MAKERERE UNIVERSITY

EFFECTS OF CYTOKINE GENE POLYMORPHISMS ON IL-10, TNF-α LEVELS AND OXIDATIVE STRESS IN SICKLE CELL DISEASE PATIENTS FROM MULAGO HOSPITAL, UGANDA

BY

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DECLARATION

I, **Cissy Berrida Namuleme**, declare that this dissertation is my own original work and to the best of my knowledge, has never been submitted to any institution for a degree award.

Signature ..... Date 19/04/22

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DEDICATION

I dedicate this report to the Almighty God.

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LIST OF ACRONYMS AND ABBREVIATIONS

ARMS	Amplification Refractory Mutation System
bp	Base pairs
CRP	C-reactive protein
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide triphosphates
Hb	Hemoglobin
HbAA	Homozygous normal haemoglobin
HbAS	Heterozygous sickle haemoglobin
HbSS	Homozygous sickle cell recessive haemoglobin
SCT	Stem Cell Transplantation
IL-10	Interleukin- 10
MDA	Malondialdehyde
μL	Microliter
RBCs	Red Blood Cells
ROS	Reactive Oxygen Species
SCD	Sickle Cell disease
SCA	Sickle Cell Anemia
TGF-β	Transforming Growth Factor Beta
TNF-α	Tumor Necrosis Factor Alpha
TBA	Thiobarbituric Acid
VOC	Vaso- occlusive crisis

ABSTRACT

Sickle cell disease is a major cause of death in children under 5 years in Africa. Chronic oxidative stress and the release of inflammatory cytokines contribute to sickle cell disease pathogenesis and thus complicate the clinical management of this haemoglobinopathy. Polymorphisms in cytokine genes potentially influence increased cytokine production, a key risk factor for severe disease in sickle cell disease patients. Therefore, the purpose of this study was to investigate the frequency of cytokine gene polymorphisms and their effect on plasma levels of IL-10, TNF-α/β and malondialdehyde in sickle cell disease patients. This was a case control study, involving 178 sickle cell disease patients against 189 healthy controls. The extent of oxidative stress was evaluated using malondialdehyde by spectrophotometry while IL-10 and TNF-α/β levels were measured using the Enzyme Linked Immuno-Sorbent Assay. The Amplification Refractory Mutation System-PCR was used to genotype IL-10 and TNF-α/β gene polymorphisms in the Ugandan population. Descriptive analysis showed significantly higher (p=0.0063) median plasma levels of malondialdehyde in sickle cell disease patients (2.615μM) as compared to healthy controls (2.490μM). A similar trend was observed with median plasma levels of IL-10 in patients (20.37pg/ml) as compared to healthy controls (7.5pg/ml). The most frequent genotype for IL-10 (1082) gene polymorphism in the sampled Ugandan population was heterozygous GA. However, its homozygous GG (1082) (22.12pg/ml), the higher producer for IL-10 that was found to be significantly associated (p=0.0234) with increased plasma levels of this cytokine in sickle cell disease patients as compared to other genotypes (GA+AA) (13.94pg/ml) with the mutant allele. Similarly, higher levels of malondialdehyde were found to be significantly (p<0.0001) associated with homozygous GG (1082) for IL-10. The most frequent and only reported genotype for TNF-α/β gene polymorphisms was heterozygous GA in both patients and controls, thus no association was made for the cytokines. Therefore, results from this study show that sickle cell disease is associated with increased levels of oxidative stress that predispose patients to severe forms of the disease. Also, a protective role has been suggested for IL-10 with increased plasma levels of this cytokine standing as a potential biomarker for reduced sickle cell disease severity. In this study, a significant influence of cytokine gene polymorphisms on plasma levels of cytokines and possibly malondialdehyde has been established but not its overall effect on the clinical outcome of sickle cell disease.

CHAPTER ONE

INTRODUCTION

1.1 Background

Sub-Saharan Africa has reported 75% sickle cell disease incidence with a possible increase by 2050 (Piel *et al.,* 2013). Sickle cell disease contributes greatly to infant mortality in Africa. This violates the United Nation's (UN) Sustainable Development Goal 3 towards a reduced infant mortality (Grosse *et al.,* 2011; Ware, R.E. 2013). Uganda's 13.3% prevalence for sickle cell trait varies in eight districts. Furthermore, Uganda reports 13·2% prevalence for sickle cell trait and 0·8% for disease in children under 6 months (Ndeezi *et al.,* 2016).

Oxidative stress and inflammation contribute significantly to the pathobiology of sickle cell disease (Chaves *et al.,* 2008; Conran *et al.,* 2018). Pro-inflammatory molecules like TNF-α influence the adhesion of leukocytes and sickled erythrocytes to the endothelium. This aggregates the sickled red blood cells, neutrophils and platelets on the endothelium causing micro-occlusion, a major clinical outcome in sickle cell disease (Pathare *et al.,* 2004). Reactive oxygen species (ROS) and lipid peroxidation increase oxidative stress when released into the blood stream (Rother *et al.,* 2005).

Malondialdehyde (MDA) has been used as a biomarker of oxidant injury. Sickle cell anemia patients show elevated levels of plasma malondialdehyde (Antwi-Boasiako *et al.,* 2019; Hebbel *et al.,* 1982; Jain *et al.,* 1990; Sertac *et al.,* 1997). Accumulation of malondialdehyde disrupts the human erythrocyte membrane bilayer and thus increases the risk of developing irreversible sickle red cells (Jain *et al.,* 1990). Sickled red cells are the drivers of disease severity. However, the oxidative stress profile for the Ugandan sickle cell disease population remains unknown.

When compared to inflammatory makers, increased plasma levels of malondialdehyde and C-reactive protein correlated for both steady state and painful episode sickle cell disease phenotypes as compared to healthy controls (Bhagat *et al.,* 2012). Increased red blood cell- NADPH oxidase activity, Reactive Oxygen Species, and cytokines, TGF-β1 and endothelin-1 also correlated in sickle cell patients

(George *et al.,* 2013). Sickle cell disease pathogenesis contributes to the chronic inflammatory state seen in patients following increased cytokine production during vaso- occlusion, hemolysis, infections and perhaps oxidant injury (Nur *et al.,* 2011). Sickle cell anemia patients show augmented levels of TNF-α and IL-8 during vaso-occlusive crisis compared to steady state and significantly to healthy controls (Keikhaei *et al.,* 2013). Elevated levels of cytokines IL-10, TNF-α, TGF-β1 and IL-6 have been reported as markers of inflammation in sickle cell disease (Sarray *et al.,* 2015).

Cytokine gene polymorphisms alter serum levels of inflammatory cytokines and maybe associated with clinical heterogeneity of sickle cell disease (Cajado *et al.,* 2011). Gilli *et al.,* (2016) reports greater gene expression for IL6, IL10 and TGF-β cytokine polymorphisms in sickle cell disease. Tumour Necrosis Factor-α -308 G/A gene polymorphism predisposes sickle cell patients to stroke (Hoppe *et al.,* 2007). However, other findings showed TNF-α gene polymorphism and cerebral large vessel anomalies are not associated in sickle cell disease patients (Vicari *et al.,* 2011). A study in Brazil, reported a higher genotype frequency of 48.33% for TGF-β -509C/T gene polymorphism as compared to 17.92% for TNF-α -308G/A in sickle cell disease (Torres *et al.,* 2013).

1.2 Problem statement

Sickle cell disease still contributes substantially to infant mortality in Sub-Saharan African counties despite big treatment break throughs like the use of hydroxyurea to manage pain crisis in patients. Some of the key modifiers of sickle cell disease complicating management include increased oxidative stress, cytokine production and more recently, polymorphisms in cytokine genes. Furthermore, clinical heterogeneity in sickle cell disease patients remains a big paradox to sickle cell disease research (Conran *et al.*, 2018; Sundd *et al.*, 2019). Yet, in Uganda little has been studied on the oxidative stress and cytokine profile of sickle cell disease patients. Cytokine gene polymorphisms modify inflammatory response through altered cytokine production and as such have been associated with sickle cell disease heterogeneity and severity (Cavalcante *et al.*, 2016). Stroke has been reported for the Ugandan sickle cell disease patients but not its possible risk factors (Munube *et al.*, 2016). The TNF-α >308 G/A gene polymorphism has been identified as a risk factor for developing stroke in sickle cell patients (Hoppe *et al.*, 2007). Therefore, this study suggests to evaluate frequency of IL-10 (>1082 G/A and >819/592 C/T), TNF-α (>308 G/A) and TNF-β (>Intron1+252 G/A) gene polymorphisms and their effect on cytokine levels and or plasma malondialdehyde in sickle cell disease patients.

1.3 Objectives of the study

1.3.1 General objective

To determine the levels of oxidative stress and effect of cytokine gene polymorphisms on cytokine levels in sickle cell disease patients from Mulago hospital.

1.3.2 Specific objectives

1. To determine the levels of Malondialdehyde, IL-10 and TNF-α in sickle cell disease patients and healthy controls.
2. To determine the frequency of IL-10 and TNF-α/β gene polymorphisms in sickle disease cell patients and healthy controls.
3. To determine the association between levels of Malondialdehyde, IL-10 and TNF-α with cytokine gene polymorphisms in sickle cell disease.

1.4 Research questions

1. What is the difference in plasma levels of Malondialdehyde, IL-10 and TNF-α between sickle cell patients and controls?
2. Which IL-10 and TNF-α/β gene polymorphisms in the Ugandan sickle cell disease population are common?
3. What is the relationship between levels of Malondialdehyde, IL-10 and TNF-α with cytokine gene polymorphisms in sickle cell disease?

1.5 Significance/ Justification

The clinical outcome of sickle cell disease due to chronic hemolysis, oxidative stress and inflammation is organ damage, pain and anemia among others (Rees *et al.*, 2010). The need for hydroxyurea therapy and blood transfusion has been adopted in sickle cell disease patients following increased hospitalization and infant mortality (Opoka *et al.*, 2017). The proposed remedies for sickle cell disease include gene therapy which is still in clinical trial and stem cell transplantation treatment that has ethical issues and remains unacquainted experience for the sickle cell community in developing countries like Uganda (Serjeant *et al.*, 2003). Therefore, our study investigates the influence of IL-10 (1082 >G/A and IL-10 (819/592 >C/T), TNF-α (308G>A), TNF-β (>Intron+252G/A) gene polymorphisms on cytokine levels and oxidative stress in sickle cell disease as topics of active study and new drug targets

in the Ugandan population. This will guide the choice of potential biomarkers common to the Ugandan population for diagnosis and new drug initiations. Additional knowledge emerging from this study on the oxidative and cytokine profile for the Ugandan sickle cell disease population will contribute to the body of literature to help understand disease heterogeneity in regards to ethnicity and genetics. Lastly, our study will guide sickle cell disease research on whether there is an interaction between mediators of inflammation and oxidative stress as a potential cause of sickle cell disease heterogeneity and severity.

CHAPTER TWO

LITERATURE REVIEW

2.1 Epidemiology of Sickle cell disease (SCD)

The global burden due to sickle cell disease (SCD) is escalating because of improved survival in high prevalent countries and the migration of populations to low prevalent countries (Piel *et al.*, 2013). Sickle cell anaemia, the most common implication of the disease has a global projection of 83% for all new-borns (Piel *et al.,* 2013). In 2010, the estimated regional contribution to sickle cell anaemia by sub- Saharan Africa was 79% for new-borns but this is likely to increase to 88% in 2050 (Piel *et al.*, 2013). The highest burden of sickle cell trait ranges from 2-38% and was estimated to be in Tanzania (Weatherall *et al.*, 2001). In Uganda, the estimated prevalence of sickle cell trait is 13.3% which is representative of all the 112 districts (Ndeezi *et al.*, 2016).

2.2 Pathophysiology of sick cell disease

Sickle cell anemia is caused by inheritance of the beta-S (βS) allele from both parents (HbSS) while the sickle cell trait is due to the inheritance of both the beta-S (βS) allele, and the wild-type β-allele (Sundd *et al.*, 2019). A point mutation (GAG > GTG) in the sixth codon of the beta (β) globin gene is responsible for hemoglobin S (HbS) in sickle cell patients and the subsequent substitution of Glutamic acid for valine (Pitanga *et al.*, 2013). Under low oxygen tension, HbS polymerization causes the red cells to sickle leading to cell aggregation and hemolysis (Steinberg *et al.,* 1999).

The pathobiology of sickle cell disease is manifested by heterogeneous clinical outcome that may be influenced by ethnicity, environmental factors and more recently genetic markers of inflammation (Gilli *et al.*, 2016). An enhanced inflammatory response towards intravascular sickling in capillaries, haemolysis, cellular activation and oxidant production have been associated with impaired blood flow and sickle cell disease severity (Chiang *et al.*, 2005; Keikhaei *et al.*, 2013). Hemolysis, the primary source of free hemoglobin and consequently oxidant injury results into vaso-occlusion and increased susceptibility to infection, the key aspects for cytokine production in sickle cell disease (Conran *et al.*, 2009; Pathare *et al.,* 2003). Other clinical complications of sickle cell disease include; Organ damage, stroke, osteonecrosis,

pulmonary hypertension, acute chest syndrome and Leukocytosis among others (Balkaran *et al.*, 1992; Gladwin *et al.*, 2004; Platt *et al.*, 1994; Powars *et al.*, 2005; Sebastiani *et al.*, 2005; Steinberg *et al.*, 2009).

2.3 Clinical management of sickle cell disease

The available therapy for sickle cell disease includes; hydroxyurea and chronic blood transfusions among other clinical management options (Buchanan *et al.*, 2010; Munube *et al.*, 2016). However, lifelong dependency on blood transfusion to reduce the fraction of sickled red cells is associated with alloimmunization, autoimmunization and iron overload in patients (Yazdanbakhsh *et al.*, 2012). Hydroxyurea therapy reduces the occurrence of vaso-occlusion associated pain by decreasing the number of leukocytes, reticulocytes and lessens the expression of adhesion molecules (Lemonne *et al.*, 2015), the key factors to an inflammatory response. However, the efficacy and safety of hydroxyurea in adult patients has been questioned (Charache *et al.*, 1995; Morton *et al.*, 2015).

The current curative measures for sickle cell disease include stem cell transplant and gene therapy which has entered Phase I trials. However, effective Hematopoietic Stem Cell Transplantation (HSCT) depends on accurate matching of sickle cell patients with donors (normal or carrier) for the Human Leukocyte Antigen (HLA) to obtain an 85% disease free survival and a 97% overall survival (Panepinto *et al.*, 2007; Powars *et al.*, 2005; Walters *et al.*, 2001). Furthermore, HSCT differs among individual patients and has important adverse effects from late transplant complications such as gonadal failure and secondary malignancies (Buchanan *et al.*, 2010). Despite the ethical concerns around it, stem cell transplantation remains unacquainted experience for the sickle cell community (Meier *et al.*, 2015). This stresses the need for more research in contribution towards the development of new potential drugs such as anti-inflammatory and anti-oxidants agents to be used singly or together with the conventional regimen (Das *et al.*, 2018).

2.4 Oxidative stress and inflammation in sickle cell disease

Oxidative stress results from a depreciation in anti-oxidants and an increase in oxidant production (Amer *et al.*, 2006; Nur *et al.*, 2011). Normal red blood cells are exposed to oxidative stress because of reactive oxygen species generation as intermediates during intracellular catabolism where oxygen acts as an electron acceptor. In sickled red blood cells oxidative stress is doubled due to increased haemoglobin autoxidation (Hebbel *et al.*, 1988), cell free haemoglobin (Nagababu *et al.*, 2008), plasma enzymes and chronic pro-inflammatory state (Akohoue *et al.*, 2007). Consequently oxidant damage leads to a continuous pro-inflammatory response, lifelong oxidative stress and contributes to endothelial dysfunction and multiple organ damage (George *et al.*, 2013).

A bidirectional relationship has been proposed for oxidative stress and inflammatory state in sickle cell disease. Reactive oxygen species facilitate the activation of inflammatory mediators, expression of adhesion molecules and endothelial damage (Guzik *et al.*, 2003). Activation of the nuclear factor-κB (NF-κB) by reactive oxygen species increases gene expression and the subsequent production of pro-inflammatory cytokines (IL-1, TNF- α, IL-6) and adhesion molecules (Owusu-Ansah *et al.*, 2016). A study by Wagener *et al.*, (1997) found increased expression of adhesion molecules following haemoglobin induced- oxidative stress and TNF-α treatment in human umbilical vein endothelial cells. Thus, oxidative stress and associated pro-inflammatory response contribute significantly to the sequence of increased aggregation of sickled erythrocytes, platelets and neutrophils to the endothelium (Pathare *et al.*, 2004). This results into impaired blood flow in the capillaries and increased inflammation.

2.4.1 Malondialdehyde (MDA) levels in sickle cell disease

The highly reactive instability of reactive oxygen species limits their use as biomarkers of oxidative stress. Thus previous studies have used more stable end-products like malondialdehyde (Nur *et al.*, 2011). Malondialdehyde represents oxidation of lipids and circulating proteins and is found elevated in sickle cell disease patients in contrast to healthy controls (Jain *et al.*, 1990; Sertac *et al.*, 1997). Though, Walter *et al.*, (2006) reported contradicting results for MDA levels in sickle cell

disease patients when compared to controls. The controversy was resolved when (Rees *et al.,* 2012) and (Antwi-Boasiako *et al.,* 2019) showed high malondialdehyde levels in steady state and increasing levels in severe sickle disease phenotypes. (Bhagat *et al.,* 2012) also found a significant association between C-reactive protein, an inflammatory marker and malondialdehyde levels in sickle cell patients. Accumulation of malondialdehyde disrupts the human erythrocyte membrane bilayer (Jain *et al.*, 1990). Membrane damage increases the risk of forming irreversible sickle red cells and consequently a cascade of pathological events involving vaso-occlusion and inflammation (Jain *et al.,* 1984).

2.4.2 Cytokine gene polymorphisms in sickle cell disease

Polymorphisms in cytokine genes act as genetic factors that modulate the release of inflammatory cytokines and consequently the clinical profile of sickle cell disease. Many studies have documented the different cytokine gene polymorphisms that modify sickle cell disease severity (Cajado *et al.,* 2011; Cavalcante *et al.*, 2016; Sarray *et al.*, 2015).

2.4.2.1 Tumour necrosis factor alpha (TNF-α) gene polymorphism

Tumour Necrosis Factor-alpha is a pro-inflammatory cytokine produced mainly by monocytes/macrophages and T-cells among other cell types. The molecule is involved in endothelial cell and leukocyte activation, macrophage stimulation, leukocyte chemotaxis and recruitment (Makis *et al.,* 2000). The most frequent genotype reported for TNF-α gene polymorphism in a Brazilian population was GG (Torres *et al.,* 2013) in contrast to GA and AA in an Indian population (Sultana *et al.*, 2011). Population specific controversy has also been reported with TNF-α-308G>A gene polymorphism frequencies and stroke (Harcos *et al.*, 2006; Hoppe *et al.*, 2004; Hoppe *et al.*, 2007; Karahan *et al.*, 2005; Rubattu *et al.*, 2005; Sultana *et al.*, 2011). The TNF-α-308G>A gene polymorphism has also been emphasized in the development of splenic sequestration events in sickle cell anaemia patients (Cajado *et al.*, 2011). The absence of risk to develop a migraine with TNF-β 252G >A polymorphism has also been reported (Ghosh *et al.*, 2010).

2.4.2.2 Interleukin 10 (IL-10) gene polymorphisms

Interleukin- 10, an anti-inflammatory cytokine is mainly produced by activated CD8+ cells and others like; activated T-Helper cells, B- cells and mast cells (Moore *et al.*, 1990). Being an anti-inflammatory molecule, IL-10 inhibits the synthesis of TH1cytokines, such as TNF-α, IL-1, IL-6, and IL-8. The most frequent genotype reported for IL-10 (1082>G/A) polymorphism remains unresolved between AG and AA genotypes (Belisário *et al.*, 2015; Cavalcante *et al.*, 2016; Gilli *et al.*, 2016; Sultana *et al.*, 2011). The GG genotype for IL-10 (1082>G/A) has been associated with a three times increased risk to ischemic stroke ($P < 0.001$, OR 3.25) (Sultana *et al.*, 2011). Conversely, (Belisário *et al.*, 2015) found no significant difference between the frequency of IL-10 haplotypes in children with acute cerebral ischemia, or high-risk transcranial in contrast to those without the condition. Increased IL-10 secretion maybe indicated by IL-10 >1082 gene polymorphism or the 819 and 592 promoter haplotype-single nucleotide polymorphisms that protect against Epstein–Barr virus infection (Helminen *et al.,* 2001).

2.5 Cytokine gene polymorphisms and Plasma levels of cytokines in sickle cell disease

Cytokine-gene polymorphisms influence serum concentrations of cytokines and possibly the clinical heterogeneity in sickle cell disease (Cavalcante *et al.*, 2016). Increased plasma levels of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-8 among others have been reported for sickle cell anaemia patients in crisis and at steady state as compared to healthy controls (Pathare *et al.*, 2003; Qari *et al.,* 2012). Similarly, other studies have reported an increase in plasma levels of TNF-α in patients (Alagbe *et al.,* 2018), notably those with the Bantu/Bantu haplotype of sickle cell anaemia (Bandeira *et al.*, 2014). Positivity for the A allele in the TNF-α 308G>A gene polymorphism, particularly in the promoter region is associated with higher TNF-alpha transcript levels (Cajado *et al.*, 2011). This agreed with a previous study on cerebral malaria that reported possible influence on TNF promoter activity by the –308 G to A transition in a B-cell line (McGuire *et al.,* 1994).

However, low plasma levels of IL-10, an anti-inflammatory cytokine has been reported during crisis and high in steady state sickle cell disease patients (Sarray *et al.*, 2015).

Correspondingly, increased serum levels of IL-10 in steady state sickle cell patients have been reported and attributed to its role in the inhibition of both humoral and cell mediated immune functions (Cavalcante *et al.*, 2016; Sarray *et al.*, 2015). Decreased levels of IL-10 have been associated with osteomyelitis in patients (Sarray *et al.*, 2015). This emphasizes the protective role of IL-10 in sickle cell disease. A marked gene expression for IL-10 and TGF-β has been reported in sickle cell disease patients (Gilli *et al.*, 2016). Likewise, the GCC alleles have been associated with increased IL-10 production unlike the mutants ATA for 1082/819/592 IL-10 haplotypes in severe malaria patients (Ouma *et al.*, 2008).

CHAPTER THREE

MATERIAL AND METHODS

3.1 Study Area

Blood samples were obtained from patients who come for clinical review at Mulago hospital sickle cell clinic. The hospital is located on Mulago hill north of Kampala city (Central Uganda) and is known to handle most health cases from a highly varied ethnic population. The sickle cell clinic has over 12,000 registered patients and on average receives 200 patients in 5 days a week. Collectively, being a referral clinic also made the sickle cell clinic a suitable site for data and sample collection on patients. Healthy control samples were obtained from Blood bank, Nakasero (Central Uganda) and involved blood donations from healthy volunteers following consent from the research and ethics committee. All blood donations from blood bank were screened for Transfusion Transmissible Infections (TTIs). Additionally, the blood bank is found in the same neighbourhood as the sickle cell clinic and 50% of the blood donated has used to transfuse children. This made it a suitable site for data and sample collection.

3.2 Study Design

This was a case control study that identified participants as cases or healthy controls using the sickle cell anaemia genotyping test. Cases were recruited passively at the Mulago hospital sickle cell clinic, Central Uganda (Kampala district) from February to March, 2018 by the mother study. Cases were defined as patients with previous sickle cell test using haemoglobin electrophoresis. Healthy controls were normal volunteers positive for the normal Beta-globin gene (HbAA) from Blood bank, Nakasero, Central Uganda (Kampala district). Comparison between study groups was achieved using whole blood samples.

3.3 Sample size determination

The sample size required for this study was calculated using GraphPad StatMate 2.00 statistical, as that with a 95% power of detecting a difference between MDA means of 1.52μM (Antwi-Boasiako *et al.*, 2019) at a significant level $\alpha = 0.05$. Thus, a sample size of n=150 was used for this study.

3.4 Inclusion criteria

The case study group involved patients aged 2-19 years with prior diagnosis of sickle cell disease using Hb electrophoresis. The healthy control study group composed of healthy volunteers aged 15years and above that were positive for the normal Beta-globin gene (HbAA).

3.5 Exclusion criteria

Lysed whole blood samples and volumes of less than 3mls from paediatric patients were removed from the study. Healthy controls that were heterozygous for the sickle trait (HbAS) were excluded from both study groups but rather analysed independently.

3.6 Sample collection and preparation

After ethical clearance, a volume of 4mls of blood collected aseptically into vacutainer tubes with Ethylene-diamine tetra acetic acid (EDTA) and stored at the Molecular laboratory at the Department of Biochemistry, College of Natural Sciences were obtained for case group. Blood from healthy controls was also transported to the laboratory on dry ice. Blood from both study groups was then processed and recorded using a specific laboratory identification number. Whole blood was centrifuged at 1000×g for 10 minutes to obtain 1000µl of plasma and 500µl of buffy coat for storage at -20 $^{\circ}$C prior to laboratory analysis.

3.7 Laboratory analysis

3.7.1 Determination of Malondialdehyde (MDA) concentration in plasma

Using methods described by Devasagayam *et al*., (2003); 200µl of TCA/TBA-HCl (Trichloroacetic acid/ Thiobarabituric acid) solution and 300µl of 1M hydrochloric acid (HCl) were added to 300µl plasma in 2ml tube and the mixture put to heat at 100° C for 60 minutes on a heat block. The hot mixture was placed one ice to allow cooling for 10 -20 minutes, and there after centrifuged at 3000rpm for 2 minutes to separate the supernatant from the pellets. On addition of 300µl of 3M sodium hydroxide to the sample and centrifuging the mixture at 3000rpm for 1 minute, a pink color was observed. The absorbance of the sample was read at 540nm within 10minutes using a spectrophotometer (Thermo-electron Corporation, Aquamate). Malondialdehyde

concentration was calculated using the molar absorption extinction coefficient of $1.56\times10^{5}M^{-1}cm^{-1}$.

3.7.2 Total gDNA extraction

This was executed using methods described by Iranpur-Mobarakeh *et al*., (2010). To a 1.5ml eppendorf tube, 500µl of the buffy coat layer were added and then topped up with 1000µl of red cell lysis buffer (0.01M Tris-HCl, 320mM sucrose, 5mM $MgCl_2$, and 1% Triton X100). Sample homogenization was achieved by gentle shaking of the microfuge tube followed by spinning for 2 minutes at 7000 rpm. On discarding the supernatant, the previous steps were repeated 2-3 times to remove hemoglobin and clean the white blood cells of any residual hemoglobin. To break down the pellet further, vortexing while rinsing in red blood lysis buffer was done. To drain residual red lysis buffer, the tube was placed downwards on tissue paper for a few seconds. After, 400µl of nucleic lysis buffer were added to the tube (0.01M Tris-HCl, 11.4mM sodium citrate, 1mM EDTA and 1% SDS) and the pellet dissolved up and down with help of a pipette. Following pellet dissolution, 100µl of saturated NaCl (5M) and 600µl of chloroform were added. This was mixed on a rotating blood mixer at room temperature, centrifuged for 2minutes at 7000rpm and then 400µl of supernatant were transferred to a new 1.5ml tube. Next 800µl of cold (-20^{o}C) absolute ethanol was added, the mixture shaken gently and then vortexed on a rotating blood mixer prior to centrifugation at 12000 rpm for 1 minute to allow DNA precipitation (DNA appears as a mucus-like strand in the solution phase). The supernatant was discarded with great caution and the tube left to dry completely at room temperature. Lastly, 50µl of TE buffer (Tris-HCl and EDTA) were added to the white pellet, followed by vortexing to allow reconstitution and DNA storage at -20^{o}C for PCR analysis. Successful DNA extraction was confirmed by 1% agarose gel electrophoresis.

3.7.3 Sickle Cell Anemia genotyping using Allele specific PCR

Sickle cell disease phenotype was determined using methods as described by Waterfall *et al*., (2001). Amplification by PCR was achieved using a reaction volume of 22.0µl composed of 10.3µl of PCR water, 2.5µl of Taq buffer, 2.5µl of dNTPs, 0.2µl of Taq DNA polymerase, 2.5µl of Forward-Primer (mutant and wild type), 2.5µl

of Reverse-Primer (mutant and wild type), and 1.5µl gDNA. The primer sequences for normal and mutant β-globin genes were as follows.

Wild type –AS (5'-ATG GTG CAC CTG ACT CCT GA-3'and W.T-CP517 (5'CCC CTT CCT ATG ACA TGA ACT-3). The PCR product is 517-bp for the normal β-globin gene.

Mutant –AS (5'-CAG TAA CGG CAG ACT TCT CCA-3' and MUT-CP267 (5'-GGG TTT GAA GTC CAA CTC CTA-3') (Waterfall and Cobb, 2001). The PCR product is 267bp for the mutant β-globin gene.

The program for amplification was initial denaturation at 95 °C for 3minutes, denaturation at 95 °C for 30seconds, annealing at 64 °C for 10 seconds, 35 seconds of extension at 72 °C, and this was repeated 45 times. Lastly, final extension was set at 72 °C for 7 minutes and cooling at 4 °C. After amplification, the amplified DNA was analyzed in 1% Agarose gel for HbSS- homozygous sickle hemoglobin gene, HbAS-heterozygous gene and HbAA- normal beta-globin gene.

3.7.4 The Amplification Refractory Mutation System- PCR (ARMS-PCR)

The ARMS- PCR is an allele-specific PCR. The PCR detects known mutations involving single nucleotide polymorphisms (SNPs). In this method, amplification of test DNA by sequence- specific PCR primers depend on the presence of the target allele in the DNA sample. The ARMS-PCR utilizes allele- specific primers; wild type or mutant at 5′-end and a generic primer at 3′-end. Using agarose gel electrophoresis, the PCR amplicons include homozygosity for the mutant or wild type allele and heterozygosity for both alleles (Hanafi *et al.*, 2014; Newton *et al.*, 1989; Yang *et al.*, 2018). Design of the ARMS-PCR test requires vigorous optimization. A previous study by (Chen *et al.*, 2007) reported low sensitivity for the test due to competing PCR reactions conducted in one test tube. The PCR reactions include the control DNA fragment that has been found to out compete the mutant and occasionally the wild type alleles for primers among other reaction components. Thus, an increase in the mutant-specific primer concentration and a decrease in the generic primer concentration have been adopted for strong amplification of the mutant allele. The ARMS-PCR assay is advantageous because of easy result interpretation from the different patterns of

normal, mutant, or mixed genotypes as observed during agarose gel electrophoresis. The method is cost-efficient, time and labour saving as well.

3.7.5 Amplification refractory mutation system-PCR (ARMS-PCR) protocol

The ARMS-PCR as described by Perrey *et al*., (1999) was used to genotype cytokine gene polymorphisms. In this method, the mutant and wild type allele-specific primers were used separately to give 2 PCR reactions. The primer sequences used for; IL-10 haplotypes and TNF-α/β gene polymorphisms are described (Table 3.1) (Perrey *et al.*, 1999). Successful PCR amplification of the specific allele was checked using the human growth hormone primer sequence as the internal control. The IL-10 (1082G/A) polymorphism was genotyped using a 10µl reaction volume made of 1µl mixture of generic primer (anti-sense) and allele specific mutant or wild type primer (sense), 2.5µl of PCR water, 5µl of 1x TaqMan Mastermix, 0.2µl of $MgCL_2$, 0.2µl of dNTPs, 0.1µl of internal control primer mix (reverse and forward) and 1µl template DNA were used for ARMS-PCR. The amplification program was set as follows; initial denaturation at 95°C for 3 minutes, denaturation at 95°C for 1 minute, specific-primer annealing at 59°C for 1 minute, extension at 72°C for 1minute, all of which was run for 35 cycles. Final extension was set at 72°C for 7 minutes.

For IL-10 (819/-592C/T) polymorphism, a 10µl reaction volume of 2.5µl of PCR water, 5µl of 1x TaqMan Mastermix, 1µl of generic primer (anti-sense) and allele specific mutant or wild type primer (sense) mix, 0.2µl of $MgCL_2$, 0.2µl of dNTPs, 0.1µl of internal control primer mix (reverse and forward) and 1µl template DNA were used for ARMS-PCR. The Step- down PCR conditions were set as follows; initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 15 seconds, annealing at 65°C for 50 seconds, extension at 72°C for 40 seconds, all of which was run for 10 cycles and lastly; denaturation at 95°C for 20 seconds, specific-primer annealing at 59°C for 50 seconds, extension at 72°C for 30 seconds, for 25 cycles then final extension at 72°C for 7 minutes.

For TNF-α (308G/A) polymorphism, a 10µl reaction volume made of 0.25µl mix of generic primer (anti-sense) and allele specific mutant or wild type primer (sense), 3.125µl of PCR water, 5µl of 1x TaqMan Mastermix, 0.3µl of $MgCL_2$, 0.2µl of dNTPs, 0.125µl of internal control primer mix (reverse and forward) and 1µl template DNA

were used for ARMS-PCR. The amplification program was set as follows; initial denaturation at 95°C for 1 minute, denaturation at 95°C for 30 seconds, specific-primer annealing at 55°C for 30 seconds, extension at 72°C for 1minute, all of which was run for 30 cycles. Final extension was set at 72°C for 7 minutes.

The TNF-β Intron1+252(G/A) amplification by PCR was achieved using a reaction volume of 10µl composed of 0.25µl mix of generic primer (anti-sense) and allele specific mutant or wild type primer (sense), 3.125µl of PCR water, 5µl of 1x TaqMan Master mix, 0.3µl of $MgCL_2$, 0.2µl of dNTPs, 0.125µl of internal control primer mix (reverse and forward) and 1µl template DNA. The Step- down PCR conditions were set as follows; initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 15 seconds, annealing at 65°C for 50 seconds, extension at 72°C for 40 seconds, all of which was run for 10 cycles and lastly; denaturation at 94°C for 20 seconds, specific-primer annealing at 59°C for 50 seconds, extension at 72°C for 50 seconds, for 25 cycles then final extension at 72°C for 7 minutes.

A 2% agarose gel electrophoresis containing 0.5mg/ml of ethidium bromide was used to view the amplified products, against a 50-bp ladder (MFT).

Table 3 1: Primer sequences for cytokine gene polymorphisms using ARMS-PCR

Primer name	Primer sequence	Product size
IL-10 (-1082)	Generic primer (antisense): 5'-CAGTGCCAACTGAGAATTTGG-3' Primer G (sense): 5'CTACTAAGGCTTCTTTGGGAG-3' Primer A (sense): 5'ACTACTAAGGCTTCTTTGGGAA-3'	258bp
IL-10 (-819/-592)	Generic primer (antisense): 5'-AGGATGTGTTCCAGGCTCCT-3' Primer C (sense): 5'-CCCTTGTACAGGTGATGTAAC-3' Primer T (sense): 5'-ACCCTTGTACAGGTGATGTAAT-3'	233bp
TNF-α-308	Generic primer (antisense): 5'-TCTCGGTTTCTTCTCCATCG-3' Primer G (sense):5'-ATAGGTTTTGAGGGGCATGG-3' (TNF1 allele) Primer A (sense): AATAGGTTTTGAGGGGCATGA-3' (TNF2 allele)	154bp
TNF-β Intron1+252	Generic primer (antisense): 5'-AGATCGACAGAGAAGGGGACA-3' Primer G (sense): 5'-CATTCTCTGTTTCTGCCATGG-3' (TNFβ2 allele) Primer A (sense): 5'-CATTCTCTGTTTCTGCCATGA-3' (TNFβ2 allele)	94bp
Internal control primer	Forward primer: 5'-GCCTTCCCAACCATTCCCTTA-3' Reverse primer: 5'-TCACGGATTTCTGTTGTGTTTC-3'	429bp

3.7.5 Determining plasma levels of IL-10 and TNF-α cytokines

Plasma levels of IL-10 and TNF-α were quantified in both sickle cell disease patients and healthy controls using the human IL-10 and TNF-α Enzyme Linked Immunosorbent Assay (ELISA) kits (BD Biosciences, Pharmingen, San Diego, USA), following the manufacturer's instructions). The immunoassay was also standardized against the procured recombinant human IL-10 and TNF- α cytokines to ensure quality results. A dilution factor of 3 was used against the study population samples.

3.8 Data analysis

Data was analyzed using GraphPad prism software. Using the D'Agostino-Pearson normality test, deviation from normality was tested before further analysis of data. Thus, values for MDA concentration were expressed as median ± SEM for each study

group due to lack of a normal distribution of data. Comparison of MDA levels between groups was achieved using the T-test while the Chi-square (χ^2) and Fisher's exact tests were used for comparison of gene frequencies between groups. Descriptive analysis was used to show an association between MDA levels and gene frequencies with the T-test. The level of statistical significance was set as $P< 0.05$. Genotype frequencies were used to calculate allele frequencies and later expressed as a percentage of the total number of alleles.

3.9 Ethical consideration

This study was approved by Makerere University School of Health Sciences Research and Ethics Committee (MAKSHSREC) Reference Number MAKSHSREC-2021-90. A waiver of informed consent to use both the sickle cell disease blood samples from the mother study entitled **"Oxidative Stress in Sickle Cell Disease",** and healthy control blood samples from Blood bank, Nakasero was obtained.

CHAPTER FOUR

RESULTS

4.1 Baseline characteristics of study population

A total of 372 participants that included sickle cell patients and healthy controls were recruited and sampled in this study. The patient group consisted of 183 sickle cell disease patients (HbSS), of these 105 (57.4%) were males and 78 (42.6%) were females. The healthy control group consisted of 189 individuals (HbAA), of these 100 (52.9%) were males and 89 (47.1%) were females. A total of 9 (4.7%) individuals were identified as carriers for the sickle cell gene (HbAS) and excluded from the healthy control group. The average age for the sickle cell disease group was 9.8±0.64 years while that of the control groups was 27±0.67 years. Sickle cell disease was more common in patients below 5 years (45, 27.6%) and children aged 5 – 14 years (82, 50.3%) as compared to older patients aged above 15 years (36, 22.1%). This was in regard to 163 sickle cell disease patients that had age related data (Table 4.1).

Table 4.1: Baseline characteristics of study population

	Sickle cell disease patients		**Healthy controls**	
Age (years)	Males [N (%)]	Females [N (%)]	Males [N (%)]	Females [N (%)]
<5	24 (53.33)	21 (46.67)	0 (0)	0 (0)
5- 9	28 (57.14)	21 (42.86)	0 (0)	0 (0)
10- 14	22 (66.67)	11 (33.33)	0 (0)	0 (0)
15- 19	14 (73.68)	05 (26.32)	44 (67.69)	21 (32.31)
20- 24	03 (50)	03 (50)	22 (68.75)	10 (31.25)
>25	06 (54.54)	05 (45.45)	78 (84.78)	14 (15.22)

4.2 Levels of Oxidative stress in the study population

To determine the extent of oxidative stress in sickle cell disease, plasma levels of malondialdehyde (MDA) were analyzed in patients (N =178) and controls (N =189) using descriptive analysis. Results showed that median plasma levels of MDA were significantly higher (p= 0.0063, Mann-Whitney test) in sickle cell patients (2.615µM)

as compared to healthy controls (2.490μM) (Figure 4.1). When median MDA levels were compared across sex, no significant differences (p= 0.39, Mann-Whitney test) were noted between male (2.700μM) and female (2.475μM) sickle cell disease patients (Figure 4.2A). Similarly, comparison of median MDA levels across different age groups in sickle cell disease patients did not yield any significant difference (Kruskal-Wallis test) (Figure 4.2B). Malondialdehyde analysis (N =178) was limited by low volumes of plasma obtained from patients that were mostly anaemic.

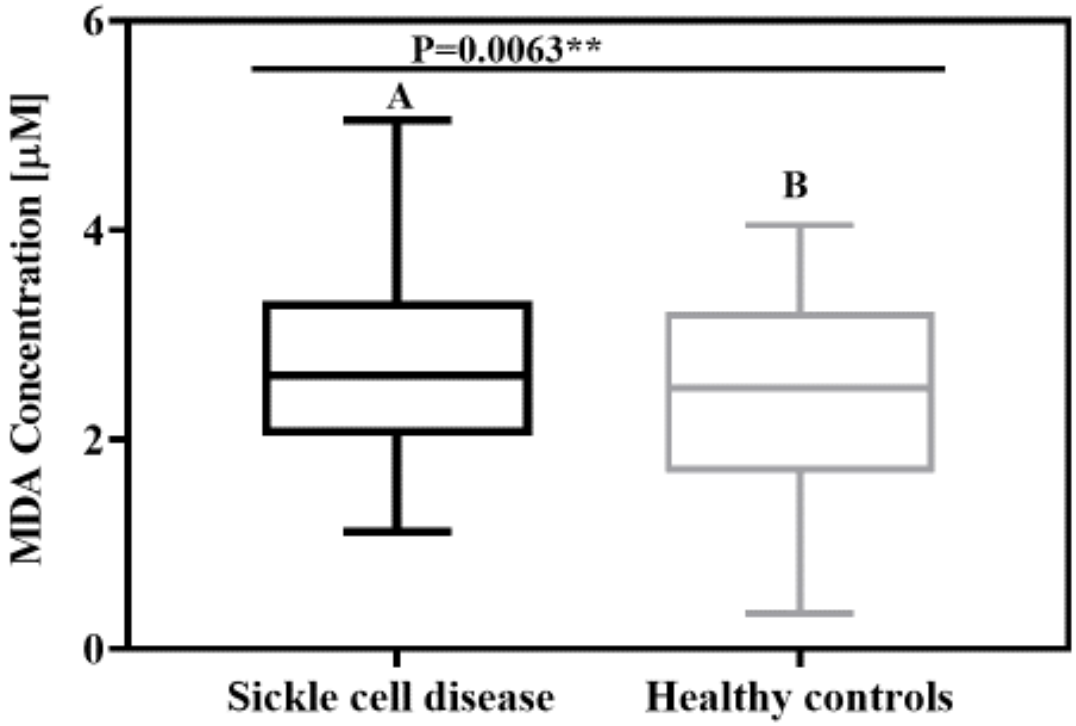

Figure 4. 1: Plasma levels of MDA in sickle cell disease patients (N=178) and healthy controls (N=189)

Boxes indicate median and inter quartile range while the whiskers define the highest and lowest values. The uppercase letters indicate significant difference across groups.

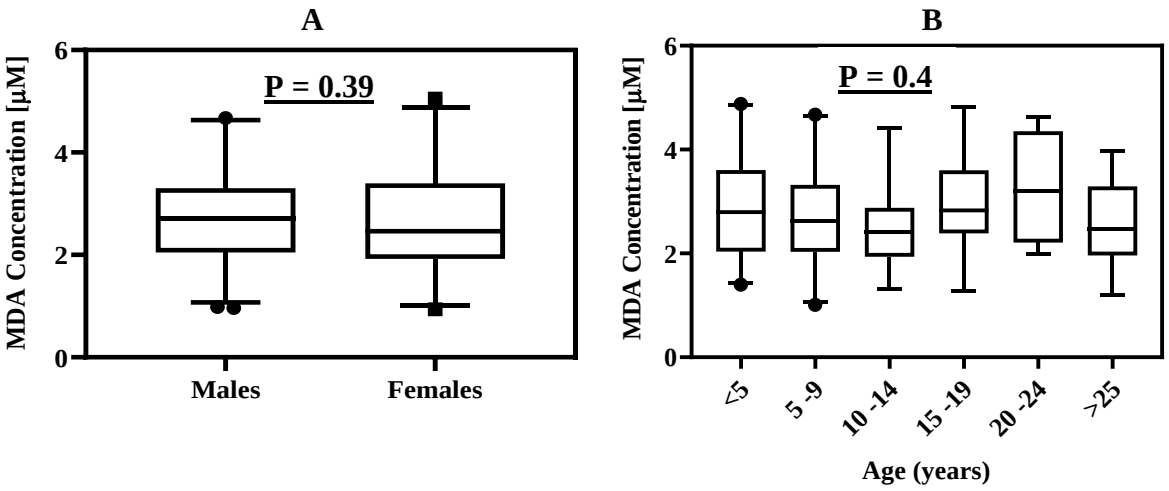

Figure 4. 2 Plasma levels of Malondialdehyde compared across sex (Plot A) and age (Plot B) in sickle cell disease patients

Boxes indicate median and interquartile range while whiskers are defined as 2.5-97.5 percentiles. Dots define outliers.

4.3 Levels of Cytokines in the study population

In order to demonstrate the role of cytokines in the modulation of sickle cell disease, we assayed IL-10 and TNF-α cytokines in the plasma of both sickle cell disease patients (N =178) and healthy controls (N =189). The detection limits for the assays were 14.2pg/ml (IL-10) and 2.7pg/ml (TNF-α). Median plasma levels for IL-10 were significantly higher (p<0.0001, Mann-Whitney test) in the plasma of sickle cell disease patients (20.37pg/ml) as compared to healthy controls (7.5pg/ml) (Figure 4.3). On the other hand, plasma levels of TNF-α were detected in 2 sickle cell disease patients and 2 healthy control samples.

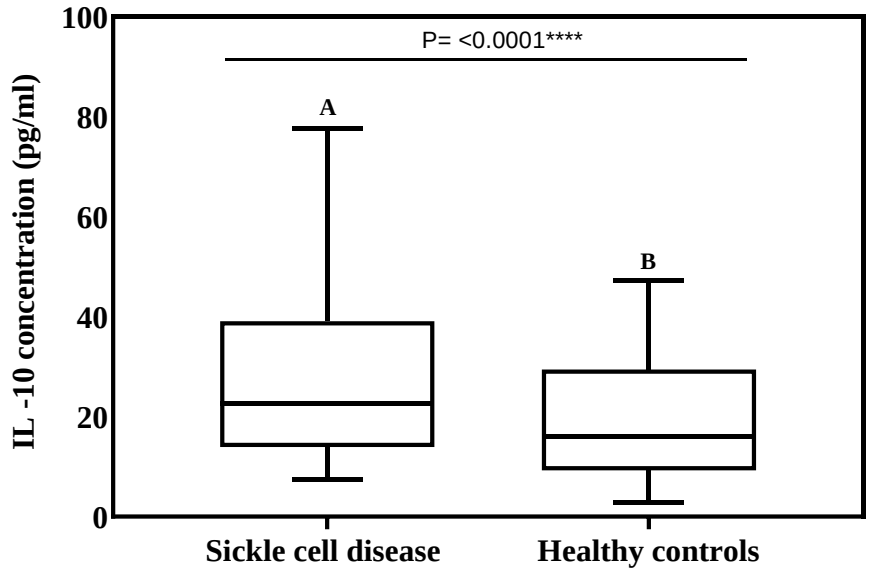

Figure 4.3 Plasma levels of IL-10 in sickle cell disease patients (N=178) and healthy controls (N=189)

Boxes indicate median and interquartile range while whiskers define the highest and lowest values. The uppercase letters indicate significant difference across groups.

4.4 Cytokine gene polymorphisms in the study population

In order to determine the frequency of three IL-10 biallelic and TNF-α/β polymorphisms in the study participants, allele specific primers for both the mutant and wild type allele were used to identify homozygous wildtype, heterozygous and homozygous mutant genotypes in sickle cell disease patients and healthy controls.

4.4.1 Polymorphisms in Interleukin- 10 (1082 >G/A and 819/592 >C/T) genes

The genotypes for IL-10 (1082 >G/A) polymorphism were deduced from the presence or absence of a 258bp amplicon specific for either G or A and both alleles (Figure 4.4). A 429bp amplicon of the Human Growth Hormone (HGH) as internal control was also observed in the gel, indicating optimal amplification of test DNA in sickle cell disease patients (N=115) and healthy controls (N= 056). Our study shows that heterozygous GA was the most common genotype for IL-10 (1082 >G/A) polymorphism in both patients (62.61%) and healthy controls (64.3%). Similarly, 35.65% of sickle cell disease patients had homozygous GG genotype while the control group scored 33.9%. The least genotype observed for IL-10 (1082 >G/A) was homozygous AA, with 1.74% in patients

and 1.8% in healthy controls (Table 4.2). However, statistical analysis did not yield any significant (p<0.05, Chi-square test) difference when IL-10 (1082 >G/A) genotype frequency was compared in patients and healthy controls. There was also no significant association between IL-10 (1082 >G/A) genotypes, GG (odds ratio=1.08, 95% confidence interval = 0.54-2.14, p= 0.87); GA (odds ratio = 0.93, confidence interval = 0.48-1.82, p= 0.87); AA (odds ratio = 0.97, confidence interval = 0.11-14.34, p>0.9999, Fisher's exact test) and sickle cell disease. The G allele for IL-10 (1082>G/A) polymorphism was more frequent in the study population as compared to the A allele.

For IL-10 (819/592 >C/T) polymorphism, genotypes were deduced from the presence or absence of a 233bp amplicon specific for either C or T and both alleles (Figure 4.5). A 429bp amplicon of the Human Growth Hormone (HGH) as internal control was also observed in the gel, indicating optimal amplification of sample DNA in both sickle cell (N=115) disease patients and healthy controls (N=056). The most frequent genotype for IL-10 (819/592 >C/T) polymorphism in both patients (99.13%) and healthy controls (100%) was heterozygous CT. The TT genotype was absent in the study population while the CC genotype was present in the patient's group (0.87%) but not in the healthy control group (0%) (Table 4.2). The genotypes for IL-10 (819/592 >C/T) polymorphism did not yield any significant (p=>0.9999, Fisher's exact test) association with sickle cell disease. The allele frequency for IL-10 (819/592 >C/T) polymorphism was comparable between C and T alleles in the study population.

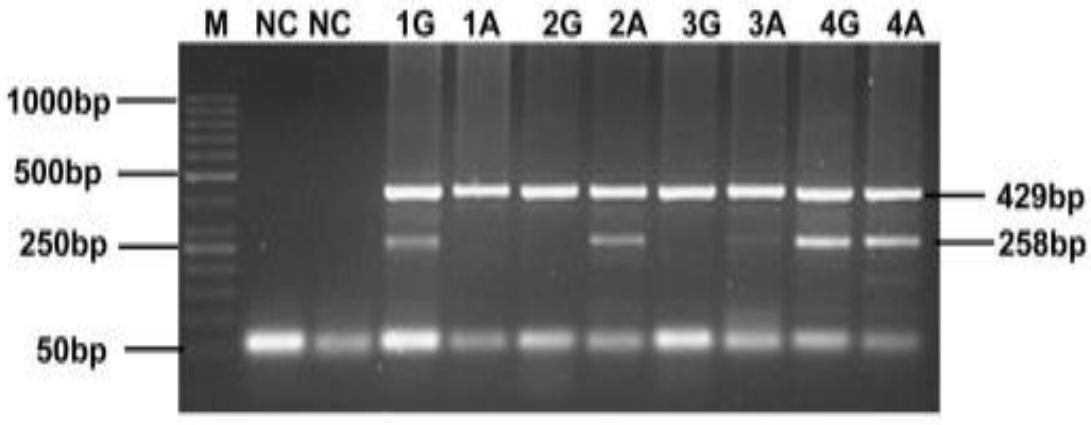

Figure 4. 4 IL-10 (1082>G/A) genotypes from ARMS-PCR amplification

From left to right: Agarose gel with a 429bp amplicon of the HGH control present in all lanes, showing optimal amplification. M= 50bp ladder, NC = Negative control; Lane

1G- 1A = Homozygous GG; Lanes 2G- 2A and 3G- 3A= Homozygous AA and Lane 4G- 4A = Heterozygous GA for IL-10 (1082 G/A) polymorphism in sickle cell disease patients.

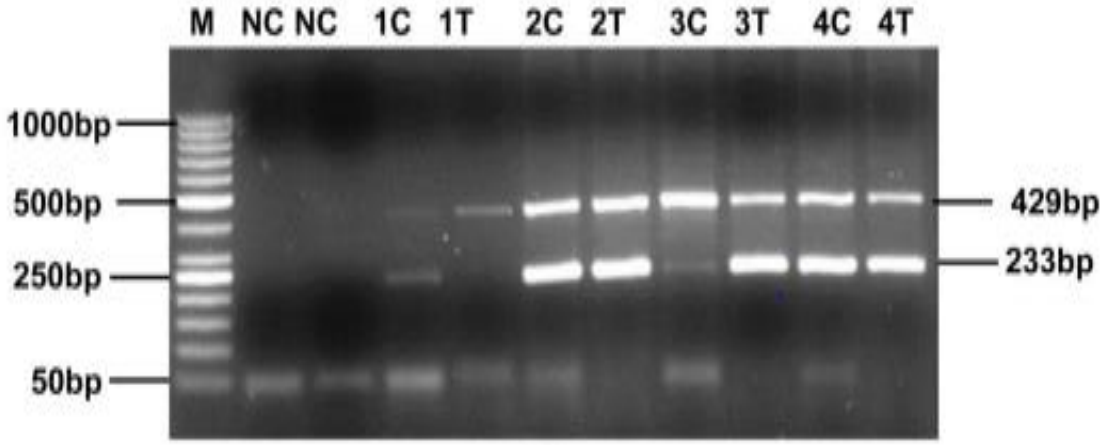

Figure 4.5 IL-10 (819/592 >C/T) genotypes from ARMS-PCR amplification

From left to right: Agarose gel with a 429bp amplicon of the HGH control present in all lanes, showing optimal amplification. M= 50bp ladder, NC = Negative control; Lane 1C- 1T = Homozygous CC; and Lanes 2C- 2T, 3C- 3T and 4C- 4T = Heterozygous CT for IL-10 (8191/592 >C/T) polymorphism in sickle cell disease patients.

Table 4. 2 Genotypic and Allelic frequency distribution of IL-10 Polymorphisms in in the study population

	Sickle cell patients		**Healthy controls**		**Statistical analysis**
Cytokine gene Polymorphism	Genotypic frequency [N (%)]	Allelic frequency	Genotypic frequency [N (%)]	Allelic frequency	*p*-value (OR, 95% CI)
IL-10 (1082G/A)					
GG	41 (35.65)	G = 0.6696	19 (33.9)	G = 0.6607	0.87 (1.08, 0.54-2.14)
GA	72 (62.61)	A = 0.3304	36 (64.3)	A = 0.3393	0.87 (0.93, 0.48-1.82) >0.99 (0.97, 0.11-14.34)
AA	02 (1.74)		01 (1.8)		>0.99
IL-10 (819/592C/T)					>0.99 (0.00, 0.00-18.48)
CC	01 (0.87)	C = 0.504	00 (0)	C = 0.5	
CT	114 (99.13)	T = 0.496	56 (100)	T = 0.5	
TT	00 (0)		00 (0)		

OR = odds ratio; CI = confidence interval

4.4.2 Polymorphisms in Tumor necrosis factor alpha /beta

The genotypes for TNF-α (308 >G/A) and TNF-β (Intron1+252>G/A) polymorphisms were deduced from the presence or absence of a 154 or 94 bp amplicon specific for either G or A and both alleles (Figure 4.6 and 4.7), respectively. A 429bp amplicon of the Human Growth Hormone (HGH) as internal control was also observed in the gel, indicating optimal amplification of sample DNA in both patients (N=115) and healthy controls (N= 056). Results from our study showed heterozygous GA (100%) as the most frequent and only genotype present in our study population for both TNF-α (308 >G/A) and TNF-β (Intron1+252>G/A) polymorphisms (Table 4.3). Using the chi-square and fisher's exact tests, the GA genotype did not show any significant (*p*= >0.9999) association with sickle cell disease. The frequency of G and A alleles for both TNF-α (308 >G/A) and TNF-β (Intron1+252>G/A) polymorphisms were also found to be comparable between patients and healthy controls.

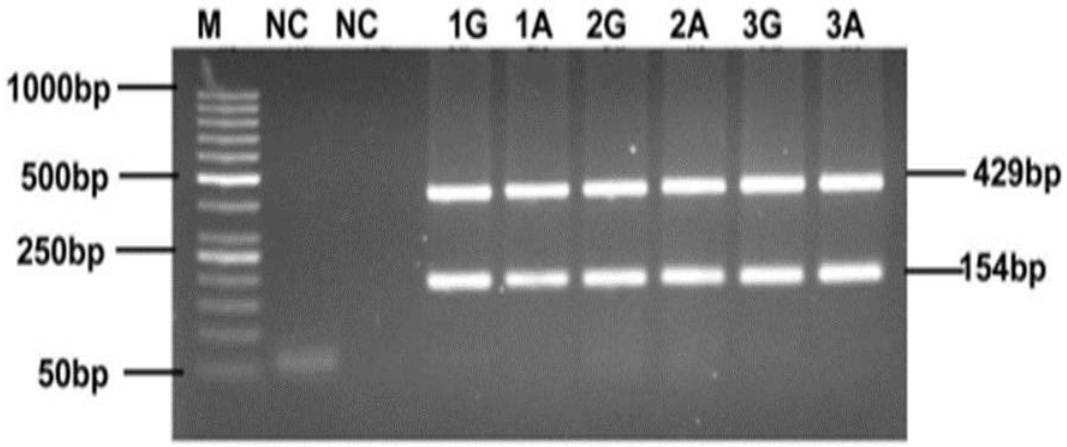

Figure 4. 6 TNF-α (308 >G/A) genotypes from ARMS-PCR amplification.

From left to right: Agarose gel with a 429bp amplicon of the HGH control present in all lanes, showing optimal amplification. M= 50bp ladder, NC = Negative control; Lanes 1G- 1A; 2G- 2A and 3G- 3A= Heterozygous GA for TNF-α (308 >G/A) polymorphism in sickle cell disease patients.

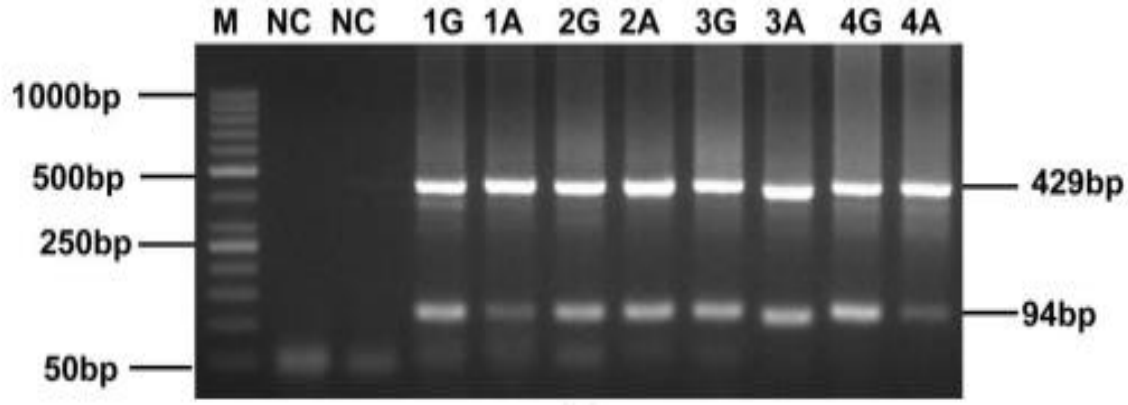

Figure 4. 7 TNF-β (Intron1+252>G/A) genotypes from ARMS-PCR amplification.

From left to right: Agarose gel with a 429bp amplicon of the HGH control present in all lanes, showing optimal amplification. M= 50bp ladder, NC = Negative control; Lanes 1G- 1A; 2G- 2A and 3G- 3A= Heterozygous GA for TNF-β (Intron1+252> G/A) polymorphism in sickle cell disease patients.

Table 4. 3: Genotypic and Allelic frequency distribution of TNF-α/β polymorphisms in the study population

	Sickle cell patients		**Healthy controls**		***p*-value**
Cytokine gene Polymorphism	Genotypic frequency [N (%)]	Allelic frequency	Genotypic frequency [N (%)]	Allelic frequency	
TNF-α (308G/A)					
GG	00 (0)	G = 0.5	00 (0)	G = 0.5	
GA	115 (100)	A = 0.5	56 (100)	A = 0.5	>0.99
AA	00 (0)		00 (0)		
TNF-β (Intron1+252)					
GG	00 (0)	G = 0.5	00 (0)	G = 0.5	
GA	115 (100)	A = 0.5	56 (100)	A = 0.5	>0.99
AA	00 (0)		00 (0)		

4.5 Association between Cytokine gene polymorphisms with plasma levels of cytokines and Malondialdehyde

To determine whether IL-10 (1082 >G/A) gene polymorphism was associated with changes in IL-10 levels, plasma concentrations of IL-10 were compared across IL-10 (GG wildtype vs GA+AA mutant) genotypic groups in sickle cell disease patients. There was a significant difference (Mann-Whitney test, p= 0.0234) in IL-10 levels across the genotypic groups (Figure 4.8C). Homozygous GG (22.12pg/ml) genotype showed higher median plasma levels of IL-10 as compared to GA+AA (13.94pg/ml) genotypes. Comparisons between IL-10 (1082 GG wildtype vs GA+AA mutant) genotypic groups with plasma levels of malondialdehyde also yielded a significant difference (Mann-Whitney test, p<0.0001) in sickle cell disease patients. Higher levels of malondialdehyde were observed in patients with GG (3.35μM) genotype as compared to those with GA and or AA (2.30μM) genotypes (Figure 4.8D). Comparisons were not made for other cytokine gene polymorphisms like IL-10 (8191/592 >C/T), TNF-α (308 >G/A) and TNF-β (Intron1+252> G/A) because their genotypes lacked the required heterogeneity in the study population (Table 4.2 and 4.3).

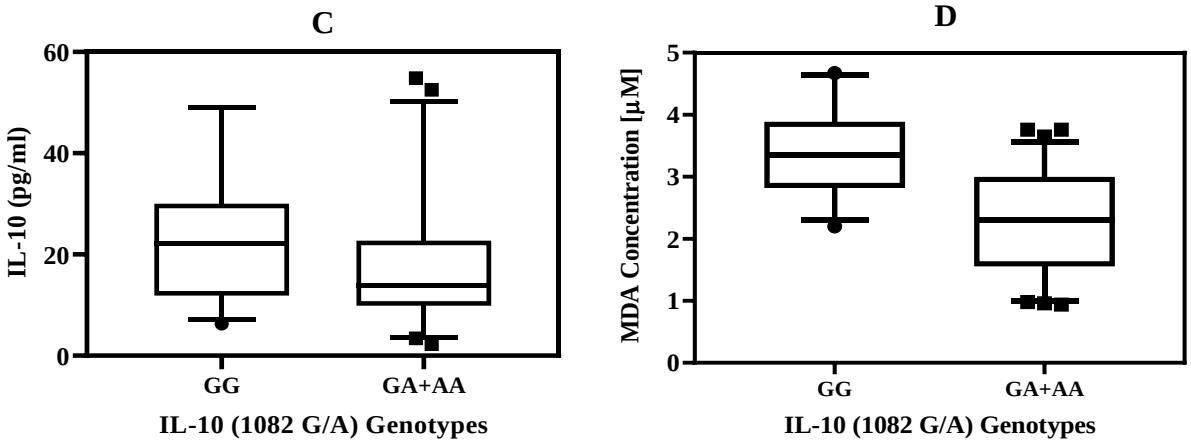

Figure 4. 8: Plasma levels of Malondialdehyde (Plot C) and IL-10 (Plot D) compared across IL-10 (1082 GG wildtype versus GA and AA mutant genotypes in sickle cell disease patients

Boxes indicate median and interquartile range while whiskers are defined as 5 - 95 percentiles.

CHAPTER FIVE

DISCUSSION

Oxidative stress and inflammation contribute significantly to the pathophysiology of sickle cell disease (Chaves *et al.,* 2008). The key sources of oxidant injury in sickle cell disease include increased levels of cell free hemoglobin following chronic hemolysis, mechanisms leading to vaso-occlusion ischemia-reperfusion and inflammation (Conran *et al.,* 2018). Similarly, activation of inflammatory cells and their signalling pathways culminates into the release of molecules that drive the inflammatory state in sickle cell disease such as cytokines, chemokines and growth factors (Conran *et al.,* 2018). Tumor Necrosis Factor alpha is a pro-inflammatory cytokine and as such is said to be generated as an early consequence of ischemia reperfusion (Solovey *et al.,* 2017) with increased levels being detected during pain crisis. While IL- 10 cytokine is known to limit the production of pro-inflammatory cytokines such as TNF-α, its found to be elevated in steady-state SCD patients [without pain] (Musa *et al.*, 2010). Recently, polymorphisms in cytokine genes have also been associated with increased cytokine production and consequently, a heterogeneous clinical outcome of sickle cell disease in patients (stroke, leg ulcers, splenic sequestration and end-organ damage among others) (Belisário *et al.*, 2015; Hoppe *et al.*, 2007). Therefore, our current research aimed at studying the relationship between sickle cell disease pathogenesis and the key modifiers of disease severity like oxidative stress, cytokines levels and cytokine gene polymorphisms in the Ugandan population.

The median plasma levels of malondialdehyde were significantly higher in sickle cell disease patients as compared to healthy controls. These results were consistent with previous studies, reporting increased levels of malondialdehyde in severe sickle cell disease (with pain crises) and steady state patients (without pain crises) as compared to healthy controls (Antwi-Boasiako *et al.*, 2019; Bhagat *et al.*, 2012). However, Walter *et al.,* (2006) reported contradicting results for MDA levels in sickle cell disease patients when compared to healthy controls. The extent of oxidative damage measured as MDA was higher in patients because of doubled generation of reactive oxygen species during intracellular catabolism and from the pathological events of a sickled red blood cell such as increased hemoglobin autoxidation (Hebbel *et al.,* 1988), increased release of cell free hemoglobin (Nagababu *et al.,* 2008) and pro-inflammatory

molecules (Akohoue *et al.*, 2007). The lack of a significant association between plasma levels of MDA with patient's sex and age affirmed that sickle cell disease pathogenesis was the primary source of increased oxidative damage (Nur *et al.*, 2011). Oxidative damage in patients usually results into endothelial dysfunction, vaso-occlusion pain, early organ damage and continuous inflammation (Chaves *et al.*, 2008).

This study also found significantly higher plasma levels of IL-10 in sickle cell disease patients as compared to healthy controls. This finding agreed with previous results that showed increased serum levels of IL-10 in steady state sickle cell disease and its possible inhibition of both humoral and cell mediated immune functions (Musa *et al.*, 2010; Sarray *et al.*, 2015). Contradictory to our results, no significant difference in serum levels of IL-10 between patients and healthy controls was reported in Brazil (Cavalcante *et al.*, 2016; Graido-Gonzalez *et al.*, 1998). Nonetheless, being an anti-inflammatory molecule, IL-10 inhibits the synthesis of TH1cytokines, such as TNF-α, IL-1, IL-6, and IL-8. The sickle cell disease patients included in this current study were asymptomatic cases. Thus, plasma levels of TNF-α were only detected in 2 sickle cell disease patients as in healthy controls. However, Graido-Gonzalez *et al.*, (1998) reported normal levels of TNF-α in patients.

The production of IL-10 can be controlled at transcriptional level and thus the 3 bi-allelic single nucleotide polymorphisms from the transcription start site, at positions 1082 >G/A, 819 >C/T and 592 >C/A have been studied (Moudi *et al.*, 2016). The IL-10 (1082>G/A) genotypic frequency reported in this current study showed no significant difference when compared between sickle cell disease patients and healthy controls. This finding was in agreement with previous studies (Cavalcante *et al.*, 2016; Gilli *et al.*, 2016). However, our sample population showed heterozygous GA as the most frequent genotype for IL-10 (1082>G/A) polymorphism followed by GG. Our results were similar to those for IL-10 (1082>G/A) genotype frequency reported by Munshi *et al*., (2010) and Belisário *et al*., (2015) in patients from India and Brazil, respectively. However, a different trend of AA as the most frequent genotype, followed by GA and GG has been reported for (1082>G/A) polymorphism in Kenya (Ouma *et al.*, 2008; Sultana *et al.*, 2011) and in Brazil (Cavalcante *et al.*, 2016; Gilli *et al.*, 2016),

respectively. The source of variation in findings are not well understood but maybe ascribed to population heterogeneity and sampling methods used.

Similarly, the IL-10 (8191/592 >C/T) genotypic frequency showed no significant difference when compared between sickle cell disease patients and healthy controls. This finding was in agreement with previous studies (Belisário *et al.,* 2015; Cavalcante *et al.,* 2016; Gilli *et al.,* 2016; Ouma *et al.,* 2008). The alleles for 592>C/ 819>C and 592A/ 819>T are inherited together (Moudi *et al.*, 2016) thus, in our current study, the IL-10 (8191/592 >C/T) single nucleotide polymorphisms were characterized together. The most frequent genotype in our sickle cell disease study population was heterozygous CT. The mutant genotype TT was absent in the sampled sickle cell disease population. A similar trend for IL-10 (8191/592 >C/T) genotype frequency was reported by Belisário *et al*., (2015) and Ouma *et al*., (2008) but not Gilli *et al*., (2016). Population heterogeneity and sampling methods used remain the possible sources of variation in results.

Plasma changes in IL-10 levels were significantly different across the IL-10 (1082 >G/A) gene polymorphism with homozygous GG genotype showing higher median levels of IL-10 as compared to GA+AA genotypes in sickle cell disease patients. Consistent with this current study, Lio *et al.*, (2002) and Turner *et al*., (1997) also showed that individuals negative for the mutant allele A at position >1082 for IL-10 had significantly higher IL-10 production as compared to those positive for the A allele. This association was independent of the (8191/592 >C/T) polymorphisms. Alternatively, Ouma *et al.,* (2008) and others also reported that the GCC haplotype was associated with increased IL-10 production unlike the ATA haplotype in patients with severe malaria (Wilson *et al.,* 2005). Enhanced binding of transcriptional factors that promote higher IL-10 production maybe favored by the presence of GCC haplotype unlike the ATA haplotype that may prime enhanced binding sites for repressors and thus reduced IL-10 production (Ouma *et al.*, 2008). As anti-inflammatory molecule, IL-10 inhibits the production of type-1 cytokines that drive the inflammatory state in sickle cell disease, and changes in the cytokine can be used in vaso-occlusion crisis prognosis (Sarray *et al.*, 2015).

The polymorphisms in the pro-inflammatory cytokine genes; TNF-α (308 >G/A) and TNF-β (Intron1+252> G/A) were also analyzed and results showed GA (100%) as the

most frequent and only genotype present in the sample sickle cell disease population. This finding agreed with Sultana *et al.* (2011) who reported GA (88.7%) as the most frequent genotype for TNF-α polymorphism but not with other studies (Cajado *et al.*, 2011; Cavalcante *et al.*, 2016; Gilli *et al.*, 2016; Torres *et al.*, 2013). Similar to our results, (Cavalcante *et al.*, 2016; Gilli *et al.*, 2016) also reported the absence of the AA in the sample sickle cell disease population. Correspondingly, our results for TNF- β (252G >A) polymorphism did not agree with Gosh *et al.*, (2010) who reported AA as the commonest genotype. The GA genotype has been described as an immediate producer while the GG genotype has been described as a low producer of TNF-α (Cavalcante *et al.*, 2016).

Similarly, plasma levels of malondialdehyde were found to be significantly different across IL-10 genotypes with homozygous GG genotype showing higher levels of malondialdehyde as compared to GA+AA genotypes for IL-10 (1082 >G/A) gene polymorphism in sickle cell disease patients. This further emphasises the multifactorial etiology that has been reported for sickle cell disease pathogenesis with different disease modifiers driving disease severity in patients (George *et al*., 2013; Owusu-Ansah *et al*., 2016; Conran *et al.*, 2018). Also, GA genotype an intermediate producer of IL-10 and malondialdehyde was most common in this study population, whether this is indicative of reduced disease severity and longer life span in patients remains unclear due to little research on the inter-relationship between the key modifiers of sickle cell disease.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Collectively, results from this study show that sickle cell disease is associated with increased plasma levels of oxidative stress and IL-10 cytokine. More so, the Genotype GG IL-10 (1082 >G/A) was significantly associated with higher levels of oxidative stress and IL-10 among patients. Also, GA and CT were the most frequent genotypes for the 3 IL-10 (1082 >G/A, 8191/592 >C/T) and TNF-α/β (308 >G/A, Intron1+252> G/A) polymorphisms in the Ugandan population however, the genotypes were not comparable between sickle cell disease patients and healthy controls.

6.2 Limitations

This current study did not consider other key drivers of inflammation and lacked substantial clinical data for complete evaluation of the effect of cytokine gene polymorphisms on cytokine levels and oxidative stress in sickle cell disease.

The study was also limited to genotyping only IL-10 and TNF-α/β gene polymorphisms among so many other cytokine gene polymorphisms that have been associated with sickle cell disease pathogenesis.

6.3 Recommendation

More studies preferably follow up studies with real time clinical data are required to better evaluate the effect of cytokine gene polymorphisms on cytokine levels and consequently sickle disease severity and early death in patients.

Lastly, more studies with a bigger sample size are needed to determine the frequency of other cytokine gene polymorphisms associated with sickle cell disease heterogeneity in the Ugandan population.

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APPENDICES

Appendix I: Ethical Clearance

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COLLEGE OF HEALTH SCIENCES
SCHOOL OF HEALTH SCIENCES
OFFICE OF THE DEAN

19th April 2021

Ms. Cissy Namuleme
Makerere University

Dear Ms. Namuleme,

Re: Approval of waiver of informed consent to use stored blood samples and remnant blood from Nakasero blood bank

In reference to your research protocol titled 'Levels of Oxidative Stress and Frequency of Cytokine Gene Polymorphisms among Sickle Cell Disease Patients in Mulago Hospital, Uganda-Ref No:2021-90', you requested the School of Health Sciences Research and Ethics Committee on the 25th of January 2021 to waive off research participant's informed consent in order to use the stored blood samples previously obtained from sickle cell patients in the previous research projects and as well as remnant blood from the routine screening of transfusion transmissible infections carried out on donated blood from Nakasero blood bank which will be used as control blood samples in the study.

The participants from whom the samples were obtained in the previous studies and those who donated blood to Nakasero blood bank cannot be traced to obtain their informed consent for the proposed study. Also, there shall be no physical contact with the individuals except using their stored blood samples and confidential information about participants except their gender and date of sample collection will be obtained and thus the results from investigator's assays will not be traced back to any of the participants. The remaining blood samples after laboratory assays will be disposed of following the waste disposal guidelines of the laboratory and institution.

The outcome of this study will help us understand the levels of oxidative stress and frequency of cytokine gene polymorphisms among sickle cell disease patients.

On behalf of the committee, I am glad to inform you that the committee granted a waiver of informed consent to use patient's stored samples basing on the reason mentioned above on the 19th of March 2021.

You may proceed with your study after getting approval from the School of Health Sciences Research and Ethics Committee.

MAKERERE UNIVERSITY
SCHOOL OF HEALTH SCIENCES
19 APR 2021
INSTITUTIONAL REVIEW BOARD
P. O. BOX 7072, KAMPALA

Yours sincerely,

Dr. Kalidi Rajab
AG. Chairperson, School of Health Sciences Research and Ethics Committee

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