MAKERERE  **UNIVERSITY**

FREQUENCY OF FACTOR V-LEIDEN, PROTHROMBIN G20210A, AND METHYLENETETRAHYDROFOLATE REDUCTASE MUTATIONS (C677T) AMONG PATIENTS IN MULAGO SICKLE CELL CLINIC

BY

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DECLARATION

I, **OROMA PATRICK**, declare that to the best of my knowledge, this dissertation is my original work and it has never been submitted to any academic institution for an award of any degree

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DEDICATION

I dedicate this work to my loving parents, whose words of encouragement and push for tenacity always rang in my ears. To my loving wife Consolate Ayoo, thank you very much for your unconditional love as well as support throughout the course of this study and not forgetting my son Kumagum Pearson who always put smiles on my face.

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LIST OF ABBREVIATIONS AND ACCRONYMS

ACS	Acute chest syndrome
AS-PCR	Allele specific polymerase chain reaction
CI	Confident Interval
DNA	Deoxyribonucleic acid
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra acetic acid
FVL	Factor V Leiden
HbS	Hemoglobin S
HWE	Hardy-Weinberg Equilibrium
MTHFR	Methylenetetrahydrofolate Reductase
NO	Nitric oxide
OR	Odd ratio
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
SCD	Sickle cell disease
SNP	Single nucleotide polymorphism
TE	Tris-EDTA buffer
VTE	Venous thromboembolism

ABSTRACT

Sickle cell diseases (SCD) is the major cause of death among children under 5 years. Some genetic polymorphisms associated with thrombophilia are known to increase the severity and frequency of this complication in sickle cell patients. This study aims to investigate the frequency of MTHFR C677T, FVL G1691A and Prothrombin G20210A gene polymorphisms among SCD patients and Healthy controls in Uganda. This was a case control study using archived clinical blood samples comprised of 123 sickle cell disease samples and 152 healthy controls samples from Mulago Sickle Cell Clinic. Genotyping was done using allele specific PCR for detection of MTHFR C677T, FVL G1691A, and Prothrombin G20210A gene mutations. The frequency of MTHFR C677T gene polymorphisms was 77.69% (101 of 130) in SCD patients and 56% (70 of 125) in healthy controls (X^2=13.375, DF=2, P=0.001). The CT (heterozygous) genotypes of MTHFR C677T variants was more likely to occur in SCD patients than controls (OR=2.739, 95%CI: 1.57-4.76, P<0.001). In this study, the overall prevalence of Prothrombin G20210A gene mutation was 6.2% (8 of 129) in SCD patients and 2.14% (3 of 140) in healthy controls (X^2=2.832, DF=2, P=0.243). There was no FVL G1691A gene polymorphism in this study population. From this study it can be concluded that, the MTHFR C677T polymorphisms was significantly high among SCD patients than healthy controls. Therefore, it may be a risk factor for severe vascular complications seen in some patients with sickle cell disease.

CHAPTER ONE

INTRODUCTION

1.1 Background

Sickle cell disease (SCD) is an autosomal recessive disorder caused by a replacement of glutamic acid by valine at the sixth position of the beta-globin gene leading to the formation of abnormal hemoglobin S(HbS). The HbS variant comes with many physical and chemical abnormalities. It polymerizes in the absence of oxygen and this subsequently leads to increase in red cell density hence damaging the cell and consequently reducing the life span of red blood cells (Belhaj *et al*., 2018). The dense and rigid red cells lead to obstruction in blood flow and is believed to be the cause of acute pain, acute chest syndrome (ACS), and pulmonary hypertension seen in patients with SCD (Rahimi & Parsian, 2011). Sickle cell disease is characterized by hemolytic anemia, increased susceptibility to infections and vascular obstruction (Nur *et al*., 2011).

Globally, hemoglobinopathies lead to substantial burden of diseases that are not properly addressed (Weatherall, 2010). The greatest burden of SCD is seen in sub-Saharan Africa where over 75% of all SCD occur and this percentage is projected to increase by 2050 (Piel *et al*., 2013). In Africa sickle cell disease leads to increase mortality in children under 5years (Piel *et al*., 2013). The burden of sickle cell disease is very high in Uganda with over 15000 babies born each year with sickle cell conditions and 13.3% of children having sickle cell traits (Ndeezi *et al*., 2016)

Much as sickle cell hemoglobin is due to a mutation in one gene, the disease outcomes varies and polymorphism in associated genes determine the individual difference seen among sickle cell anemia patients (Fawaz *et al.*, 2004). Knowing the role of these polymorphisms in the pathophysiology of SCD may help in proper management of the disease.

Patients with SCD show increased platelet activation, thrombin generation and fibrinolysis during pain episodes. Both platelets and erythrocytes contribute to procoagulant activities promoting generation of thrombin which leads to the development of thrombo-occlusive complication seen in SCD patients. Clinical complication of SCD that are most likely to have a thrombotic component includes; vaso-occlusive painful events, cerebrovascular accidents (stroke), avascular necrosis, priapism, retinopathy and pulmonary infarction. Because of the significance of vascular complications in the pathophysiology of SCD, a number of genetic polymorphisms in different

genes associated with thrombophilia have been studied as potential modifiers of SCD (Nishank *et al*., 2013; Pandey *et al*., 2012).

Genetic risk factor for venous thrombosis includes mutations in the genes that encode antithrombin, protein C and protein S, and the factor V Leiden and factor II G20210 A mutations. Moreover, plasmatic risk indicators, such as hyperhomocysteinemia and elevated concentrations of factors II, VIII, IX, XI and fibrinogen, have also been documented. This extensive list of genetic and acquired factors serves to illustrate that a single cause of venous thrombosis does not exist and that this condition should be considered as a complex or multifactorial trait (Franco & Reitsma, 2001). However, Factor V Leiden G1691A (Mutation in Factor V gene), Prothrombin G20210A (Mutation in Prothrombin gene), and Methylenetetrahydrofolate reductase (MTHFR C677T) mutation has been found to be a major risk factors in the pathogenesis of sickle cell diseases (Bertina *et al*., 1994; Kujovich, 2011; Hanson *et al*., 2001).

FVL G1691A mutation cause Factor V resistance to activated protein C and consequently increasing the risk of deep vein thrombosis (Hirmerova *et al*., 2014), Prothrombin G20210A mutation is associated with increased plasma level of Prothrombin, which lead to an increase in thrombin generation hence increasing the risk of thrombosis (Kangne *et al.*, 2015), and the C677T variant of the MTHFR gene has been correlated with a 50% decrease in the activity of the key enzyme in homocysteine (Hcy) metabolism and consequent increase in plasma Hcy levels, which is known to cause damage to endothelial cells and hence increasing the risk of thrombosis (Hatzlhofer *et al.*, 2012). The Prothrombin G20210A mutation increases the relative risk of venous thrombosis by three-folds (Abdullah *et al*., 2010).

This aim of this study was to determine the prevalence of the FVL G1691A mutation, Prothrombin gene G20210A variant, and MTHFR (Methylenetetrahydrofolate reductase) C677T polymorphisms among patients with sickle cell disease and healthy controls in Mulago Sickle cell clinic.

1.2 Problem statement

The burden of sickle cell disease is very high in Uganda with over 15000 babies born each year with sickle cell disease and 13.3% of children having sickle cell traits (Ndeezi *et al*., 2016). Sickle cell disease presents with many unpredictable complications such as: vaso-occlusive painful events, cerebrovascular accidents (stroke), avascular necrosis, priapism, retinopathy and pulmonary hypertension leading to difficulty in diseases management (Kato *et al*., 2009).

Although sickle hemoglobin is due to a mutation in one gene, the disease manifestation varies and polymorphism in related genes dictates the individual differences seen among sickle cell anemia patients (Fawaz *et al*., 2004). Genetic polymorphisms such as MTHFR C667T, FVL G1691A and Prothrombin G20210A associated with thrombophilia are known potential modifier to SCD manifestation (Pandey *et al*., 2012; Nishank *et al*., 2013)). Unfortunately, there is a general lack of information regarding the occurrence of these genetic polymorphisms among sickle cell patients in Uganda and so it is difficult to predict the likely outcome of sickle cell crises among sufferers of this condition in Uganda. This lack of information also makes it difficult to plan a proper health management scheme for sickle cell disease condition.

1.3 General objective

To establish the occurrence of known genetic polymorphisms associated with thrombophilia in sickle cell disease individuals and healthy controls in Uganda.

1.4 Specific objectives

1. To determine the frequencies of MTHFR C677T, Prothrombin G20210A and FVL G1691A gene polymorphisms among patients with SCD and healthy individuals in Uganda.
2. To determine the association between the occurrence of MTHFR C677T, Prothrombin G20210A and FVL G1691A gene polymorphisms and sickle cell disease.

1.5 Justification

This study will provide information on which of the genetic polymorphism is common among sickle cell disease patients in Uganda

This will provide the basis for identifying which patients is susceptible to severe sickle cell diseases manifestation

CHAPTER TWO

LITERATURE REVIEW

2.1 Epidemiology of sickle cell disease

Sickle cell disease (SCD) is a genetic disorder affecting approximately 5% of the world's population(Queiroz & Lima, 2013; Serjeant & Ndugwa, 2003). In (2008) United Nations estimates that there are between 20 and 25 million people worldwide living with SCD, of which 12–15 million live in Africa. It is estimated that 75–85% of children born with SCD are born in Africa, where mortality rates for those under age 5 range from 50% to 80% (Mulumba & Wilson, 2015). The highest frequency of sickle cell traits in Africa occur between latitude 15^0 North and 20^o South, where the prevalence range between 10% and 40% of the population (Mulumba & Wilson, 2015)

In Uganda, the prevalence is between 4-45%, with the highest percentage coming from areas where malaria is endemic(Okwi *et al*., 2010). The burden of sickle cell disease is very high in Uganda with over 15000 babies born each year with sickle cell condition and 13.3% of children having sickle cell traits (Ndeezi *et al*., 2016).

2.2 Pathophysiology of sickle cell disease

Sickle cell disease is a disorder which is characterized by abnormal, rigid and sickle shape red blood cells. Sickling decreases cell's flexibility and results in a risk of various life-threatening conditions characterized by recurrent vaso-occlusion with consequent body pain, chronic hemolysis and end organ damage(Platt *et al*., 1994; Steinberg, 1998). This characteristic is due to a mutation in the β-globin gene where glutamic acid is replaced by valine at position 6 of the β-globin chain. This subsequently led to reduced solubility of hemoglobin molecules in low oxygen medium resulting in polymers formation which reduced red cells flexibility. (Adewoyin, 2015; Merghani *et al.,* 2015; Yamaja Setty *et al*., 2001).

These biochemical changes result in the two primary pathologies of the disease: Vasooclusion and chronic hemolysis. Chronic hemolysis leads to the depletion of haptoglobin and hemopexin, the scavengers for free hemoglobin and heme, resulting in increased levels of these two molecules with subsequent decreased nitric oxide (NO) bioavailability. Normally, NO relaxes the endothelium and maintain vascular tone (vasodilators). Reduced level of NO lead to vasopasm. This is the underlying basis of vasculopathic complications such as cerebrovascular disease,

priapism, and pulmonary hypotension and leg ulcers seen among sickle cell diseases patients(Adewoyin, 2015; Sparkenbaugh & Pawlinski, 2017).

The SCD is an inherited disease that is autosomal recessive and only persons with homozygous recessive genes of sickle cell (SS homozygotes) will manifest the disease, while the heterozygotes (AS) are carriers (Okwi *et al*., 2010).

The role of inherited thrombophilia in the pathogenesis of sickle cell thromboses has been reported in many studies where the frequency of thrombophilic mutations of FVL, Prothrombin G20210A, and Methylenetetrahydrofolate Reductase (MTHFR) C677T and their association with incidence, and recurrence of thromboembolism among SCD patients have been examined(Hatzlhofer *et al*., 2012; Isma'eel *et al*., 2006; Nishank *et al*., 2013; Pandey *et al*., 2012).

Even though there is genetic similarity at the site of the sickle hemoglobin mutation, this disease does not affect all patients with sickle cell anemia equally. Secondary genetic factors and some other acquired erythrocyte and vascular damage are likely to be the major components of the pathophysiology of sickle cell (Steinberg, 1998).

2.3 Vascular thrombosis

The various clinical heterogeneity of SCD is associated with two main pathogenetic processes; chronic haemolysis and vascular occlusion. Infarctive events in SCD result from erythrostasis caused by rigid sickled cells in numerous vascular beds especially organs with slow blood flow such as the spleen and the bone marrow. Capillaries are about 2-3 microns in diameter. Sickle cells due to loss of flexibility are incapable of travelling through the microvasculature, hence vessel obstruction (Adewoyin, 2015).

Vascular thrombosis is the main pathophysiological feature of sickle cell disease (Samarah & Srour, 2018). Vasculopathy of sickle cell disease has been implicated in the development of pulmonary hypertension, stroke, leg ulceration and priapism and severe hemolysis (Kato *et al*., 2009)

2.4 Genetic Polymorphism associated with thrombophilia in sickle cell disease

Venous thrombosis, whose main clinical presentations include deep vein thrombosis and pulmonary embolism, represents a major health problem worldwide. Numerous conditions are

known to predispose to venous thrombosis and these conditions are commonly referred to as risk indicators or risk factors. Genetic risk factor for venous thrombosis includes mutations in the genes that encode antithrombin, protein C and protein S, and the factor V Leiden and factor II G20210 A mutations. Plasmatic risk indicators, such as hyperhomocysteinemia and elevated concentrations of factors II, VIII, IX, XI and fibrinogen, have also been recognized. This extensive list of genetic and acquired factors serves to illustrate that a single cause of venous thrombosis does not exist and that this condition should be considered as a complex or multifactorial trait (Franco & Reitsma, 2001). However, Factor V Leiden G1691A (Mutation in Factor V gene), Prothrombin G20210A (Mutation in Prothrombin gene), and Methylenetetrahydrofolate reductase (MTHFR C677T) mutation has been found to be a most common risk factors link with thrombosis among sickle cell diseases patients (Bertina *et al*., 1994; Kujovich, 2011; Hanson *et al*., 2001).

2.4.1 MTHFR C677T polymorphism and Sickle cell diseases

MTHFR, an enzyme encoded by the MTHFR gene which is located on short arm of chromosome 1 at position 36 (Goyette *et al*., 1994), catalyzes the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is a co-substrate for homocysteine remethylation (Merghani *et al*., 2015). The C677T variant of the MTHFR gene is known to decrease the activity of the key enzyme in homocysteine metabolism by 50% (Hatzlhofer *et al*., 2012). Elevated level of plasma homocysteine may be caused by a deficiency of folate or by reduced activity of key enzymes in homocysteine-methionine metabolism; 5, 10-methylenetetrahydofolate Reductase (Castro *et al*., 2006).

Elevated plasma total homocysteine concentration is a recognized risk factor for vascular and thrombotic diseases in the general population. Raised homocysteine level have a detrimental effect on endothelial tissues and could contribute to organ damage observed in sickle cell disease individual as early as the first year of life(Al-Saqladi *et al*., 2010).

A meta-analysis has shown different distributions of MTHFR C677T gene polymorphisms among the diverse ethnic groups residing in different geographical part of the world (Yadav *et al.*, 2017). In the study, the worldwide frequencies of T allele and TT genotype were found to be 24.0 % (95 % CI 21.7–26.5) and 7.7 % (95 % CI 6.5–8.9) respectively. In deep analysis, the lowest frequency of T allele was seen among Africans (10.3 %; 95 % CI 3.8–16.8), and the highest was seen among

Europeans (34.1 %; 95 % CI 31.9–36.3). The frequencies of T allele in the North India were 11 %. Generally, the analysis showed that the frequency of MTHFR C677T gene polymorphisms is highest among the Caucasian (Yadav *et al*., 2017). Similarly, a study done in Texas identify the lowest frequency of the MTHFR C677T mutations among African Americans (11.9%), 32.7% in Caucasians, 47.7% in Ashkenazi Jews, and the highest frequency was reported among the Hispanic population (47.9%) (Matalon *et al*., 2002). They concluded that the difference is not only due to environmental factors but also the diversity in the origin and relatedness of various ethnic groups across the world.

Similarly, the allele frequency database from the 1000 genomes project shows low frequency of T alleles for MTHFR C677T gene polymorphisms among Africans (9%), Asian (30%), Europeans (36%) and the highest among Mexicans (47%) (Contreras-Cubas *et al*., 2016)

A study done among the general population in Zambia detected low frequencies of MTHFR C677T gene polymorphisms (8.6%). They concluded that the frequency of MTHFR C677T mutations was generally low in African population (Atadzhanov *et al*., 2014)

In the study conducted by Moreira Neto *et al* (2006) in Brazil, the presence of the MTHFR 677T allele was associated with the occurrence of vascular complications in SCD. However, this association was not significant if each complication is considered independently. Considering all the four complications together, the OR was 4.8 (95% CI: 1.3-17.3, P = 0.020). In conclusion, they suggested that MTHFR C677T genetic polymorphism is perhaps the major risk factor for vascular occlusion among SCD patients (Moreira *et al*., 2006).

A study conducted in Tunisia shows a significant association between Prothrombin G20210A and MTHFR C677T mutations and sickle cell anemia (homozygous Hb S) in Tunisian patients and a significant association between the MTHFR C677T mutation and Hb S/b0-thal. The PRT G20210A mutation was identified in a heterozygous state in all patients with a significantly higher frequency in homozygous Hb S patients (17.14%) than in controls (3.0%) [P=0.009; OR=6.68; 95% CI (1.3–43.12)]. The frequency of the mutant G20210A allele differed significantly between the homozygous Hb S and control groups [P=0.01; OR=6.15; 95% CI (1.26–38.79)]. A high frequency of the MTHFR C677T mutation was seen in sickle cell disease patients (76.56%) compared to healthy subjects (40.0%). The frequency of the MTHFR C677T mutation was significantly higher among SCD patients than in normal subjects (Belhaj *et al*., 2018).

Higher frequency of mutant variants of MTHFR gene (28.0% heterozygotes and 14.6% homozygotes) was found among SCD patients in Central India as compared to normal individuals (Nishank *et al*., 2013). The SCD patients having mutation in the MTHFR and FVL genes showed higher incidence of pain in chest, abdomen and bone joints as well as reliability on blood transfusion than those SCD patients having the non-mutated form of these thrombotic genes. Hence MTHFR C677T and FVL G1691A polymorphisms may be risk factors for increased vascular complications in SCD patient (Nishank *et al*., 2013).

In conclusions, many investigators found high frequency of MTHFR C677T gene polymorphisms among sickle cell disease patients and generally low prevalence of MTHFR C677T gene mutation among Africans. However very few studies regarding MTHFR C677T have been done among Africans(19.53%)(Shafia *et al*., 2018).

2.4.2 Prothrombin G20210A polymorphism (rs1799963) and Sickle cell diseases

The Prothrombin G20210A gene mutation involves substitution of guanine by adenine at nucleotide position 20210 of the Prothrombin gene. The mutation is associated with an increased plasma concentration of Prothrombin, which leads to an increased potential for thrombin generation (Abdullah *et al*., 2010).

DNA sequencing on the Prothrombin gene for patients with unexplained venous thromboembolism (VTE) discovered a single missense mutation (Guanine to Adenine; G-A) at nucleotide position 20210, which is located in the 3′ untranslated region of the Prothrombin gene. This Prothrombin G20210A mutation exist in the noncoding region of Prothrombin gene, and hence it does not affect the actual structure of the Prothrombin molecule and it does not affect its function as a strong clotting factor when activated into thrombin (Franco *et al*., 1999; Poort *et al*., 1996). Prothrombin G20210A mutation has been found to cause high levels of blood Prothrombin (by one-third above normal; 133%), which is more than the extra 15% needed to develop VTE. Prothrombin G20210A gene mutation is known to increased mRNA and protein expression for Prothrombin(Ceelie *et al*., 2004).

High frequency of prothrombin G20210A polymorphism was identified in heterozygous state among sickle cell patients (17.4%) than in controls (3.0%). The difference was very significant with [(p=0.009; OR=6.68; 95% CI (1.26-38.79)]. The proportion of the mutant G20210A allele

differed significantly between the homozygous HbS and healthy controls [(P=0.01; OR=6.15; 95% CI (1.26-38.79)] (Belhaj *et al*., 2018).

A study conducted among Palestinian patients on the distribution of prothrombin G20210A mutations between SCD patients and healthy controls shows no significant difference and hence not associated with SCD (Samarah & Srour, 2018). Study on larger number of patients and controls was suggested in order to define a guideline. Similarly, there was no prothrombin G20210A mutation among Indian sickle cell patients (Pandey *et al*., 2012).

In the general populations, high frequencies of prothrombin G20210A mutation was seen among Hispanic (15%), in Caucasians (9.7%), among Africans (1.1-2.2%) and the lowest was seen among Asian population (0-0.6%) (Jadaon, 2011; Shafia *et al*., 2018).

2.4.3 Factor V –Leiden (G1691A) polymorphism (rs6025) and Sickle cell diseases

Factor V Leiden is a mutated form of human factor V, which is known to causes an increase in blood clotting. The FVL is a point mutation where Guanine is substituted by Adenine at position 1691 in exon 10 of the factor V gene. With this mutation, protein C, an anticoagulant protein is not able to bind normally to factor V, leading to hypercoagulable state(De Stefano & Leone, 1995). FV G1691A is linked with venous and, less frequently, arterial thromboembolic events. The risks are projected to be up to 5-10-folds for heterozygous and 50-100-foldS for homozygous adults respectively (Dahlback, 1997).

A study done in Egypt shows that heterozygous factor V Leiden was significantly higher in the SCD patients (30 %) compared to controls (16 %). Factor V Leiden conferred increased risk of vaso -occlusive crises in SCD patients (OR 1.7, 95 %; CI 1.01–3.43) (Hamdy *et al*., 2013).

In a study conducted among Palestinian patients with sickle cell disease, the presence of Factor V Leiden G1691A polymorphism is highly associated with the occurrence of joints pain, chest and as well as regular dependency on blood transfusion compared to SCD patients with wild type allele. Age and sex adjusted regression analysis revealed a significant association between FVL and sickle cell anemia with an odd ratio (OR) of 5.6 (95% confidence intervals [CI] of 1.91-39.3, P=0.039) in SS patients. The frequency of FVL and its significant association with sickle cell anemia could be a major risk factor for developing occlusive crisis among SCD patients. The

authors suggested a larger number of patients and controls in order to define a specific guideline (Samarah & Srour, 2018).

Rahimi *et al* (2008) found a significant association between Factor V Leiden mutation and sickle cell anemia among Iranians patients, with odd ratios (OR) of 6.5(95% confidence intervals [CI] 1.19-35.33, P=0.03) in SS patients(Rahimi et al., 2008). They suggested more studies to be done on association between venous thrombophilia and Factor V Leiden mutation.

A study conducted among Indians with sickle cell disease shows a significantly high frequency of FVL heterozygous (p=0.02) compared to healthy controls leading to the fact that this polymorphism could be associated with increased risk of thrombosis among SCD patients (Pandey *et al*., 2012).

The frequencies of FVL gene mutation was substantially low among sickle cell patients and controls in Western India, affecting only 3 (2.38%) of the SCD patients and none among healthy controls, hence low risk associated with FVL in the population(Kangne *et al*., 2015). In Eastern Saudi Arabia, there was no difference in the frequencies of FVL between SCD patients and controls (P=0.174, OR=3.81, 95% CI 0.75-14.6) (Fawaz *et al*., 2004).

A review by Shafia et al (2018) shows no FVL gene polymorphisms among African population and the highest frequency was seen among Arabs (52%) and Caucasians.

CHAPTER THREE

MATERIALS AND METHOD

3.1 Study design

This study was case control study and was conducted between July 2020 and April 2021. Archived clinical blood samples which were obtained from Mulago sickle cell clinic from the previous studies were used in this study

3.2 Study site and setting

The hospital is located on Mulago Hill in Northern part of the city of Kampala in Kawempe division, Mulago I parish. It is approximately 5km North East of Kampala's central business area. The analysis of samples was done in the Molecular Biology Research Laboratory located at the Department of Biomolecular resource and Biolab Sciences, College of Veterinary Medicine, Animal Resources and Biosecurity (COVAB), Makerere University.

3.3 Study population

A total of 275 archived clinical blood samples were analyzed in this study. 123 were positive SCD samples and 152 were healthy controls

3.4 Eligibility criteria

3.4.1 Inclusion criteria for patients

Confirmed sickle cell samples and confirm healthy controls after genotyping

3.4.2 Exclusion criteria

Samples which were heterozygous for SCD were excluded from the study

3.5 Sample size determination

The sample size was calculated according to (Israel, 1992) using the equation;

$$n_0 = \frac{Z^2 pq}{e^2}$$

Where n_0 is the sample size, Z^2 is abscissa of the normal curve that cuts off an area α at the tails (a **Z** value of 1.96 for 95% confidence interval is used), ***p*** is the estimated percentage prevalence of SCD in Uganda which is between 4-45%(Serjeant & Ndugwa, 2003). P was taken as 0.1 and ***e,*** the desired level of precision of 0.05.

From the formula, n_0 was calculated as 138. However, 123 patients and 152 healthy individuals were sampled.

3.6 Laboratory methods and procedures

3.6.1 Sample preparation

DNA was extracted from the white buffy coat layer (leukocyte) which were removed from whole blood and stored at -20^{o}C.

3.6.2 DNA extractions procedures

Total genomic DNA was extracted from the stored buffy coat obtained from whole blood during sample preparation. Starting with 500µl of the buffy coat in the 1.5ml Eppendorf tube, 1000µl of red cell lysis buffer was added and shaken gently until the mixture was fully homogenized. The tube was spun for 2 minutes at 7000 revolutions per minute.

The supernatant was discarded and the remaining sediment rinsed with red cell lysis buffer two times in order to obtained a white pellet of white blood cells free from residual hemoglobin. To the pellets in the Eppendorf tube, 400µl of lysis buffer was added followed by 100µl of saturated sodium chloride (5M) and 600µl of chloroform.

The pellet was then mixed fully by pipetting up and down at room temperature and then centrifuged for 2 minutes at 7000 revolutions per minute. From the supernatant, 400µl was transferred to the new 1.5 ml Eppendorf tube followed by addition of 800µl of cold absolute ethanol and then vortexing.

The mixture was spun down for 1 minute at 12000rpm to precipitate out the DNA, the supernatant was then discarded carefully and the tube left to dry at room temperature. DNA was suspended in 50µl of Tris-EDTA buffer (TE buffer) and stored at -20^{o}C until it was used for genotyping.

3.6.3 Genotyping

3.6.3.1 Molecular diagnosis of sickle cell anemia using allele specific PCR

Two sets of primers were used in this study: the wild-type primers amplifying only in the presence of wild type allele and the mutant primers amplifying only in the present of mutant allele. The primers were arranged in a bi-directional orientation in such a way that both primers terminate at the mutation site (Waterfall, 2001). The wild-type primers yield a product size of 517bp (HbAA) while the mutant primers yield a product size of 267bp (HbSS). For the heterozygote

(HbAS/carrier) both amplicons (517bp and 267bp) are produced(Waterfall *et al*., 2009). This method has inherent PCR controls in such a way that at least one allele specific fragment much be yielded per reaction under optimal PCR conditions.

Polymerase chain reaction (PCR) was performed using the primers listed in Table 1 below. PCR was performed in a total volume of 12.5µL reaction mixture comprising of 6.25 µL of Quick-Load Taq 2X Master Mix (New England Biolabs), 5.25 µL PCR water and 1 µL of the template DNA. The thermo cycler was programmed to perform 35cycles using PCR conditions stated in Tables 1 below for determination of sickle cell anemia status (Waterfall *et al*., 2009).

Table 1 Primers and PCR conditions for sickle cell genotyping using Allele Specific PCR

Primers	**Primer Sequence**	**PCR product Size (bp)**	**Reference**
MUT Primers	F: 5'-CAG TAA CGG CAG ACT TCT CCA-3' R: 5'-GGG TTT GAA GTC CAA CTC CTA-3'	267	(Waterfall *et al*., 2001)
WT Primers	F: 5'-ATG GTGCACCTG ACT CCT GA-3' R: 5'-CCC CTT CTT ATG ACA TGA ACT-3'	517	

MUT =Mutant, WT= Wild Type, R=Reverse, F= Forward

3.6.3.2 Genotyping of MTHFR C677T FVL G1691A and Prothrombin G20210A Polymorphisms using allele specific PCR.

The primers used in this study and the reactions conditions are shown in table 2 below. Two matching reactions were performed, one amplifying the wild type alleles and the other amplifying the mutant alleles to determine the single nucleotide polymorphisms. Thermo cycler was programmed to perform 35cycles using PCR conditions stated in tables 2 below(Angelini *et al*., 2002).

Table 2 Primers and PCR conditions for MTHFR, FV, and Prothrombin genes

Primers	Primer Sequence	Product Size (bp)	Reference
MTHFR-RW **MTHFR-RM** **MTHFR-CF**	5'-AAG GAG AAG GTG TCT GCG GGC GC-3' 5'-AAG GAG AAG GTG TCT GCG GGC GT-3' 5'-AAG ATC CCG GGG ACG ATG GGG-3'	127	(Garakanidze *et al*., 2018)
Prothrombin-RW **Prothrombin-RM** **Prothrombin-CF**	5'-CAC TGG GAG CAT TGA GGA TC 5'-CAC TGG GAG CAT TGA GGA TT 5'-TCT AGA AAC AGT TGC CTG GC-3'	340	(Angelini *et al*., 2002)
FVL-RW **FVL-RM** **FVL-CF**	5'-CAG ATC CCT GGA CAG ACG-3' 5'-CAG ATC CCT GGA CAG ACA-3' 5'-TGT TAT CAC ACT GGT GCT TAA-3'	174	
FIX-Forward **FIX-Reverse**	5'-CTC CTG CAG CAT TGA GGG AGA TGG ACA TT-3' 5'-CTC GAA TTC GGC AAG CAT ACT CAA TGT AT-3'	Used as internal controls 250	Ranguelov *et al*., 2002

RW=Reverse wild Primer, RM=Reverse mutant primer, CF=Common Forward Primer, FVL=Factor V Leiden, MTHFR=Methylenetetrahydrofolate Reductase, FIX=Factor IX

3.6.3.3 Visualization of the PCR products by agarose gel electrophoresis

To identify the polymorphisms, the PCR products was separated on a 2% ethidium bromide-stained agarose gel, along with a 50bp DNA ladder, provided by Bioline (USA). An initial voltage

of 50V was used till the DNA moved out of the wells and started migrating and then a voltage of 100V was applied (Gayathri et al., 2016) ;(Nishank *et al.,* 2013; Hamdy *et al.,* 2012). Expected bands for each genotype was identified as shown in table above.

3.6.3.4 Quality Control

Negative control reactions where DNA is replaced- with nuclease free water as well as positive controls was included in all genotyping procedures for quality control purposes. Human factor IX (FIX) was used as an internal control to assess the quality of extracted DNA.

3.7 Data analysis

Data was entered using Microsoft excel version 2013 and analyzed using Stata version 14.0. Difference between the cases (SCD patients) and controls were compared using chi-squared test. The chi-squared test was used to determine if the genotype frequencies were in agreement with those expected by the Hardy-Weinberg equilibrium. The genotype specific risks were estimated as odd ratio (OR) and its 95% CI determined. Haplotypes based case-controls analysis was done using SHEsis online software(http://analysis.bio.x.cn)(Li *et al*., 2009). A P- value of <0.05 was considered statistically significant.

3.8 Ethical Consideration

This study was approved by Makerere University School of Health Sciences Research and Ethics Committee (MAKSHSREC-2021-102). Waiver of informed consent to use stored samples from sickle cell patients were obtained (Appendix I)

CHAPTER FOUR

RESULTS

4.1 Baseline characteristics

This was a case control study consisting of 275 participants of which, 123 (44.7%) were SCD patients and 152 (55.3%) healthy controls. This study was conducted to determine the frequency of MTHFR C677T, Prothrombin G20210A and FVL G1691A gene polymorphisms amongst sickle cell patients and Healthy Individuals at Mulago Sickle Cell Clinic.

Amongst SCD patients, 65(52.8%) were males and 58 (47.2%) were females. The healthy group consist of 74 (51.77%) male and 78 (48.23%) female.

Table 3: Base line characteristics of the study participants

Variable	SCD patients, N (%)	Healthy Controls, N (%)
Sex		
Female	58 (47.2)	78 (48.23)
Male	65(52.8)	74 (51.77)
Age groups		
<5years	31(25.20)	12(7.89)
5-9years	46(37.39)	15(9.87)
10-14 years	28(22.76)	12(7.89)
15-19 years	11(8.94)	14(9.21)
20-24 years	2(1.63)	15(9.87)
>25 years	5 (4.07)	84 (55.26)

SCD=sickle cell disease

4.2. PCR results for Sickle cell genotyping

One hundred twenty-three samples were positive for HbSS identifying them as sicklers and one hundred fifty-two controls were identified as having the normal (HbAA) hemoglobin.

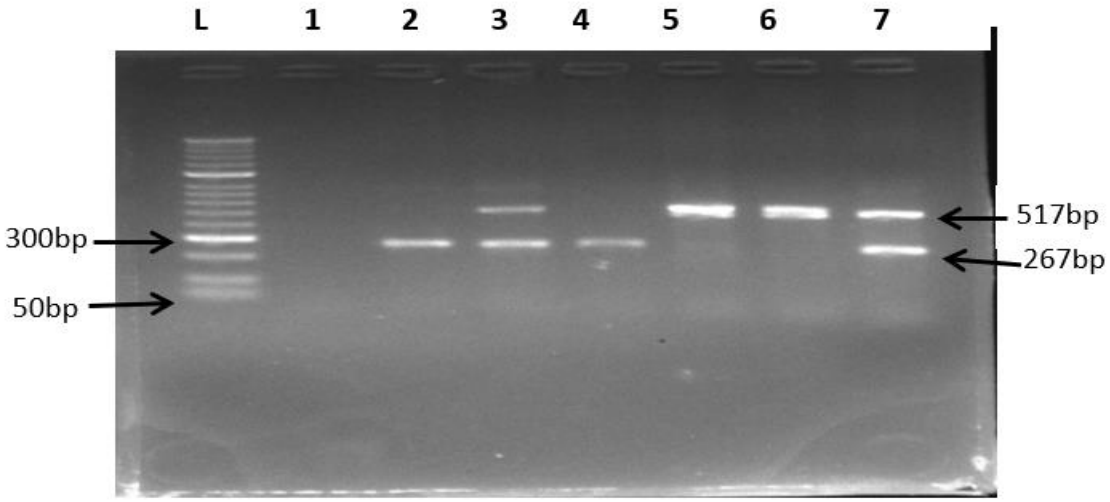

Figure 1: A 2% representative gel for sickle cell diagnosis using allele specific PCR.

Lane L is the 50bp molecular ladder, Lane 1-Negative control, Lanes 2 and 4 are samples with HbSS (sickle hemoglobin), Lanes 3 and 7 are those with HbAS (carriers), Lanes 5 and 6 are those with HbAA (Normal Hemoglobin).

4.3(a) Prevalence of MTHFR C677T gene Polymorphisms

The MTHFR C677T gene polymorphisms were determined by allele specific PCR (Figure 2). The frequency of MTHFR C677T gene mutations is depicted in table 4 below. Among the SCD patients, 9 (7.8%) patients were homozygous (TT) for MTHFR C677T mutation and 78 (67.8%) patients were heterozygous (CT) for the mutation. While among the controls, 7(5.6%) individuals were homozygous (TT) for MTHFR C677T gene mutation and 63(50.4%) were heterozygous (CT) for the mutation. Of the 275 samples, 38 failed PCR amplifications for MTHFR C677T gene polymorphisms detections

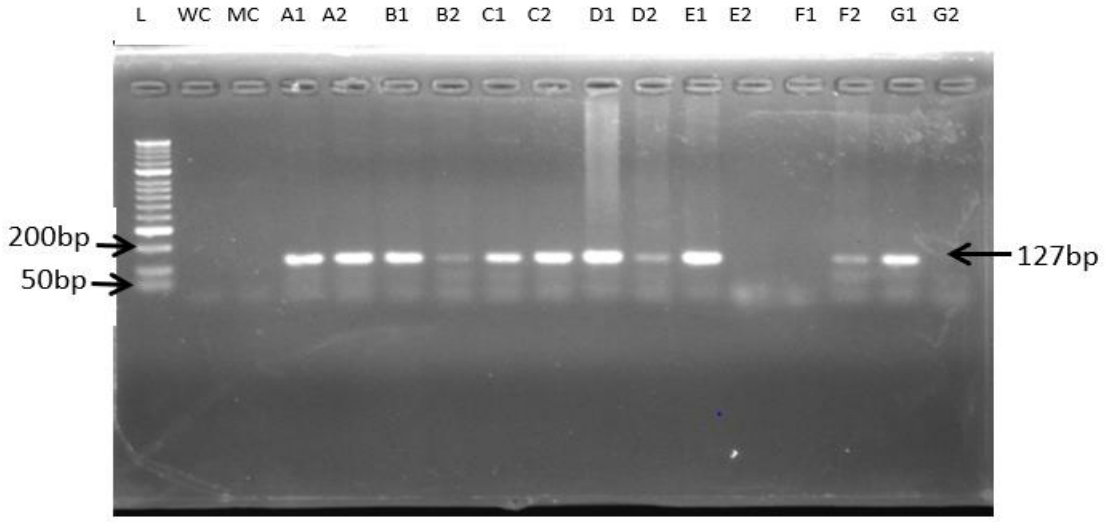

Figure 2: A 2% representative gel for the MTHFR C677T gene polymorphisms
Lane-L represents a 50bp molecular ladder, WC and MC are negative controls, Lanes A1A2, B1B2, C1C2 and D1D2 are heterozygous Individuals (CT) for MTHFR C677T mutations, Lanes E1E2 and G1G2 are normal homozygous dominants (CC) and Lane F1F2 represent Individual who are homozygous recessive (TT) for MTHFR C677T mutations.

To investigate whether there is a difference between the frequency of MTHFR C677T gene mutations among SCD patients and controls, a chi-square test was conducted. All the assumptions were met. Table 4 below shows the Pearson chi-square results and indicate that there is a significant different in the frequency of MTHFR C677T gene polymorphisms between SCD patients and controls (X^2=10.23, DF=2, P=0.006). There were high frequencies of MTHFR C677T mutation in sickle cell disease patients 87(75.6%) compared to normal individuals 70 (56%).

To test for Hardy-Weinberg equilibrium, a chi-square test was conducted. The Pearson chi-square results is shown in table 4 below and it indicate that our observations were significantly different from our expectations, assuming Hardy-Weinberg equilibrium both in Cases and Controls respectively (X^2=17.905, df=1, P<0.0001; X^2=4.2, df=1, P=0.04).

Table 4: Table showing the alleles and genotype frequencies for MTHFR C677T gene polymorphisms in cases and controls

	MTHRFR C677T	Cases (SCD Patients) (freq)	Controls (freq)	X^2	P-value
Genotypes	CC	28 (0.243)	55 (0.440)	10.23	0.006
	CT	78 (0.678)	63 (0.504)		
	TT	9 (0.078)	7 (0.056)		
Alleles	C	134(0.583)	173(0.692)	6.2	0.012
	T	96 (0.4170	77 (0.308)		

SCD=Sickle cell disease, HWE=Hardy Weinberg equilibrium, X^2=Chi-square, C=wild allele, T=Mutant allele

Hardy-Weinberg equilibrium test for the case: X^2=17.905, df=1, P<0.001

Hardy-Weinberg equilibrium test for the control: X^2=4.2, df=1, P=0.04

4.3 (b). The occurrences of MTHFR C677T mutations in SCD patients and controls

Logistic regression was used to determine the likelihood of MTHFR C677T mutations between case and controls as indicated in table 5 below. The CT genotypes for MTHFR C677T polymorphisms were 2.7 times more likely to occur in SCD patients than the controls. This was statistically significant (OR=2.739, 95%CI: 1.57-4.76, P<0.001). **currences of MTHFR C677T genotypes in SCD patients and Controls**

Variable					
	Control	SCD	OR	95% CI	P-value
MTHFR C677T					
CC	55(44.0)	28(24.3)	1.00	-	-
CT	63(50.40)	78(67.8)	2.639	1.476-4.261	<0.001
TT	7(5.60)	9(7.8)	2.509	0.833-7.563	0.067

Abbreviation: MTHFR= Methylenetetrahydrofolate reductase, CC-homozygous normal, CT-heterozygous, TT-homozygous mutant, OR=odd ratio, SCD=sickle cell disease, CI=confident intervals.

4.4(a) Prevalence of Prothrombin G20210A mutation

The Prothrombin G20210A mutation was determined by allele specific PCR (Figure 3). The frequency of homozygous (AA) genotypes and heterozygous (GA) genotypes among SCD patients were 2 (1.8%) and 5 (4.4%) respectively. Meanwhile in controls, the frequency of homozygous (AA) genotypes and heterozygous genotypes were 1(0.71%) and 2(1.43%) respectively as indicated in table 6 below. To test for difference in frequency among SCD patients and controls, a chi-square test was conducted. And the frequency of Prothrombin G20210A mutations were not statistically different between sickle cell patients and controls (X^2=2.72, DF=2, P=0.256).

Table 6 below shows the allele and genotype frequencies of Prothrombin G20210A gene polymorphisms between SCD patients and the controls. To test for Hardy-Weinberg equilibrium, a chi-square test was conducted. The Pearson chi-square results is shown in table 6 below and it indicate that the observed genotypes frequencies were significantly different from the expected frequencies, assuming Hardy-Weinberg equilibrium both Cases and Controls respectively (X^2=20.067, df=1, P<0.0001; X^2=34, df=1, P<0.0001).

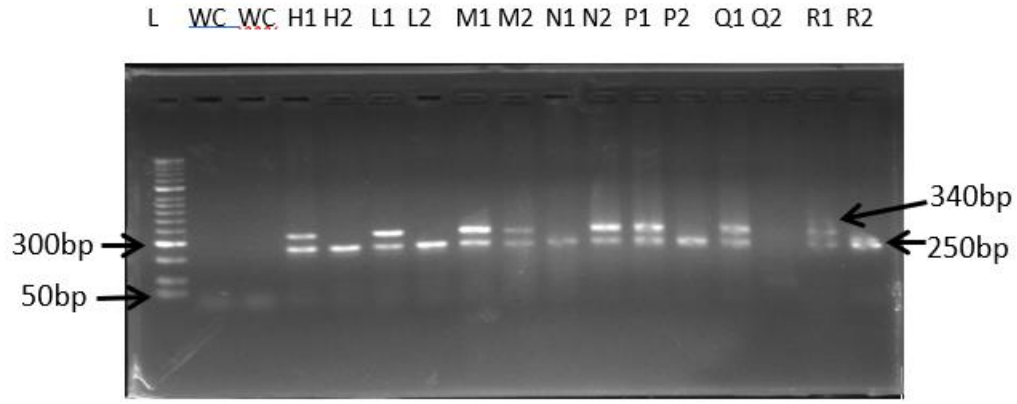

Figure 3: A 2% representative gel for Prothrombin G20210A gene polymorphisms

L- 50bp DNA ladder, WC and Mc are negative controls, (H1H2, L1L2, P1P2, R1R2) are normal homozygous individuals (GG), M1M2 represent heterozygous individual (GA) and N1N2 represent homozygous mutant individual **(AA).**

Table 6: Table showing the alleles and genotypes frequencies for Prothrombin G20210A gene polymorphisms in cases and controls.

	Prothrombin G20210A	Cases (SCD Patients) (freq)	Controls (freq)	X^2	P-value
Genotypes	GG	106 (0.938)	137(0.979)	2.72	0.256
	GA	5 (0.044)	2 (0.014)		
	AA	2 (0.018)	1 (0.007)		
Alleles	G	217(0.960)	276(0.986)	3.3	0.07
	A	9 (0.04)	4(0.014)		

SCD=Sickle cell disease, HWE=Hardy Weinberg equilibrium, X^2=Chi-square, G=wild allele, A=Mutant allele

Hardy-Weinberg equilibrium test for the case: X^2=20.067, df=1, P<0.0001

Hardy-Weinberg equilibrium test for the control: X^2=34, df=1, P<0.0001

4.4 (b). The occurrences of Prothrombin G20210A mutations in SCD patients and controls

Logistic regression was used to determine the likelihood of Prothrombin mutations among SCD patients as indicated in table 7 below. There was no difference in the frequency of GA and AA genotypes between sickle cell patients and controls as depicted in the table 7 below.

Table 7: The Occurrences of Prothrombin G20210A genotypes in SCD patients and

Variable					
	Controls	SCD	OR	95% CI	P-value
Prothrombin G20210A					
GG	137(97.86)	121(93.80)	1.00	-	-
GA	2(1.43)	5(3.88)	2.83	0.539-14.85	0.219
AA	1(0.71)	3(2.33)	3.39	0.348-33.087	0.292

SCD=Sickle cell disease, OR=odd ratio, CI=Confident Intervals

4.5 Prevalence of FVL G1691A mutation

The frequency of FVL G1691A gene polymorphisms was determined by allele specific PCR (Figure 4 below). There was no FVL G1691A gene mutations identified among SCD patients and the controls group. All the participants was having the wild type genotypes (GG) as shown in table 8 below. Due to low frequency seen, the relationship between these gene polymorphism and other factors was not considered.

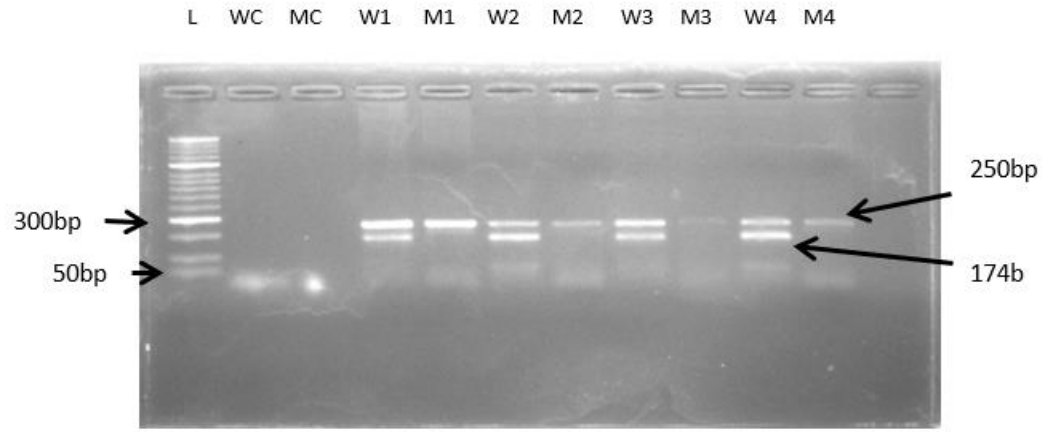

Figure 4: A 2% representative gel for FVL G1691A polymorphisms

L-50bp DNA ladder; WC and Mc are negative controls; W1M1, W2M2, W3M3 and W4M4 are normal homozygous (GG) genotypes for FVL G1691A.

Table 8: Frequency of FVL G1691A genotypes

Variable					
	Controls	SCD	OR	95% CI	P-value
FVL G1691A					
Genotypes					
GG	134(49.26)	120(50.74)	1.00	-	-
GA	0(0)	0(0)	-	-	-
AA	0(0)	0(0)	-	-	-

SCD=sickle cell disease, CI=confident intervals, FVL=factor-V- Leiden, OR=odd ratio

4.6. Haplotype analysis of MTHFR C677T, Prothrombin G20210A and FVL G1691A gene polymorphisms.

Haplotype analysis was done using SHEsis software. As shown in table below, the haplotype CGG and TGA was significantly high among the cases than in healthy controls (OR=0.657, 95%CI: 0.435-0.992, P=0.045 & OR=1.5, 95%CI: 1.01-2.99, P=0.045 respectively).

Table 9: The distribution of haplotypes between Cases (SCD patients) and healthy controls.

Haplotypes			Case(freq)	Control(freq)	OR (95%CI)	p-values
C	G	A	5.82(0.028)	3.99(0.020)	-	-
C	G	G	115.18(0.6)	135(0.66)	0.657(0.435-0.992)	0.045
T	G	G	3.18(0.015)	0.01(0.00)	-	-
T	G	A	81.82(0.4)	62.99(0.312)	1.5(1.01-2.99)	0.045

Global haplotypes associations X^2=4.02, df=1, P=0.045002(Pearson's)

Haplotypes were arranged in the order of MTHFR C677T, FVL G1691A, Prothrombin G20210A respectively

Note: All those haplotypes with frequency<0.05 was ignored in the analysis

CHAPTER FIVE

DISCUSSION

The aim of the study was to determine the frequency of MTHFR C677T, Prothrombin G20210A, and FVL G1691A gene polymorphisms among sickle cell patients and healthy controls in Uganda. And to further understand the relation of these genetic polymorphisms with sickle cell disease. In the study, MTHFR C677T genetic polymorphisms appear to be associated with sickle cell disease. This is shown by increased frequency of the CT and TT genotypes among sickle cell disease patients. The MTHFR C677T mutation was found among 77.69% of sickle cell disease patients and 56.0% in controls groups. The occurrences of CT genotype for MTHFR C677T variants was more likely in SCD patients than controls (P<0.001; OR 2.739; 95%CI, 1.576-4.761). This was the first study on MTHFR C677T gene mutation in Uganda and this finding was comparable to study done by Belhaj *et al* (2018) in Tunisia, which identify high frequencies of MTHFR C677T mutations among sickle cell patients (76.56%) than in healthy controls (40.0%) groups (Belhaj *et al*., 2018). The high frequencies of MTHFR C677T gene mutations in this study could affect not only people with SCD but also other diseases associated with thrombosis such as cardiovascular diseases. MTHFR C677T mutation impair MTHFR functions leading subsequent rise in homocysteine levels and hence increasing the risk of vascular complications(Hatzlhofer *et al*., 2012). Much as homocysteine levels was not investigated in this study, Al-Saqladi *et al* found that raised homocysteine level causes detrimental effects on endothelial tissues and consequently resulting into atherosclerosis which is the main cause of organ failure seen in SCD patients ((Al-Saqladi *et al*., 2010).

In contrast to this study, the allele frequency database from the 1000 genomes project shows low frequency of T alleles among Africans (9%), Asian (30%), Europeans (36%) and the highest among Mexicans (47%)(Contreras-Cubas *et al*., 2016). Worldwide, a meta-analysis study shows that the lowest frequency of T alleles was found among Africans (10.3%), 19.7% in Asians, 20.5% in Australians, 27.8% in South Americans, 31.2% in North Americans, and the high was found among Europeans (34.1%). Likewise, the frequency of TT genotypes was low among Africans 2.4%, followed by Asians 5.5%, and high among Americans and Europeans (11.9% and 11.6%) respectively (Yadav *et al.*, 2017). Therefore, the frequency of MTHFR C677T mutations is low among Africans and high in Europeans. However, the present study reported high frequencies of

MTHFR C677T gene mutations and the difference could be due to some environmental genetic factors which was not investigated in this study and perhaps increasing the sample size in the subsequent study is important to justify the increment of MTHFR C677T in this population.

This study has found low frequency of Prothrombin G20210A polymorphisms between the SCD patients (6.21%) and controls (2.14%). This result was in comparison with a study done in Eastern Saudi Arabia where no significant difference was observed between patients and controls on the prevalence of Prothrombin G20210A (P=0.397) (Fawaz *et al*., 2004). Globally other related study indicate that the lowest frequency of Prothrombin is seen among Asians (0-0.6%), followed by Africans (1.1-2.2%), Caucasians (9.7%), and 15% among Hispanic populations (Jadaon, 2011; Shafia *et al.*, 2018). A similar study done among Palestinians found 6.83% overall prevalence of Prothrombin G20210A which is comparable to this study and the distributions of this mutations between SCD patients and controls was not significantly different (Samarah & Srour, 2018). Even though the frequency of Prothrombin G20210A mutations are very low in this study, individuals with this mutation are at a very high risks of developing thrombotic complications and will therefore need proper health management.

There was completely no FVL G1691A gene mutations in the present study. This result was in comparison with a study done in Eastern Saudi Arabia where no significant difference was observed between patients and controls on the prevalence of FVL G1691A (P= 0.174) (Fawaz *et al*., 2004). A review done by Shafia *et al* (2018) on the prevalence of FVL G1691A among different populations was in agreement with this study. They reported no FVL mutation among Africans and the highest prevalence was seen among Arab (52%), followed by 19.53% in Caucasians (Shafia *et al.*, 2018). In contrast to the present study, Hamdy *et al* (2013) found significantly high frequencies of FVL gene mutation among Egyptian sickle cell patients (30%) than in healthy controls (16%)(Hamdy *et al*., 2013). This suggest that, FVL must have originated among people of Arab origins. Therefore, geographical locations and ethnicity may have a big impact on the distributions of FVL G1691A gene polymorphisms and therefore making it less significant in Ugandan population.

The present study provides a landmark for more investigation on effect of genetic polymorphisms associated with thrombophilia in our population. Much as this study found no significant difference on the frequencies of FVL and Prothrombin G20210A among sickle cell patients and healthy controls, this could be due to small sample size in the study attributed to lack of fund. Therefore, larger samples size in the subsequent studies is needed in order to confirm or rule out the contributions of MTHFR C677T, Prothrombin G20210A and Factor V Leiden in the pathogenesis of sickle cell disease. Despite these limitations, this study provides the first information on the frequency of thrombophilia associated gene polymorphisms in the country.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

- From this study, it can be concluded that MTHFR C677T gene polymorphisms is more common among SCD patients than healthy control. This is evidence by high frequency of MTHFR C677T gene among SCD patients
- The allele frequency of Prothrombin G20210A mutations was very low in this study populations and its distribution between SCD patients and controls was not significant.

6.2 RECOMMENDATIONS

- There is need to do more screening for MTHFR C677T and Prothrombin G20210A mutations among the high-risk individual in order to guide management of thrombotic complications in the affected individuals since this was a pilot study and the coverage was small
- More studies involving larger sample sizes and among people of different ethnic background is necessary in order to define specific guidelines this is because our samples size was small due to limited fund

REFERENCE

Abdullah, W. Z., Kumaraguru, S., Ghazali, S., & Yusoff, N. M. (2010). Factor V Leiden and Prothrombin G20210A Mutations Among Healthy Indians in Malaysia: Table 1. *Laboratory Medicine*, *41*(5), 284–287. https://doi.org/10.1309/LM9W9L8GQPCZVAYO

Adewoyin, A. S. (2015). Management of sickle cell disease: A review for physician education in Nigeria (Sub-Saharan Africa). *Anemia*, *2015*. https://doi.org/10.1155/2015/791498

Al-Saqladi, A. W. M., Harper, G., Delpisheh, A., Fijnvandraat, K., Bin-Gadeem, H. A., & Brabin, B. J. (2010). Frequency of the MTHFR C677T polymorphism in Yemeni children with sickle cell disease. *Hemoglobin*, *34*(1), 67–77. https://doi.org/10.3109/09687630903554111

Angelini, A., Di Febbo, C., Rullo, A., Di Ilio, C., Cuccurullo, F., & Porreca, E. (2002). New method for the extraction of DNA from white blood cells for the detection of common genetic variants associated with thrombophilia. *Pathophysiology of Haemostasis and Thrombosis*, *32*(4), 180–183. https://doi.org/10.1159/000070424

Atadzhanov, M., Mwaba, M. H., Mukomena, P. N., Lakhi, S., Mwaba, P., Rayaprolu, S., Meschia, J. F., & Ross, O. A. (2014). Frequency of APOE, MTHFR and ACE polymorphisms in the Zambian population. *BMC Research Notes*, *7*(1), 1–10. https://doi.org/10.1186/1756-0500-7-194

Belhaj Nefissi, R., Doggui, R., Ouali, F., Messaoud, T., & Gritli, N. (2018). Factor V Leiden G1691A, Prothrombin G20210A, and MTHFR C677T and A1298C Mutations in Patients With Sickle Cell Disease in Tunisia. *Hemoglobin*, *42*(2), 96–102. https://doi.org/10.1080/03630269.2018.1451340

Bertina, R. M., Koeleman, B. P. C., Koster, T., Rosendaal, F. R., Dirven, R. J., De Ronde, H., Van Der Velden, P. A., & Reitsma, P. H. (1994). Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature*. https://doi.org/10.1038/369064a0

Castro, R., Rivera, I., Blom, H. J., Jakobs, C., & Tavares de Almeida, I. (2006). Homocysteine metabolism, hyperhomocysteinaemia and vascular disease: An overview. In *Journal of Inherited Metabolic Disease*. https://doi.org/10.1007/s10545-006-0106-5

Ceelie, H., Spaargaren-van Riel, C. C., Bertina, R. M., & Vos, H. L. (2004). G20210A is a functional mutation in the prothrombin gene; effect on protein levels ans 3′-end formation. *Journal of Thrombosis and Haemostasis*. https://doi.org/10.1111/j.1538-7836.2003.00493.x

Contreras-Cubas, C., Sánchez-Hernández, B. E., García-Ortiz, H., Martínez-Hernández, A., Barajas-Olmos, F., Cid, M., Mendoza-Caamal, E. C., Centeno-Cruz, F., Ortiz-Cruz, G., Jiménez-López, J. C., Córdova, E. J., Salas-Bautista, E. G., Saldaña-Alvarez, Y., Fernández-López, J. C., Mutchinick, O. M., & Orozco, L. (2016). Heterogenous distribution of MTHFR gene variants among mestizos and diverse amerindian groups from Mexico. *PLoS ONE*, *11*(9), 1–13. https://doi.org/10.1371/journal.pone.0163248

D. Hatzlhofer, B. L., Bezerra, M. A. C., Santos, M. N. N., Albuquerque, D. M., Freitas, E. M., Costa, F. F., Araújo, A. S., & Muniz, M. T. C. (2012). *MTHFR* Polymorphic Variant C677T

Is Associated to Vascular Complications in Sickle-Cell Disease. *Genetic Testing and Molecular Biomarkers*, *16*(9), 1038–1043. https://doi.org/10.1089/gtmb.2011.0361

Dahlback, B. (1997). Resistance to activated protein C as risk factor for thrombosis: molecular mechanisms, laboratory investigation, and clinical management. *Semin.Hematol.* https://doi.org/10.1002/uog.14939

De Stefano, V., & Leone, G. (1995). Resistance to activated protein C due to mutated factor V as a novel cause of inherited thrombophilia. *Haematologica*, *80*(4), 344–356.

Fawaz, N. A., Bashawery, L., Al-Sheikh, I., Qatari, A., Al-Othman, S. S., & Almawi, W. Y. (2004). Factor V-Leiden, prothrombin G20210A, and MTHFR C677T mutations among patients with sickle cell disease in Eastern Saudi Arabia. *American Journal of Hematology*, *76*(3), 307–309. https://doi.org/10.1002/ajh.20087

Franco, R. F., Trip, M. D., Ten Cate, H., Van den Ende, A., Prins, M. H., Kastelein, J. J. P., & Reitsma, P. H. (1999). The 20210 G → A mutation in the 3'-untranslated region of the prothrombin gene and the risk for arterial thrombotic disease. *British Journal of Haematology*. https://doi.org/10.1046/j.1365-2141.1999.01149.x

Franco, R. F., & Reitsma, P. H. (2001). Genetic risk factors of venous thrombosis. *Human Genetics*, *109*(4), 369–384. https://doi.org/10.1007/s004390100593

Garakanidze, S., Costa, E., Bronze-Rocha, E., Santos-Silva, A., Nikolaishvili, G., Nakashidze, I., Kakauridze, N., Glonti, S., Khukhunaishvili, R., Koridze, M., & Ahmad, S. (2018). Methylenetetrahydrofolate Reductase Gene Polymorphism (C677T) as a Risk Factor for Arterial Thrombosis in Georgian Patients. *Clinical and Applied Thrombosis/Hemostasis*, *24*(7), 1061–1066. https://doi.org/10.1177/1076029618757345

Hamdy, M. S. E. D., Gouda, H. M., Shaheen, I. A. M., Khorshied, M. M., & Tomerak, R. H. (2013). Prevalence of factor V Leiden (G1619A) and prothrombin gene (G20210A) mutation in Egyptian children with sickle cell disease. *Comparative Clinical Pathology*, *22*(4), 697–702. https://doi.org/10.1007/s00580-012-1469-7

Hanson, N. Q., Aras, O., Yang, F., & Tsai, M. Y. (2001). C677T and A1298C polymorphisms of the methylenetetrahydrofolate reductase gene: Incidence and effect of combined genotypes on plasma fasting and post-methionine load homocysteine in vascular disease. *Clinical Chemistry*. https://doi.org/10.1093/clinchem/47.4.661

Hatzlhofer, B. L. D., Bezerra, M. A. C., Santos, M. N. N., Albuquerque, D. M., Freitas, E. M., Costa, F. F., Araújo, A. S., & Muniz, M. T. C. (2012). MTHFR polymorphic variant C677T is associated to vascular complications in sickle-cell disease. *Genetic Testing and Molecular Biomarkers*. https://doi.org/10.1089/gtmb.2011.0361

Hirmerova, J., Seidlerova, J., & Subrt, I. (2014). The association of factor V Leiden with various clinical patterns of venous thromboembolism-the factor V Leiden paradox. *Qjm*, *107*(9), 715–720. https://doi.org/10.1093/qjmed/hcu055

Isma'eel, H., Arnaout, M. S., Shamseddeen, W., Mahfouz, R., Zeineh, N., Jradi, O., & Taher, A.

(2006). Screening for inherited thrombophilia might be warranted among Eastern Mediterranean sickle-βeta-0 thalassemia patients. *Journal of Thrombosis and Thrombolysis*, *22*(2), 121–123. https://doi.org/10.1007/s11239-006-8953-3

Jadaon, M. M. (2011). Epidemiology of activated protein C resistance and Factor V Leiden mutation in the Mediterranean region. *Mediterranean Journal of Hematology and Infectious Diseases*, *3*(1), 1–11. https://doi.org/10.4084/MJHID.2011.037

Kangne, H. K., Jijina, F. F., Italia, Y. M., Jain, D. L., Nadkarni, A. H., Ghosh, K. K., & Colah, R. B. (2015a). The prevalence of factor V Leiden (G1691A) and methylenetetrahydrofolate reductase C677T mutations in sickle cell disease in Western India. *Clinical and Applied Thrombosis/Hemostasis*, *21*(2), 186–189. https://doi.org/10.1177/1076029613495308

Kato, G. J., Hebbel, R. P., Steinberg, M. H., & Gladwin, M. T. (2009). Vasculopathy in sickle cell disease: Biology, pathophysiology, genetics, translational medicine, and new research directions. *American Journal of Hematology*. https://doi.org/10.1002/ajh.21475

Kujovich, J. L. (2011). Factor v Leiden thrombophilia. In *Genetics in Medicine*. https://doi.org/10.1097/GIM.0b013e3181faa0f2

Li, Z., Zhang, Z., He, Z., Tang, W., Li, T., Zeng, Z., He, L., & Shi, Y. (2009). A partition-ligation-combination-subdivision em algorithm for haplotype inference with multiallelic markers: Update of the SHEsis (http://analysis.bio- x.cn). *Cell Research*. https://doi.org/10.1038/cr.2009.33

Matalon, R. K., Tyring, S. K., Szucs, S., Rady, P. L., Grady, J., Hudnall, S. D., Nitowsky, H., & Kellner, L. H. (2002). Genetic polymorphisms of methylenetetrahydrofolate reductase (MTHFR) and methionine synthase reductase (MTRR) in ethnic populations in Texas; a report of a novel MTHFR polymorphic site, G1793A. *American Journal of Medical Genetics*. https://doi.org/10.1002/ajmg.10122

Merghani, M., Mahmoud, R. M. O., Hamid, I. M. M., & Abdalla, M. H. A. (2015). *research article methylenetetrahydrofolate reductase (mthfr c677t) polymorphism in sudanese patients with sickle cell anaemia 1 Ghada*. *3*, 3–6.

Moreira Neto, F., Lourenço, D. M., Noguti, M. A. E., Morelli, V. M., Gil, I. C. P., Beltrão, A. C. S., & Figueiredo, M. S. (2006). The clinical impact of MTHFR polymorphism on the vascular complications of sickle cell disease. *Brazilian Journal of Medical and Biological Research*, *39*(10), 1291–1295. https://doi.org/10.1590/S0100-879X2006001000004

Mulumba, L. L., & Wilson, L. (2015). Sickle cell disease among children in Africa: An integrative literature review and global recommendations. *International Journal of Africa Nursing Sciences*, *3*(2015), 56–64. https://doi.org/10.1016/j.ijans.2015.08.002

Ndeezi, G., Kiyaga, C., Hernandez, A. G., Munube, D., Howard, T. A., Ssewanyana, I., Nsungwa, J., Kiguli, S., Ndugwa, C. M., Ware, R. E., & Aceng, J. R. (2016). Burden of sickle cell trait and disease in the Uganda Sickle Surveillance Study (US3): A cross-sectional study. *The Lancet Global Health*, *4*(3), e195–e200. https://doi.org/10.1016/S2214-

109X(15)00288-0

Nishank, S. S., Singh, M. P. S. S., & Yadav, R. (2013). Clinical impact of factor V Leiden, prothrombin G20210A, and MTHFR C677T mutations among sickle cell disease patients of Central India. *European Journal of Haematology*, *91*(5), 462–466. https://doi.org/10.1111/ejh.12190

Nur, E., Biemond, B. J., Otten, H. M., Brandjes, D. P., & Schnog, J. J. B. (2011). Oxidative stress in sickle cell disease; pathophysiology and potential implications for disease management. In *American Journal of Hematology*. https://doi.org/10.1002/ajh.22012

Okwi, A. L., Byarugaba, W., Ndugwa, C. M., Parkes, A., Ocaido, M., & Tumwine, J. K. (2010). An up-date on the prevalence of sickle cell trait in Eastern and Western Uganda. *BMC Blood Disorders*. https://doi.org/10.1186/1471-2326-10-5

Pandey, S. K., Meena, A., Kishor, K., Mishra, R. M., Pandey, S., & Saxena, R. (2012). Prevalence of factor v Leiden G1691A, MTHFR C677T, and prothrombin G20210A among Asian Indian sickle cell patients. *Clinical and Applied Thrombosis/Hemostasis*, *18*(3), 320–323. https://doi.org/10.1177/1076029611425830

Piel, F. B., Hay, S. I., Gupta, S., Weatherall, D. J., & Williams, T. N. (2013). Global Burden of Sickle Cell Anaemia in Children under Five, 2010-2050: Modelling Based on Demographics, Excess Mortality, and Interventions. *PLoS Medicine*. https://doi.org/10.1371/journal.pmed.1001484

Platt, O. S., Brambilla, D. J., Rosse, W. F., Milner, P. F., Castro, O., Steinberg, M. H., & Klug, P. P. (1994). Mortality In Sickle Cell Disease -- Life Expectancy and Risk Factors for Early Death. *New England Journal of Medicine*. https://doi.org/10.1056/NEJM199406093302303

Poort, S. R., Rosendaal, F. R., Reitsma, P. H., & Bertina, R. M. (1996). A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood*. https://doi.org/10.1182/blood.v88.10.3698.bloodjournal88103698

Queiroz, R. F., & Lima, E. S. (2013). Oxidative stress in sickle cell disease. *Revista Brasileira de Hematologia e Hemoterapia*. https://doi.org/10.5581/1516-8484.20130008

Rahimi, Z., & Parsian, A. (2011). Sickle cell disease and venous thromboembolism. *Mediterranean Journal of Hematology and Infectious Diseases*, *3*(1). https://doi.org/10.4084/MJHID.2011.024

Rahimi, Z., Vaisi-Raygani, A., Nagel, R. L., & Muniz, A. (2008). Thrombophilic mutations among Southern Iranian patients with sickle cell disease: High prevalence of factor V Leiden. *Journal of Thrombosis and Thrombolysis*. https://doi.org/10.1007/s11239-007-0069-x

Ranguelov, R. D., Rosenthal, N., Bromley, C., & Vasef, M. A. (2002). Detection of factor V Leiden and prothrombin gene mutations in patients who died with thrombotic events: A retrospective allele-specific multiplex polymerase chain reaction study. *Archives of Pathology and Laboratory Medicine*, *126*(10), 1193–1196. https://doi.org/10.1043/0003-9985(2002)126<1193:DOFVLA>2.0.CO;2

Samarah, F., & Srour, M. A. (2018). Factor V Leiden G1691A and prothrombin G20210A mutations among Palestinian patients with sickle cell disease. *BMC Hematology*, *18*(1), 1–7. https://doi.org/10.1186/s12878-018-0097-0

Serjeant, G. R., & Ndugwa, C. M. (2003). Sickle cell disease in Uganda: A time for action. *East African Medical Journal*. https://doi.org/10.4314/eamj.v80i7.8724

Shafia, S., Zargar, M. H., Khan, N., Ahmad, R., Shah, Z. A., & Asimi, R. (2018). High prevalence of factor V Leiden and prothrombin G20101A mutations in Kashmiri patients with venous thromboembolism. *Gene*, *654*(November 2017), 1–9. https://doi.org/10.1016/j.gene.2018.02.031

Sparkenbaugh, E., & Pawlinski, R. (2017). Prothrombotic aspects of sickle cell disease. *Journal of Thrombosis and Haemostasis*, *15*(7), 1307–1316. https://doi.org/10.1111/jth.13717

Steinberg, M. H. (1998). Pathophysiology of sickle cell disease. *Bailliere's Clinical Haematology*, *11*(1), 163–184. https://doi.org/10.1016/S0950-3536(98)80074-7

Waterfall, C. M., Cobb, B. D., Breslauer, D. N., Maamari, R. N., Switz, N. A., Lam, W. A., & Fletcher, D. A. (2009). Single tube genotyping of sickle cell anaemia using PCR-based SNP analysis. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0006320

Weatherall, D. J. (2010). The inherited diseases of hemoglobin are an emerging global health burden. *Blood*, *115*(22), 4331–4336. https://doi.org/10.1182/blood-2010-01-251348

Yadav, U., Kumar, P., Gupta, S., & Rai, V. (2017). Distribution of MTHFR C677T Gene Polymorphism in Healthy North Indian Population and an Updated Meta-analysis. *Indian Journal of Clinical Biochemistry*, *32*(4), 399–410. https://doi.org/10.1007/s12291-016-0619-0

Yamaja Setty, B. N., Koneti Rao, A., & Stuart, M. J. (2001). Thrombophilia in sickle cell disease: The red cell connection. *Blood*. https://doi.org/10.1182/blood.V98.12.3228

APPENDIX I: Waiver of informed consent to used stored samples sickle cell patients

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COLLEGE OF HEALTH SCIENCES
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19th April 2021

Mr. Patrick Oroma

Makerere University

Dear Mr. Oroma,

Re: Approval of waiver of informed consent to use stored samples from sickle cell patients

In reference to your research protocol titled 'Factor V-Leiden, Prothrombin G20210A, and Methylenetetrahydrofolate Reductase mutation (MTHFR C677T) among sickle cell disease patients in Mulago Sickle Cell Clinic -Ref No:2021-102', you requested the School of Health Sciences Research and Ethics Committee on the 25th of March 2021 to waive off research participant's informed consent in order to use the stored samples previously obtained from sickle cell patients in previous research projects.

The participants from whom the samples were obtained in the previous studies cannot be traced to obtain their informed consent for the proposed study and there shall be no physical contact with the individuals except using their stored samples.

The outcome of this study will help us understand the effect of genetic polymorphisms associated with thrombophilia on sickle cell disease manifestation.

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.

Yours sincerely,

MAKERERE UNIVERSITY
SCHOOL OF HEALTH SCIENCES
★ 14 APR 2021 ★
INSTITUTIONAL REVIEW BOARD
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Dr.Kalidi Rajab
AG. Chairperson, School of Health Sciences Research and Ethics Committee