sclerosis (FSGS) was diagnosed in 6 patients (54%), systemic lupus erythematosus (SLE) was diagnosed in 3 patients (27%), HIV-associated nephropathy (HIVAN) was diagnosed in 1 patient (9%) and minimal change disease (MCD) was diagnosed in 1 patient (9%).

Focal segmental glomerular sclerosis is characterised by segmental scarring involving a part of the glomerulus and affects some of the glomeruli. Patients with FSGS can present with the clinical manifestations of nephrotic syndrome or with haematuria, hypertension, or renal insufficiency (Shabaka, Tato Ribera and Fernández-Juárez 2020). There have been reports of the association between FSGS and FD as far back as 2005 when Svarstad *et.al.* (2005) described the presence of FSGS and vascular changes in a male and female with FD. He concluded that FSGS has the potential role as a marker of progressive renal disease in some Fabry patients (Svarstad *et al.* 2005). Fabry disease and FD share similar pathophysiological characteristics since both cause podocyte damage. Recent studies by Hasbal et.al. revealed that alpha-Gal-A enzyme levels in patients with FSGS were lower than in patients on haemodialysis (2.88±1.2 mmol/L/h versus 3.79±1.9 mmol/L/h, p<0.001) (Hasbal *et al.* 2020). In our study, 23 patients (11.5%) were diagnosed with FSGS. In the haemodialysis group (CKD stage 5D), only 1 patient (1.7%) was diagnosed with FSGS with alpha-Gal levels >5000pg/ml. In the pre- renal group (CKD stage 1-5), 22 patients (22%) were diagnosed with FSGS. Only 1 patient (4.5%) (CKD stage 2) from this group had a level of 3271pg/ml. The remaining patients (n=21) (95%) had levels <1600pg/ml. Out of the 21 patients, 6 patients (29%) had alpha-Gal levels below the cut-off of 500pg/ml. This is comparable with studies conducted by Hasbal *et.al*. (Hasbal *et al.* 2020). In a retrospective study of 21 patients with FD conducted by Rusu *et.al.* (2022), patients were classified into 2 groups. The first group was categorised according to the presence of the combined endpoint (50% decrease of estimated glomerular filtration rate (eGFR) from baseline, kidney failure (KF), end-stage kidney disease (ESKD), or death and mortality) and the second group reached the combined endpoint outcome. Investigations revealed the presence of segmental sclerosis in all patients with the combined endpoint and only 33% of patients without the combined endpoint (Rusu *et al.* 2022).

In this study, systemic lupus erythematosus (SLE) was diagnosed in 3 patients (27%) with low alpha-Gal enzyme levels <500pg/ml. All 3 patients had eGFR values >60ml/min/1.73m2. Overlapping organ involvement is evident in the course of FD and SLE (Kiykim *et al.* 2020). The accumulation of Gb3 causes alterations in the lymphocyte cell membranes enabling an environment suitable for autoimmune disease (Neto et al. 2019). The presence of zebra bodies during histological examination is usually associated with FD. However, a study conducted by Manabe *et.al*. (2021*)* biopsied 5 patients with lupus nephritis during hydroxychloroquine treatment. Zebra body formation and kidney phospholipidosis were evident in the biopsies. None of the patients had clinical manifestations of FD or any family history of FD. No genetic studies were performed to confirm the diagnosis of FD. A comparison of the number and size of the zebra bodies to FD confirmed a likely diagnosis of hydroxychloroquine-associated phospholipidosis (Manabe *et al.* 2021). SLE-associated autoantibodies have been reported in FD. In a study by Kiykim *et.al.* (2020), 76 juvenile SLE patients (mean age 16±3.3 years; range, 8 to 23.5 years) were tested for FD by GLA sequencing. There were no positive cases found (Kiykim *et al.* 2020). In our study, the median MSSI score of the 3 patients diagnosed with SLE was 8.3. The median age was 35.3 years. Hearing loss in both ears was reported by one patient with SLE.

In our study, only 1 patient (9%) presented with HIVAN with alpha-Gal levels <200pg/ml. There is little that is known regarding the association between FD and HIV-associated nephropathy (HIVAN). It has been established, however, that sphingolipid accumulation is evident in glomerular diseases such as HIVAN (Hasbal *et al.* 2020). In the present study, the patient was a 50-year-old male who reported hypertension and anhidrosis. His MSSI score was 10 and eGFR >60ml/min/1.73m2.

Only 1 patient (9%) was diagnosed with minimal change disease in this current study. Minimal-change disease (MCD), otherwise known as lipoid nephrosis or nil disease, is the most common cause of idiopathic nephrotic syndrome in children (Maas, Nijenhuis and van der Vlag 2022). It is characterised by intense proteinuria leading to oedema and intravascular volume depletion. Light microscopic findings reveal normal glomeruli or mild mesangial proliferation with negative immunofluorescence and no immune complex deposition while electron microscopy typically demonstrates diffuse effacement of the epithelial cell foot processes (Meyrier and Niaudet 2018). Nonsense mutation has been reported in FD with nephrotic syndrome developing secondary to minimal change disease (Fujisawa *et al.* 2019). In the present study, the patient

was an 18-year-old male with CKD stage 2 and eGFR >60ml/min/1.73m2. His MSSI score was 8, however, alpha-Gal levels were <200pg/ml. The patient did not present with any other clinical manifestations of FD.

Limitations and Challenges

There were several challenges and limitations in this study. The patient sample size should have been larger owing to the statistics indicating the low prevalence of FD. The study required only males to be enrolled. The male population in the haemodialysis units from the 3 provincial hospitals could have been larger. The patient numbers at the renal clinics were limited due to the COVID-19 lockdown restrictions, therefore, enrolment and blood sampling was delayed. Visitation as the principal investigator was restricted by the hospital management due to lockdown regulations, delaying the enrolment process. The research was being conducted concurrently at the renal clinic and patients were hesitant to provide blood samples as Covid was a major contributing limiting factor through the entire process of the research study. A larger control group of healthy males would have been more effective for better outcomes, however, the sample size for both groups was approved by the supervisors and statistician.

Limitations of the study included analysing only the alpha-Gal-A enzyme concentration levels. Also, since this was a cross-sectional study, the patients' condition and presentation of clinical symptoms were documented for that day.