T.C.
REPUBLIC OF TURKEY
HACETTEPE UNIVERSITY
GRADUATE SCHOOL OF HEALTH SCIENCES

DETERMINATION OF LIPID METABOLISM ALTERATIONS AND THEIR ASSOCIATION WITH DISEASE PATHOGENESIS IN SICKLE CELL DISEASE

Dr. Ahmet YALÇINKAYA

Program of Biochemistry
DOCTOR OF PHILOSOPHY THESIS

ANKARA
2020

ACKNOWLEDGMENTS

"Research is what I'm doing when I don't know what I'm doing."

Wernher von Braun

It is impossible to express the degree of gratitude I feel towards my thesis supervisor, Assoc. Prof. Dr. Yeşim Er Öztaş, for her unparalleled scientific expertise, open-door attitude, patience, encouragement, and unwavering support during the planning, conduct, analysis and writing of my studies.

It goes without saying that I am deeply indebted to each and every one of our faculty members who spared their valuable time and resources during the course of this research. I am especially thankful to Prof. Dr. Hatice Asuman Özkara for her kind leadership, Prof. Dr. Incilay Lay for her decisive support in analyses and extensive contributions in my studies, Prof. Dr. Mutay Aslan for generously opening her laboratory for part of our research, Prof. Dr. Selma Ünal for her clinical guidance, Prof. Dr. Erdinç Devrim for his encouraging outlook, and Prof. Dr. Zeliha Günnur Dikmen and Prof. Dr. Aslı Pınar who were central to the clinical chemistry investigations. I also wish to express my deepest gratitude towards Prof. Dr. Besim Öğretmen for everything that I experienced and learned in his laboratory at the Medical University of South Carolina.

I am whole-heartedly thankful and consider myself in great debt to my friends and colleagues who critically contributed to my research and were always available for assistance, both scientifically and otherwise. R.A. Müslüm Gök, Dr. Çiğdem Çiçek and R.A. Mehmet Özcan for always being there when I needed assistance, R.A. Burcu Eser for her key contributions to my study, and, last but not least, Reşat, İbrahim, Megan and Max for their devotion in teaching and training me.

Without doubt, my thanks also extend to my family for their enduring support, my father for his visionary guidance since I was but a child, and especially my brother Ali for taking the thankless role of being the 'executive' among siblings.

My wife, Rumeysa, with her patience and encouragement at my worst, pride and delight at my best, in addition to her critical contributions to my research and relentless belief in my capabilities, reserves the right to a thank you that cannot be expressed in words. I am also thankful for my daughters, Meryem and Feyza, who many a night fell asleep while waiting for their father to return home from work.

I would also like to thank TÜBİTAK and Hacettepe University for providing the necessary funding for these studies, and their contributions to my development.

ABSTRACT

YALCINKAYA A., Determination of Lipid Metabolism Alterations and Their Association with Disease Pathogenesis in Sickle Cell Disease, Hacettepe University Graduate School of Health Sciences, Department of Medical Biochemistry, Doctor of Philosophy Thesis, Ankara, 2020. Sickle cell disease (SCD) is a hemoglobinopathy that causes severe anemia and is characterized by lipid abnormalities, oxidative stress and chronic inflammation. It is caused by a single point-mutation in the beta globin gene. Even though the biochemical disease pathophysiology was established more than half a century ago, there are still many questions surrounding the influence of these basic changes on various characteristics seen in patients. We measured parameters associated with lipid profile / lipid metabolism alterations, oxidative stress and chronic inflammation in pediatric patients with SCD and aimed to determine the relationships between these markers, and to assess whether various bioactive lipids (sphingosine 1-phosphate, oxysterols and ceramides) were associated with disease characteristics. The results of our study showed significant associations between anemia-related parameters, oxidative stress, inflammation and lipid profile. When the changes in lipid metabolism and some specific markers of oxidative stress and inflammation were assessed in detail, we came upon results that strongly suggested a significant change in the function of the HDL particle in patients, indicating that the unfavorable environment in the circulation could be leading to major HDL dysfunction. Furthermore, oxysterol and ceramide levels were found to be influential on especially hemolysis parameters and the cholesterol levels of patients. The findings of this study, especially those concerning the relationships between lipid metabolism and other parameters, indicate that SCD pathophysiology is remarkably and significantly related to lipid metabolism and bioactive lipids.

Keywords: sickle cell disease, oxidative stress, inflammation, oxysterols, ceramides.

Supported by Hacettepe University Scientific Research Projects Coordination Unit: Project code: TSA-2016-7276; and TÜBİTAK Fellowships and Grant Programmes (BİDEB): Grant: 2214-A, Term: 2018/2.

ÖZET

YALÇINKAYA A., Orak hücre hastalığında lipit metabolizmasındaki değişimlerin araştırılması ve hastalık patogeneziyle ilişkilendirilmesi, Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü, Tıbbi Biyokimya Anabilim Dalı, Doktora Tezi, Ankara, 2020. Orak hücre hastalığı (OHH), şiddetli anemiye neden olan ve lipid anormallikleri, oksidatif stres ve kronik inflamasyon ile karakterize bir hemoglobinopatidir. Beta globin genindeki tek bir nokta mutasyonundan kaynaklanır. Hastalığın biyokimyasal patofizyolojisi yarım asır önce açığa kavuşturulmuş olsa da, yukarıda listelenen temel değişikliklerin hastalarda görülen çeşitli özellikler üzerindeki etkisini çevreleyen birçok soru vardır. Orak hücre hastalığı olan pediyatrik hastalarda lipit profili / metabolizması, oksidatif stres ve kronik inflamasyon ile ilişkili parametreleri ölçmek suretiyle bu belirteçler arasındaki ilişkileri belirlemeyi ve çeşitli biyoaktif lipitlerin (sfingozin 1-fosfat, oksisteroller ve seramidler) hastalığın karakteristikleriyle ilişkili olup olmadığını değerlendirmeyi amaçladık. Çalışmamızın sonuçları anemi ilişkili parametreler, oksidatif stres, inflamasyon ve lipit profili arasında anlamlı ilişkiler olduğunu göstermiştir. Lipid metabolizmasındaki değişikliklerin yanı sıra oksidatif stres ve inflamasyonun bazı belirteçleri ayrıntılı olarak değerlendirdiğimizde, dolaşımdaki olumsuz etkilerin HDL partikülünün işlevini ve yapısını olumsuz şekilde etkilediğini gösteren önemli sonuçlar elde ettik. Ayrıca, oksisterol ve seramid düzeylerinin özellikle hemoliz parametreleri ve hastaların kolesterol düzeyleri üzerinde etkili olduğunu tespit ettik. Özellikle lipit metabolizması ve diğer parametreler arasındaki ilişkileri gösteren sonuçların değerlendirilmesi sonucu, OHH patofizyolojisinin lipit metabolizması değişiklikleri ve biyoaktif lipitlerin etkileriyle belirgin ve anlamlı bir şekilde ilişkili olduğu tespit edildi.

Anahtar Kelimeler: orak hücre hastalığı, oksidatif stress, inflamasyon, oksisteroller, seramidler.

Destekleyen Kurumlar: Hacettepe Üniversitesi Bilimsel Araştırma Projeleri Koordinasyon Birimi (HÜBAP): TSA-2016-7276. TÜBİTAK BİDEB Doktora Sırası Araştırma Burs Programı (2214-A, 2018/2).

TABLE OF CONTENTS

SYMBOLS AND ABBREVIATIONS

ACS	Acute chest syndrome
Apo A1	Apolipoprotein A1
Apo B	Apolipoprotein B
CerS	Ceramide synthase
C-triol	Cholestane-3β,5α,6β-triol
FFA	Free fatty acids
GC-MS/MS	Gas chromatography tandem mass spectrometry
Hb	Hemoglobin
HbA	Hemoglobin A
HbC	Hemoglobin C
HbF	Fetal hemoglobin
HbS	Hemoglobin sickle
HbSS	Homozygous sickle cell disease
HbSß$^+$	Hemoglobin sickle beta thalassemia
HDL	High density lipoprotein
HDL-C	High density lipoprotein cholesterol
Hpg	Haptoglobin
7-KC	7-ketocholesterol
LCAT	Lecithin cholesterol acyl transferase
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
MPO	Myeloperoxidase
NO	Nitric oxide
ROS	Reactive oxygen species
S1P	Sphingosine 1-phosphate
SAA	Serum amyloid A
SCA	Sickle cell anemia
SCD	Sickle cell disease
TC	Total cholesterol
TG	Triglycerides
VLDL	Very low density lipoprotein
VLDL-C	Very low density lipoprotein cholesterol

FIGURES

TABLES

1. INTRODUCTION

Hemoglobin (Hb) is the carrier of oxygen in the blood of almost all vertebrates and also some invertebrates (1, 2). It is a tetrameric metalloprotein with a quaternary structure comprised of four globin protein subunits, each carrying a heme group that directly interacts and binds a single oxygen molecule (O_2). The globin proteins determine the type of Hb formed, which show significant changes from embryonic development stages to the fetal period and adulthood (3).

Hemoglobin A1 (HbA_1, often referred to as HbA) becomes the predominant Hb tetramer soon after birth (up to 97–99%), replacing fetal Hb (HbF) (4). Hemoglobin A2 is also present throughout life (1–3%); however, its physiological importance has not been elucidated (5). The structural characteristics of Hb are an essential part of its function, as the structure shows significant changes in order to adapt to ever-changing conditions in the blood, such as pH, oxygen saturation, Hb-binding molecules, and concentration of other gases including nitric oxide (NO), carbon dioxide (CO_2) and carbon monoxide (CO) (6-8).

Hemoglobin A is a Hb type that includes alpha and beta globin chains (two of each), which are encoded by the α-globin and ß-globin genes, respectively. Various Hb variants are formed due to mutations in these genes –most of which do not cause disease (9). However, some mutant forms cause problems in the function of Hb and lead to conditions termed as hemoglobinopathies (10). Diseases which cause reduced production of Hb (be it normal or abnormal Hb) are known as thalassemias (11). Both sets of diseases usually result in anemia, albeit at varying severity. Moreover, patients may have thalassemia and hemoglobinopathies at the same time in the presence of respective mutations in their genes. The most well-known and severe hemoglobinopathy is sickle cell disease (SCD). Patients with SCD develop severe chronic hemolytic anemia and have recurrent painful vaso-occlusions (crises) in (usually) small capillaries which adversely affect quality of life and cause significant morbidity and mortality (12). The disease results from the production of a hemoglobin type termed as Hb Sickle (HbS) due to a single beta-globin mutation, first identified by Linus Pauling et al. in 1949 (13).

The genetic mutation in SCD is thought to have evolved in humans due to malaria. Widespread deforestation in sub-Saharan Africa and other areas provided a fertile environment for the propagation of the mosquito species, *falciparum malaria*, leading to a fast-paced spread of malaria infection which is carried by the females of the species. Individuals with sickle cell trait inherently had a survival advantage over those without the mutation and lived to reproduce. Whereas, those who had homozygote mutations (in both ß-globin genes) did not survive to reproduce. Therefore, the gene was passed to the descendants of individuals that had sickle cell trait as it prevented early death from malaria (14).

Sickle cell disease is the first disease to have its molecular origin identified (15). However, after almost a century of studies, some pathophysiological characteristics of the disease still remain elusive (16). Patients with SCD have significant metabolic and hormonal changes, including inflammatory activation, alterations in lipid homeostasis, dysfunctions in the endocrine system, and developmental disturbances (17). They also suffer from severe clinical manifestations which often require hospitalization and cause significant mortality as well as financial burden (18-20).

A select group of SCD patients may be cured with bone marrow transplants (21), while recent developments have shown promise with genetic treatments (22, 23). However, hydroxyurea (hydroxycarbamide) remains as the only widely-used FDA-approved treatment of SCD (24). Hydroxyurea exhibits its effect by stimulating HbF production; thereby replacing HbS in erythrocytes. It is also believed to increase the amount of bioavailable NO which is normally depleted in SCD patients (25, 26). However, this treatment does not address the biochemical foundation of the disease and can only be considered an indirect way of reducing sickling via decreasing the amount of HbS per erythrocyte.

1.1. Aims

Sickle cell disease causes an increase in oxidative stress levels, activates inflammation and leads to endothelial dysfunction as well as resulting in significant changes in the levels of various lipids. These changes may influence the severity of

disease and could have critical effects on disease pathophysiology. Particularly, even though abnormalities in lipid profile and metabolism are well known, until recently, few studies explored the significance of these alterations and their impact (or relationship) with the pathophysiology of SCD. Exploring the characteristics of these changes and determining relationships between these seemingly separate pathways of pathology (lipid metabolism abnormalities, hemolysis, oxidative stress and inflammation) may provide invaluable data in the elucidation of SCD pathophysiology.

Therefore, it is apparent that the measurement of lipid levels, oxidative stress and inflammatory parameters and determination of relationships with other disease parameters and the clinical findings of the disease will increase our understanding of SCD pathophysiology and may also reveal new targets for therapy. Our aim in the first step (first group of children with SCD) was to determine and identify the correlations between various biochemical parameters in steady state SCD children, to evaluate relationships with clinical manifestations of SCD, and to determine if any of the parameters could be used as a tool for long-term risk stratification in patients. In the second group of patients, we evaluated the levels of oxysterols (oxidized derivatives of cholesterol) with an aim to determine whether oxidative stress-induced production of oxysterols had any significant effects on the hemolysis or lipid changes that are characteristic of SCD. We also determined the levels of several ceramide species in order to assess whether their effects on eryptosis (or other parameters) had any significant relationship with the various characteristics of SCD.

2. GENERAL INFORMATION

2.1. Hemoglobin

Humans produce various types of Hb throughout life, particularly during the early developmental stages. Hemoglobin is a construct of four globin proteins (hence the term 'tetramer') that come together via very specific bonds and connections to form a final structure capable of carrying oxygen via the heme group(s) in the structure. Therefore, the type of Hb is solely dependent on the type of globin proteins that comprise its structure (27). Various Hb types have been shown and described in humans (Table 2.1.).

Table 2.1. Normal hemoglobin types with regard to developmental stage.

Name of Tetramer	Chains	Development stage	Comment
Hb E Gower 1	2 zeta, 2 epsilon $\zeta_2\varepsilon_2$	Only in the embryonic stage	The primary embryonic hemoglobin, unstable.
Hb E Gower 2	2 alpha, 2 epsilon $\alpha_2\varepsilon_2$	Prominent in the embryonic stage, also present in fetal life	Higher stability compared to Gower 1, explored for reactivation in severe hemoglobinopathies and/or thalassemia due to similar properties with HbA
Hb E Portland I	2 zeta, 2 gamma $\zeta_2\gamma_2$	Embryonic stage, also present in fetal life	
Hb E Portland II	2 zeta, 2 beta $\zeta_2\beta_2$	Embryonic stage, also present in fetal life	Very unstable
HbF Hemoglobin F	2 alpha, 2 gamma $\alpha_2\gamma_2$	Fetal life. Very low amounts later on in life.	The primary fetal hemoglobin. Higher affinity to oxygen. Remains as the majority hemoglobin from 8-10 weeks of gestation until 2-3 months of age. Constitutes approximately 1% of hemoglobin in adults
HbA_1 Hemoglobin A_1	2 alpha, 2 beta $\alpha_2\beta_2$	Production begins during fetal life, significantly increases after birth.	The primary adult hemoglobin. Takes over from HbF around 2-3 months of age and becomes the major hemoglobin type throughout life (95-99%).
HbA_2 Hemoglobin A_2	2 alpha, 2 delta $\alpha_2\delta_2$	After birth and in low amount.	Very low proportion compared to HbA (1-3%). Significance unknown.

The zeta and epsilon chains are produced during early embryonic development by the yolk sac with very low production after 6-8 weeks of gestation. They are therefore termed as 'embryonic hemoglobin chains' and are often referred to as 'Hb E' –not to be confused with the Hemoglobin type, HbE.

2.2. Definition

Sickle cell disease represents a large group of Hb disorders in which the primary Hb (HbA) is replaced with HbS which is produced due to a Glu6Val missense mutation in the 7th (previously defined as the 6th) codon of at least one of the two ß-globin genes (28). The disease is inherited autosomal recessively; that is, if one ß-globin gene is normal (defined as HbAS), patients rarely exhibit clinical manifestations and the condition is named as sickle cell trait; whereas, if both inherited genes have the mutation (HbSS), the condition is termed as sickle cell anemia (SCA), leading to severe disease and complications (29).

Patients may also have other mutations in Hb-producing genes, which cause compound hemoglobinopathies that are named in accordance with the type and name of the variant and demonstrate varying severity (e.g., HbC with HbS is named as HbSC) (30). The genotype of a patient, either with homozygote/heterozygote SCD or with compound mutations, has a critical role in disease severity as it significantly alters the underlying biochemical pathophysiology, and thus, disease complications (31, 32). Many acute clinical manifestations seen throughout the disease worsen patients' quality of life and may lead to various morbidities (33, 34). Whereas, periods in which patients have not had any crises or other debilitating acute manifestations are defined as steady state; however, the underlying pathophysiological mechanisms remain active throughout these 'steady' periods (35). The criteria that must be fulfilled for steady state disease will be detailed later in this work.

To conclude, the term "sickle cell disease" is utilized when addressing a number of different conditions where the Glu6Val mutation is present; therefore, SCD actually identifies a large group of hemoglobinopathies, including those with any other globin mutations or thalassemia types (36). Additionally, the term SCD is used rather frequently (often erroneously) to identify the most severe hemoglobinopathy (that remains compatible with life) within the group: homozygous SCD (HbSS) which is normally named as an 'anemia', as in: SCA (37). If one must strictly adhere to correct terminology, the patients included in our study would have to be defined with SCA and sickle beta thalassemia as definite diagnoses. Throughout this work, we will be using the term SCD to describe both sets of disease evaluated in this study (SCA and

sickle beta thalassemia) for the sake of simplicity; however, the true form of the disease will be accurately given at all times by defining patients with HbSS or HbSß$^{+}$ when and where necessary.

2.3. Epidemiology

Globally, around 5–7% of the population carry genes that cause different types of hemoglobinopathies (38). The genetic mutation responsible for SCD is believed to originate from the tropic and sub-tropic regions of the 'old world' in which malaria was historically endemic, such as sub-Saharan Africa, countries that are in the Mediterranean basin, countries in the Arabian Peninsula extending to Mesopotamia, and the Indian subcontinent. It was passed on to later generations due to the advantage of sickle cell trait in the presence of malaria infection which is carried by the mosquito species: *falciparum malaria* (39). As such, almost all regions in which malaria is (or was) endemic have a significant burden of hemoglobinopathies today, even if malaria is not a public health problem anymore (40). Among these hemoglobinopathies, some may not cause significant clinical findings, while others (such as SCD) result in severe disease. The characteristics of several commonly-known hemoglobinopathies are given in Table 2.2. Migration, either voluntary or forcibly, distributed the gene throughout the globe, increasing carriers of the disease in various populations, which has led to the geographic distribution of SCD, especially in the American continent (41).

As the disease is inherited, the number of carriers (sickle cell trait) in the population determines the incidence and prevalence of SCD, and thus, its burden on the population. Some countries and regions show marked increases in the frequency of sickle cell trait, especially in regions that have a history of malaria, which evidently increases the number of children born with SCD and the size of the population with SCD. On a global scale, it is estimated that there are around 300 million carriers (that is, those carrying the disease with minimal clinic in the form of trait), while annually 250–300 thousand children are born with SCD (42).

Table 2.2. Hemoglobin types that cause hemoglobinopathies.

Name of Tetramer	Chains and Mutation	Conditions(s)	Comment
Most prominent forms			
Hemoglobin S	2 alpha, 2 beta ß6 Glu->Val	Sickle cell trait: HbAS Sickle cell anemia: HbSS Sickle cell disease: (e.g. HbSC, HbSD, HbSß$^{+}$)	Less soluble than HbA and forms long polymers when deoxygenated. Frequent in Africa. Minimal clinic in trait, very severe disease in homozygous and compound forms and in the presence of thalassemia.
Hemoglobin C	2 alpha, 2 beta ß6 Glu->Lys	Trait, homozygous and compound.	Less soluble than HbA and forms hexagonal crystals. Similar geographic distribution with HbS. Causes minimal to mild anemia in trait and homozygous forms. Severe disease when compound with HbS. Subtypes with additional mutations but similar clinic exist (Hemoglobin C Harlem).
Hemoglobin D	2 alpha, 2 beta ß121 Glu->Gln	Trait, homozygous and compound.	Most common form is termed as HbD Los Angeles. Cannot be distinguished from HbS with usual electrophoresis. Trait is asymptomatic, homozygous form causes mild anemia. Severe disease with HbS.
Hemoglobin E	2 alpha, 2 beta ß26 Glu->Lys	Trait, homozygous and compound.	Electrophoretic mobility similar to HbC. Frequent in the Indian sub-continent. Severe disease when compound with HbS or in cases with thalassemia.
Other less common types			
Hemoglobin H	4 beta (no mutation)	By-product of alpha thalassemia.	Observed in patients with alpha thalassemia. Is not necessarily a direct culprit of disease as it is produced due to absence of α chains.
Hemoglobin Koln	2 alpha, 2 beta ß98 Val->Met	Trait, homozygous and compound.	Causes excessively high affinity to oxygen which limits oxygen supply to tissues, leading to erythrocytosis. However, hemolysis is also present which prevents polycythemia. Minimal clinic in trait, incompatible with life in homozygous form.
Hemoglobin Chesapeake	2 alpha, 2 gamma α92 Arg->Leu		Unlike the majority of hemoglobinopathies, the mutation is on the α chain in Hb Chesapeake. It is a mutated form of HbF and was the first high-oxygen affinity hemoglobin variant to be discovered. Causes erythrocytosis.
Hemoglobin Kansas	2 alpha, 2 beta ß102 Asn->Thr		Low-oxygen affinity hemoglobin. Low oxygen storage results in cyanosis; however, tissues normally have sufficient oxygen supply to function.

In Africa, sickle cell trait prevalence ranges between 10–45% (43). Although some tribes and communities are known have higher prevalence in some smaller regions, Nigeria has the single highest overall burden of SCD (44). Most recent data from Nigeria has shown that 3.8 million Nigerians have SCD, while approximately 90–120 thousand children are born with the disease and 23-30% of the population are carriers (HbAS) (45, 46).

In Turkey, the frequency of the sickle cell mutation is estimated to be between 0.3–0.6%; however, prevalence is higher in cities that are in the Mediterranean region (47). Data from the Ministry of Health puts the number of SCA patients at 1200 nationwide; however, this number may reflect an underestimation, as carrier frequency is reported to be between 10–13.6% in highly populated cities such as Adana, Hatay and Mersin, which are located on the far east shoreline of the Mediterranean region of Turkey (48).

In Brazil, studies indicate that the disease is more frequent in regions where the proportion of African descendants is higher. In these regions, 1 in 1000 children are born with SCD, while the trait condition is seen in 1–6% of the population (49).

In the United States of America (USA), SCD represents the most prevalent genetic blood disorder with approximately 80,000–140,000 people suffering from SCD (50). Studies have estimated that the disease occurs in 1 out of 345–500 African Americans, while the same value was 1 out of 1,000–16,000 in Hispanic Americans. Also, it was estimated that 1 out of every 13 African Americans were carrying the gene (51, 52). Due to the burden of the disease in the USA, screening in the neonatal period is being utilized (53). The number of children that have the gene among African Americans (73 in 1000 live births) and Hispanic Americans (7 in 1000 live births) suggests that the condition will remain as a significant health problem in the foreseeable future (52).

The global burden of SCD can be easily observed by the geographic distribution of the birth frequency of babies with SCD (Figure 2.1.) (54).

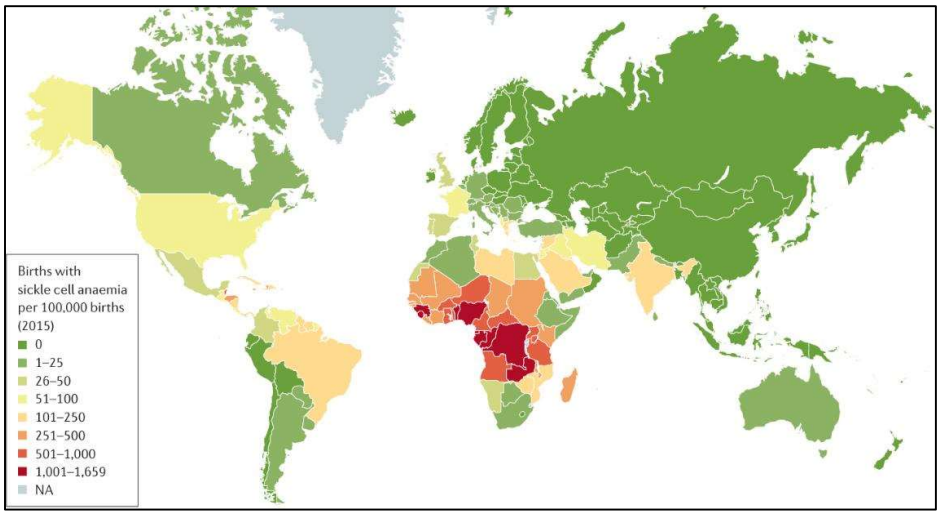

Figure 2.1. Frequency of births with homozygous SCD around the world (54).

2.4. Pathophysiology

2.4.1. Biochemical Basis of the Disease

The molecular basis of the disease is quite simple: The Glu6Val mutation in the ß-globin gene replaces a single Glutamic acid (hydrophilic) residue with a Valine (hydrophobic) residue (55); however, the consequences of this change are not so simple. The first and foremost result of this change is the reduction in the solubility of HbS (56). In hypoxic conditions, acidosis, and under other stress suffered by the erythrocyte which increase the proportion of deoxygenated Hb, the hydrophobic valine located on the exterior of the HbS structure can interact with the leucine and phenylalanine residues of the ß-globin subunit of another Hb, forming polymers that gradually increase in size and deform the characteristic shape of the erythrocyte (57). This morphological change of the erythrocyte was first observed by James B. Herrick who introduced the term ‘sickle’ in 1910, hence giving the disease its name (58).

There are various physical and chemical factors involved in polymer formation, including temperature, pH, levels of 2,3-bisphosphoglycerate, oxygenation and deoxygenation of the Hb, and presence of other Hb variants (59). These factors may also effect the clinical severity of the disease (60). Considering the fact that increased HbF proportion (which reduces polymerization and the stability of

polymers) decreases the severity of the disease (61, 62), it is evident that Hb polymerization and the stability of these polymers critically contribute to disease severity. Deformed or ‘sickled’ erythrocytes lose water, become rigid and are therefore prone to rupture, especially when passing through capillaries, resulting in hemolysis and blockage of vessels (63). The effects of constant hemolysis are not limited to anemia, patients with SCD also have significant alterations in inflammatory activity, oxidative stress and endothelial function (Figure 2.2.) (64).

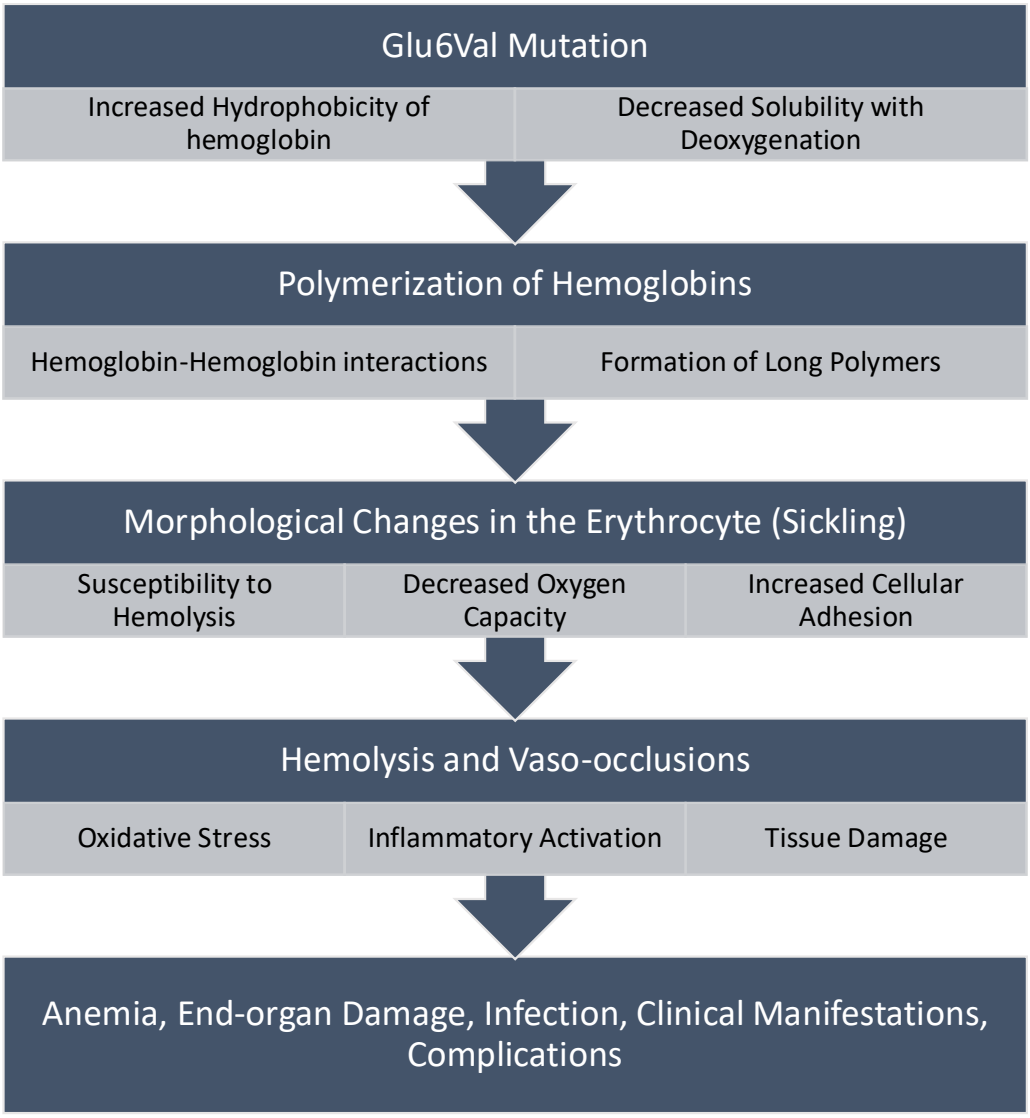

Figure 2.2. Summary of the Pathophysiology of SCD.

2.4.2. Vaso-occlusions, Endothelial Function and Inflammation

If HbS production (and polymerization) is to be considered as the foundation of the disease, vaso-occlusions can be identified as the critical building blocks of the broad range of clinical pathologies seen in SCD (65). Vaso-occlusions can manifest in nearly all areas of the body and can cause ischemia, which may lead to tissue/organ damage as well as severe pain. Other processes such as inflammation and endothelial function are also severely affected as a result of vaso-occlusions. During vaso-occlusive events, the increased rigidity (reduced deformability) of erythrocytes causes

them to be ruptured in small vessels, limiting or completely cutting the supply of oxygen to distal tissues (Figure 2.3.) (66). While the ruptured erythrocytes release Hb, heme and iron into the bloodstream causing endothelial damage and oxidative stress, the hypoxia in distal tissues results in ischemic damage. In the face of constant oxidative and ischemic insult, the inflammatory response is activated and continues at a steady state. The result is chronic inflammation which causes endothelial dysfunction; thus forming a vicious cycle (67).

In SCD, various measures of endothelial function, including flow-mediated dilatation, arterial diameter changes, endothelial independent dilatation and wall shear stress, were found to be adversely altered (68, 69). Therefore, the dysfunction of the endothelium in patients with SCD apparently contributes to disease pathophysiology and the development/progression of vaso-occlusive episodes.

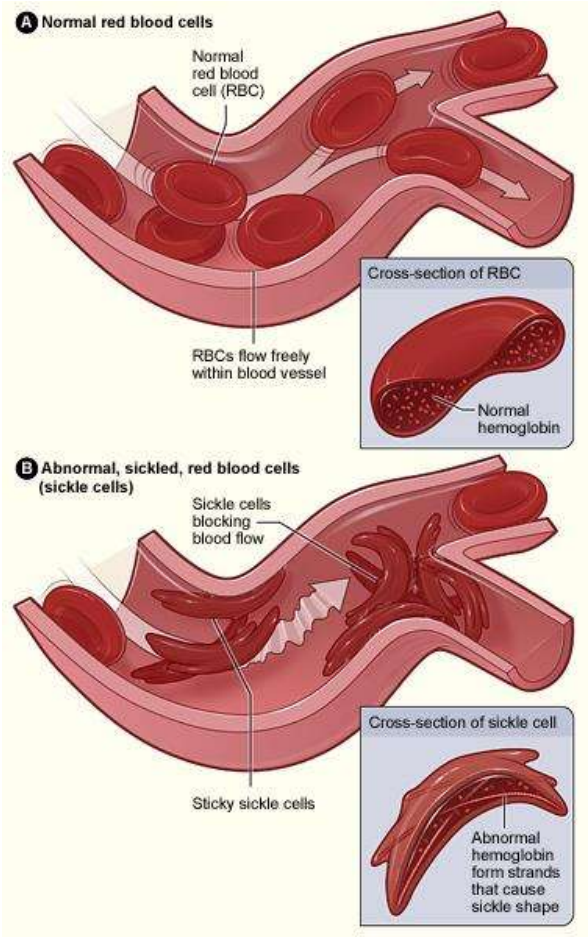

Figure 2.3. Healthy and Sickle Erythrocytes in the Circulation (70).

2.4.3. Nitric Oxide

Having explained the fundamental features of SCD pathophysiology, we may shift our focus to factors that significantly contribute to these features, the most prominent being the depletion of NO in circulation. Nitric oxide, produced by endothelium, has significant influence on vascular and endothelial function, including but not limited to vasodilation, reduction of leukocyte adhesion, regulation of vascular tone, and scavenging of compounds that cause oxidative stress (71, 72). Phagocytes also produce NO in large amounts during the immune response against infectious pathogens (73). Therefore, alterations in the bioavailability of NO have significant consequences on factors that have been shown to have critical importance in SCD pathology (endothelial function, inflammation and leukocyte adhesion). The levels of

NO and L-arginine are reportedly significantly reduced in patients with SCD, especially during vaso-occlusions (74, 75). This is a direct result of increased hemolysis, as Hb, heme and reactive oxygen species (ROS) react with NO, reducing its concentration in the circulation (76, 77). Considering that NO depletion has been associated with the pathophysiology of SCD, various studies have explored ways to increase NO levels and reduce destruction (78-81), and increase tissue response to available NO (82, 83).

2.4.4. Adhesion

The increased adhesion of the cellular components of the circulation (erythrocytes, leukocytes, platelets and endothelium) is another important feature that contributes to SCD pathophysiology. Various studies have shown increased "sticky" characteristics in erythrocytes, other circulatory cells and the endothelium, which have been associated with membrane-related changes in young erythrocytes (reticulocytes), leukocytes and other cellular components including platelets and endothelial cells (84-88). Erythrocyte adhesion to the endothelium (89) and formation of cell-cell aggregates (such as platelet-neutrophil) (90) adversely affect blood flow which is followed by increased sickling of erythrocytes due to the hypoxic environment in capillaries, thereby contributing to vaso-occlusions (91). Various receptors, molecules and factors, including CD36, vascular and intercellular adhesion molecules, integrins, selectins and lutherian protein, have been shown to be associated with erythrocyte, leukocyte and endothelial adhesion in SCD (89, 92-94).

2.4.5. Membrane Damage

Phospholipids are a crucial part of the lipid bi-layers that form cellular membranes. Despite being discovered over 60 years ago (95, 96), the Kennedy pathway (CDP-choline pathway) and Lands' cycle remained as poorly researched topics until recent studies provided data regarding their role in membrane maintenance and phospholipid production and repair. The Kennedy pathway is utilized for the production of phospholipids, while the Lands' cycle performs phospholipid remodeling and repair duties in cells (97). However, due to the lack of membrane-bound organelles, the erythrocyte cannot produce phospholipids through *de novo*

synthesis via the Kennedy cycle. Therefore, phospholipid recycling via the Lands' cycle remains as the only option for membrane maintenance in the erythrocyte. The Lands' cycle essentially functions through two membrane-bound enzymes; one of which is phospholipase A2, while the other is actually a larger group of enzymes, termed as lysophospholipid acyltransferases, which perform de-acylation and re-acylation of phospholipid species (98). Although abnormalities in membrane phospholipid composition (99) and organization (100) of sickle erythrocytes were shown in earlier studies, for many years, little –if any– research was performed in this field. Recent studies however have shown that impairment of the Lands' cycle may promote sickling through increased levels of lyso-phosphatidylcholine in erythrocytes (101). The roles of lipids in membrane stability and remodeling may indicate that the alteration of lipid profile is a significant contributor to the complex pathophysiology of SCD.

Damage to membranes, especially erythrocyte membranes, is not limited to the absence of functional repair mechanisms. The proteins, lipoproteins and lipids in circulation and cellular membranes suffer from constant oxidative attacks, leading to the oxidation of proteins, and the production of oxidized forms of lipids and lipoproteins (102) in the membranes of sickle erythrocytes. Oxidation of lipids (especially cholesterol) in cellular membranes have been shown to adversely affect membrane deformability, rheology and function (103, 104). Sickle erythrocytes are particularly susceptible to oxidation due to the imbalance between anti-oxidative and oxidative mechanisms or processes (102, 105). Previous studies have shown increased cholesterol and lipid oxidation in SCD membranes, which have been identified by the direct comparison of lipids isolated from membranes (103), and the measurement of alterations in malondialdehyde (106, 107), thiobarbituric acid reactives (108, 109), anti-oxidant levels (107, 110), trace elements (111), and various other compounds (109).

2.4.6. Erythrocyte Survival and Eryptosis

Similar to the apoptosis seen in other cells, erythrocytes also have a process of programmed cell death –conveniently called eryptosis (112). However, eryptosis seems to have more of a role than just the clearance of cells at senescence; because,

erythrocytes already have a normal removal process which occurs in the liver and spleen when they are around 120 days old (113). Furthermore, a process termed neocytolysis, which carries out the destruction of young (10 to 15-day old) erythrocytes in subjects that are adapting (or more specifically, re-adapting) to lower altitudes, has also been described as an erythropoietin-controlled mechanism through which erythrocyte levels are 'fine-tuned' (114). However, neocytolysis is activated under reduced levels of erythropoietin; thus, it is highly unlikely to have a part in the loss of erythrocytes in SCD.

Under physiological conditions and in the absence of altitude-related changes, the removal of erythrocytes from the circulation relies on the age-related natural loss of erythrocyte deformability, and the subsequent destruction in the spleen and liver (115); however, this mechanism has also been related to phosphatidylserine (PS) exposure –which is a well-defined trigger leading to eryptosis (116). Therefore, the presence of the eryptotic process alone seems to indicate that erythrocytes may be severely damaged through mechanisms other than loss of deformability in the circulation (even in healthy individuals); thus, causing the emergence of eryptosis as a process that enables the programmed death of such cells. Therefore, in brief, eryptosis is a mechanism by which the overall health of the erythrocyte population is ensured by the removal of dysfunctional or compromised cells before their natural death occurs in the liver or spleen. It is most commonly recognized by the exposure of PS in the erythrocyte structure and the subsequent increase in intracellular calcium levels, which are followed by 'membrane blebbing', cell shrinkage and death (Figure 2.4.) (117, 118).

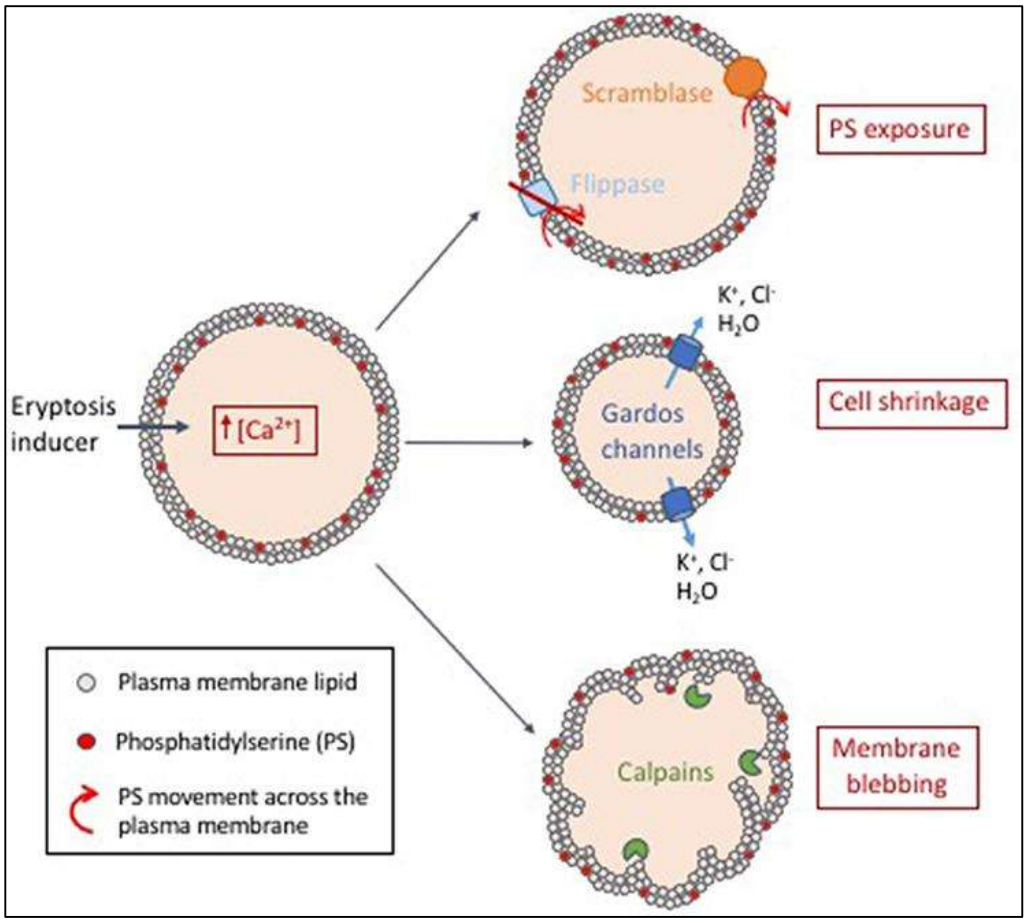

Figure 2.4. Brief representation of the activation of eryptosis (118).

Current studies on this topic have shown that oxidative stress may contribute to eryptosis by increasing PS externalization in healthy erythrocytes (119). Furthermore, such a relationship has also been demonstrated in SCD erythrocytes (120). Considering that erythrocytes that are in the process of eryptosis have been shown to have increased adhesive properties (121), and the fact that eryptosis is reportedly stimulated in SCD (120), it is apparent that eryptosis may be influential on anemia in SCD. In line with this hypothesis, it has been established that ceramides (particularly C6 and C16 ceramides) are associated with the activation and progression of eryptosis (122). Therefore, determining the levels of ceramide species and their relationships with hemolysis and oxidative parameters in patients with SCD may yield important clues concerning the role of eryptosis in SCD.

2.5. Clinical Picture and Complications

Patients with SCD suffer from chronic and severe hemolytic anemia, have vaso-occlusions which may cause immense pain, have reduced splenic function, are susceptible to infections, show gradual deterioration of organ function, and have shorter life expectancy (123, 124). The progression of disease or sudden acute problems may lead to various complications that are often life-threatening and could worsen prognosis (Figure 2.5.). The severity of the disease is closely associated with the patient's genotype and co-occurrence of other anemia-causing diseases such as ß-thalassemia; however various climate- and season-related factors, such as temperature, wind speed, humidity, air quality, altitude and socio-economic factors have also been shown to have important effects on disease characteristics and frequency of complications (39).

As described before, the erythrocytes of SCD patients are prone to rupture, making anemia an obvious and unavoidable hallmark of the disease. The erythrocytes of SCD patients have an average life-span of around 10 to 15 days which is very low in comparison to normal erythrocytes which boast a life span of 120 days (125). Hemoglobin levels among patients who have not suffered from recent hemolytic crises are usually between 7.5–10 g/dL, while hematocrit is around 20–30% (126, 127). To compensate for the loss, erythropoietin levels and erythrocyte production is increased, which leads to an increased reticulocyte count. Apart from red blood cell indices, significant increases in platelet count and mild increases in white blood cell count (WBC) have been shown (128).

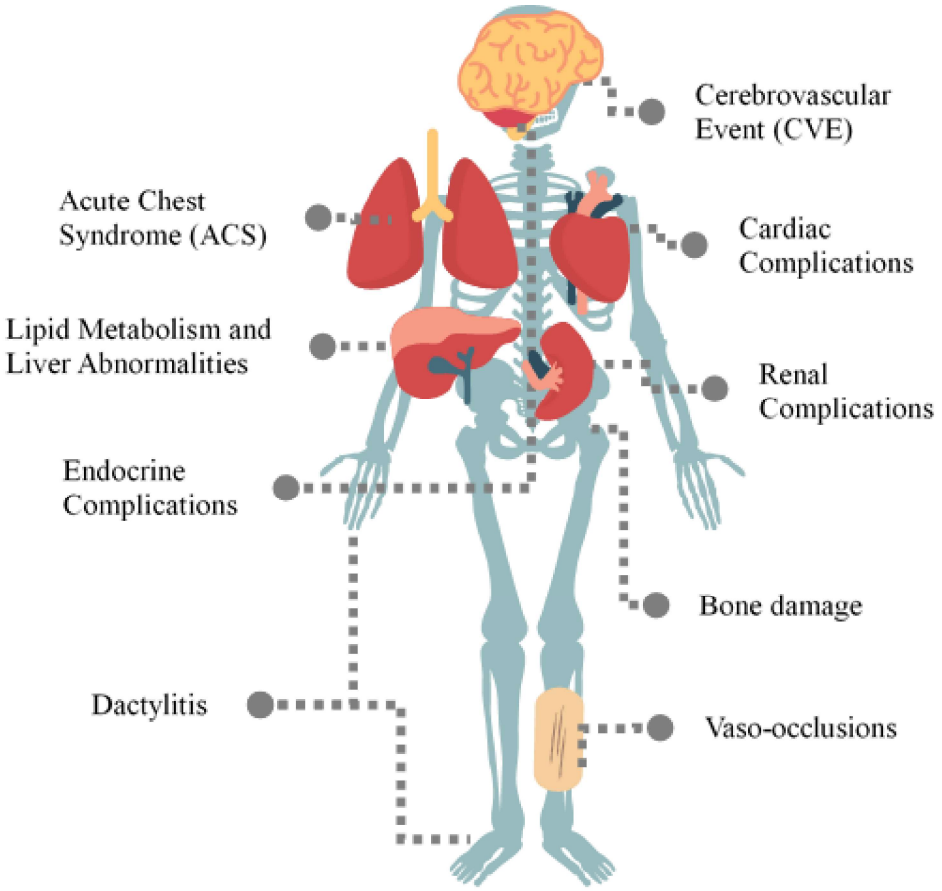

Figure 2.5. Clinical manifestations and complications of SCD.

Sickle cell disease progresses with varying types and degrees of complications which usually cause significant morbidity and mortality. Patients who have not suffered any complications recently (usually accepted as within the last 3 months) are defined as being in the 'steady state' of the disease.

2.5.1. Defining Steady State

Correct definitions are at the basis of any disease, because through the accurate identification of any conditions, physicians are able to make decisions on the diagnosis, assessment, prognosis and treatment of patients. The accuracy of these definitions carry even more weight than usual in SCD since acute and chronic complications may progress swiftly if unchecked. As such, for clinicians, and also for researchers who aim to establish and investigate baseline characteristics in order to be able to reveal pathophysiological mechanisms and their relationships with clinical

findings and outcomes, the identification of a steady state is critical. Therefore, the definition of steady state in SCD is bound to the fulfilment of some important criteria:

1. The patient should not have a history of painful episodes within at least the prior 4 weeks.
2. There should be no admission to the hospital that may be associated with painful episodes within the last 2-3 days.
3. Ideally, the closest date of transfusion should be at least 3 months ago; however, the percentage of HbA may be utilized as a measure to determine whether transfused blood levels remain significant (should be less than 10%).
4. The patient should not have any infections or inflammatory activation within the prior 4 weeks.
5. Ideally, the patient should not be receiving antibiotic treatment or chronic medications for any reason within the prior 3 weeks.

2.5.2. Acute and Chronic Complications

The acute complications of SCD include infections, sequestration crises and vaso-occlusive events which are life-threatening problems without appropriate medical care (129, 130). Chronic complications include metabolic and endocrine changes, chronic pain, anemia, growth retardation and progressive problems with many organs and systems including the eyes, brain, lungs, kidneys, heart and skeletal system (see Figure 2.5.) (131, 132). Interestingly, patients with higher Hb levels have been shown to suffer from vaso-occlusions more frequently, while the frequency of nephropathy, stroke and pulmonary hypertension were reported to be higher in those with lower Hb levels (133, 134). The higher frequency of vaso-occlusions among those with higher Hb may be attributed to increased formation of Hb polymers in those with higher Hb per cell. As a matter of fact, SCD patients with α-thalassemia or relative iron deficiency (both of which reduce Hb per cell) are suggested to have fewer sickling episodes (135, 136), supporting the prior hypothesis. However, apart from these minor changes that somewhat alter the frequency of manifestations, it is well established that the major clinical manifestations of SCD (see Figure 2.5.) are observed in the majority of patients (137).

2.6. Laboratory Findings

2.6.1. Hemolysis

Intravascular hemolysis is the most prominent finding in SCD. All parameters associated with intravascular hemolysis are chronically altered in SCD, including erythrocyte count and the levels of Hb, bilirubin and haptoglobin, among others (63). Lactate dehydrogenase (LDH) is an enzyme which is present in all human tissues and its levels are utilized as a marker of unspecific tissue damage (138). However, in SCD, LDH is accepted as an accurate indicator of intravascular hemolysis (139). The levels of LDH have been associated with increased organ damage, worse prognosis, higher frequency of ACS, and pulmonary hypertension (140).

Although well-known to be a product of both extravascular and intravascular hemolysis, bilirubin deserves a more detailed mention with regard to its characteristic changes in hemolysis that are associated with not only hemolysis itself, but also liver function, through its liver-dependent metabolism and reliance on albumin as a carrier in the circulation (141). Hemolysis results in the release of Hb and heme which immediately begin to undergo catabolism and various forms of chelation-like processes that are collectively aimed at sparing tissues from the severe oxidative properties of these molecules (142, 143). The heme-oxygenase enzyme is a vital part of these defense mechanisms as it catabolizes the protoporphyrin IX ring of heme, converting it to what is known as biliverdin. This reaction is followed by the formation of bilirubin with the enzyme biliverdin reductase (144). Bilirubin (termed as indirect or unconjugated bilirubin at this time) is then captured by albumin and carried to the liver for further processing into what is known as ‘conjugated bilirubin’, a compound that is used for the production of bile (141).

2.6.2. Chronic Inflammation

Leukocytes

The levels of leukocytes, especially neutrophils, are increased in the steady state of SCD due to the underlying chronic activation of inflammation (145-147). High neutrophil counts have been associated with worse prognosis and higher frequency of

clinical manifestations such as ACS and vaso-occlusive events (148, 149). Whereas reduced neutrophil count has been associated with better clinical (150) and laboratory findings, including higher average Hb level and lower HbS proportion (151). Notably, patients receiving hydroxyurea were found to have lower neutrophil levels and also lower neutrophil activation demonstrated by lower expression of adhesion proteins and decreased production of H_2O_2 (152). Several recent studies in murine models have also shown that preventing the formation of neutrophil-platelet aggregates are important for the prevention of vaso-occlusions SCD (153, 154). As such, the adhesion/aggregation of neutrophils and/or platelets have been investigated with results showing that they are important contributors to SCD pathophysiology. Due to these suggested relationships, there have been studies investigating their candidacy as targets for therapeutic intervention in patients with SCD (155).

Myeloperoxidase

The enzymes that play a critical role in neutrophil function have also been implicated in the pathophysiology of SCD. For instance, the enzyme myeloperoxidase (MPO) produces hypochlorous acid (HClO) (156) that results in elevated oxidative stress, while its inhibition was shown to attenuate vaso-occlusions (157). As neutrophils and MPO also contribute to the formation of ROS, the relationship between neutrophil activation and MPO levels may be a critical factor and could identify an important link between inflammatory activity and the development of an oxidative micro-environment (158, 159). Oxidative stress and its role in SCD pathophysiology will be discussed in detail later.

Serum Amyloid A

Serum Amyloid A (SAA) has been defined to be an acute phase protein which replaces apolipoprotein A1 (Apo A1) in the structure of high density lipoprotein (HDL) during inflammatory activation (160). Considering that SCD is characterized with chronic inflammation, the levels of SAA in the blood and HDL structure would be expected to increase, which may cause changes in the function of HDL (161, 162). Several studies have pointed out that SAA levels, along with other acute phase response elements and markers of inflammation, are significantly elevated among

patients with SCD, even when symptoms are absent (163). Furthermore, a previous study reported that SAA was a biomarker of painful episodes in SCD (164).

Sphingosine 1-phosphate

Sphingosine 1-phosphate (S1P) has been recognized as a critical bioactive sphingolipid with various crucial vascular and cellular effects (Figure 2.4.).

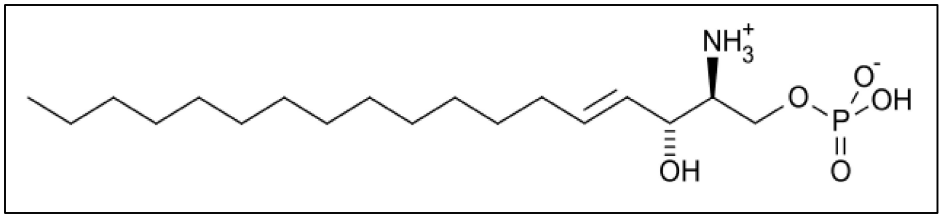

Figure 2.6. Molecular structure of sphingosine 1-phosphate.

Sphingosine 1-phosphate is carried and stored by the erythrocyte, platelets and the HDL particle, and contributes to their physiology, as well as having various atheroprotective properties including reduction of damage to the endothelium by increasing NO synthesis in the endothelium (165, 166). In a few recent studies, S1P levels were shown to be increased in SCD mice; moreover, increased S1P concentration was reportedly associated with increased sickling (167). As S1P normally has protective effects on HDL and endothelium, the nature of this increase in SCD and its associations with disease pathophysiology remain to be determined.

Other parameters associated with inflammation

Various other inflammatory markers have been shown to increase in the steady state of disease and also in the presence of crises in patients with SCD (168, 169). Among these various markers, the enzyme chitotriosidase which is secreted by activated macrophages for the purpose of breaking glycosidic bonds in chitin structure, reveals itself to be an interesting parameter as it has been associated with malaria infection (170). To our knowledge, there are no studies that have assessed chitotriosidase levels in SCD.

2.6.3. Lipid Metabolism and Lipid Profile

Patients with SCD have various metabolic and endocrine abnormalities, some due to the disease itself and some due to treatments (17, 171). Lipid abnormalities in SCD patients have been extensively studied and demonstrated since the 1960s.

The most prominent of these abnormalities is undoubtedly decreased cholesterol levels that are often lower than reference ranges (hypocholesterolemia) (172-175). In terms of concentrations in lipoprotein fractions, cholesterol concentrations in the HDL and LDL particles have also been demonstrated to be reduced (176, 177). The definite cause of hypocholesterolemia in SCD remains unknown; however, authors have reported that hypocholesterolemia is partially caused, or associated with various mechanisms, including hemolysis (178), vascular dysfunction (179), lipoprotein oxidation (180, 181), increased clearance of LDL (102), inflammation and the subsequent pro-inflammatory transformation of HDL (178, 182). Therefore, the typical characteristics of SCD, namely hemolysis, inflammation and oxidative stress could contribute to the hypocholesterolemia seen in patients with SCD.

Publications throughout the world unanimously agree that the lipid profiles of patients with SCD show significant alterations and have explored their importance (183-193). Increased levels of triglycerides (194, 195) and phospholipids (196), and decreased levels of lecithin-cholesterol acyltransferase (LCAT) (197), Apo A1 (197-199), and apolipoprotein B (Apo B) (197, 198) are among the well-established findings in SCD. Lipids and their physiological equilibrium are crucial for life due to their unquestionable role in membrane structure and rheology (200). Therefore, changes in lipid equilibrium and metabolism may be a part of SCD pathophysiology.

It is also important to broaden our perspective when evaluating these changes, as HDL cholesterol (HDL-C), usually recognized as the ‘good cholesterol’ due to its anti-inflammatory properties, shows remarkable changes when inflammation is present in the circulation and may be a source of pro-inflammatory effects when Apo A1 (main protein in HDL structure) is damaged by oxidative stress (201). Furthermore, as mentioned before, SAA is known to replace Apo A1 in the structure of HDL during inflammation (202, 203). Considering that patients with SCD have significant chronic

inflammation, the formation of pro-inflammatory HDL may exert adverse effects in patients with SCD. However, this hypothesis remains to be tested and determining whether HDL and/or its alterations contribute to SCD pathophysiology also remains as an important question.

2.6.4. Oxidative Stress

Hemoglobin is a pro-oxidant molecule that generates ROS (204); however, erythrocytes are normally armed with various anti-oxidant mechanisms, including catalase, glutathione peroxidase and superoxide dismutase, which protect the erythrocyte and other tissues from potentially injurious levels of oxidative stress (205). However, in SCD, the activation of chronic inflammation, recurrent ischemia-reperfusion injury, depletion of NO, presence of cell-free Hb, heme and iron, and even the increased autoxidation of HbS itself cumulatively result in increased oxidative burden (159, 206, 207). As such, the sickle erythrocyte, which is already under significant stress due to formation of HbS polymers, membrane abnormalities and increased adhesion, is faced with another survival challenge: oxidative damage. The oxidative stress-related injury is not limited to erythrocytes, as free Hb, heme and iron released from the erythrocytes oxidize lipids, lipoproteins, endothelium and the cellular components in the blood (208), leading to *in vivo* production of ROS and/or pro-oxidant compounds (159). Studies which have evaluated the levels of antioxidant enzymes in SCD have reported reduced levels of glutathione peroxidase and catalase (209, 210), while conflicting findings exist for superoxide dismutase with reports of lower (211), and also surprisingly higher levels (212) compared to controls. However, superoxide dismutase, catalase and glutathione peroxidase all have to function normally in order to reduce oxidative stress (213). For instance, superoxide dismutase performs the first step of conversion (O_2^- to H_2O_2), which does not necessarily cause a significant reduction in oxidative stress as H_2O_2 is also pro-oxidant. Therefore, an increase in superoxide dismutase levels cannot reliably reduce oxidative effects without adequately functioning catalase and glutathione peroxidase.

Apart from the erythrocyte and blood, the vasculature and other particles in circulation, such as HDL and LDL, are exposed to oxidative damage. As mentioned before, oxidation of the HDL particle transforms the normally anti-inflammatory HDL

to a pro-inflammatory state. Pro-inflammatory HDL levels have been shown to be correlated with LDH levels in SCD and were elevated in those with pulmonary hypertension compared SCD patients that did not have pulmonary hypertension (214). Furthermore, the LDL of SCD patients was shown to have higher susceptibility to oxidation (215), while it has been demonstrated that oxidized LDL was elevated among individuals with SCD (216). Thus, it is feasible to assume the existence of other mechanisms by which oxidative stress contributes to disease pathophysiology in SCD.

Measuring total oxidative stress (or burden) is very difficult as ROS have very short half-life; thus complicating measurement and causing inaccuracies (217). However, measurement of total antioxidant capacity is a relatively easier task. Various studies that have assessed antioxidant levels in SCD patients have shown marked decreases compared to controls (64, 218), while studies which directly measured ROS levels or compounds associated with oxidative stress (such as malondialdehyde) showed significant increases (106, 219). Furthermore, excessively reduced antioxidant capacity leads to worse prognosis in SCD (218).

Human blood has various other mechanisms (apart from those detailed above) to deal with pro-oxidant molecules, such as haptoglobin –which binds Hb, and hemopexin –which scavenges heme (220, 221). However, these defense mechanisms may be overloaded due to the chronic hemolysis in SCD. In this context, antioxidant treatments in SCD have been explored for their efficacy; while some have shown promise in mostly *in vitro* studies (222, 223), the majority of clinical studies have failed to demonstrate positive effects (224, 225).

2.6.5. Oxysterol Species

Oxysterols are oxygenated derivatives (sometimes referred to as by-products) of cholesterol. Non-enzymatically produced oxysterols are a result of interactions between cholesterol or cholesterol by-products and various compounds including ROS and lipid peroxides (226).

The alteration of cholesterol levels in SCD may affect the type and level of oxysterols formed through both enzymatic and non-enzymatic pathways; however,

quantification of non-enzymatically produced oxysterols may be a relatively reliable method of determining the levels of oxidative stress (227). Furthermore, oxysterol species also have various effects on cellular structure and several physiological processes, including lipid bi-layer (in)stability, endothelial (dys)function, cholesterol production, eryptosis, inflammation and ROS production (228-231). Therefore, the levels of non-enzymatically produced oxysterols, in addition to showing oxidative stress, may contribute to pathophysiological mechanisms in SCD. For this purpose, determination of the levels of 7-ketocholesterol (7-KC, which is directly produced in response to oxidation) and cholestane-3β,5α,6β-triol (C-triol, which is enzymatically produced from 5,6 epoxycholesterol) are reportedly sufficient in determining oxysterol production via non-enzymatic processes (232, 233). Even though C-triol itself is produced enzymatically from 5,6α- and 5,6ß- epoxycholesterols (234, 235), this does not change the fact that it is an accurate marker of non-enzymatically produced oxysterols since the 5,6 epoxycholesterols represent the majority of non-enzymatically produced oxysterols as they are readily formed in the presence of oxidative stress (236). The molecular structures of these oxysterols are depicted in Figure 2.5., adapted from (237).

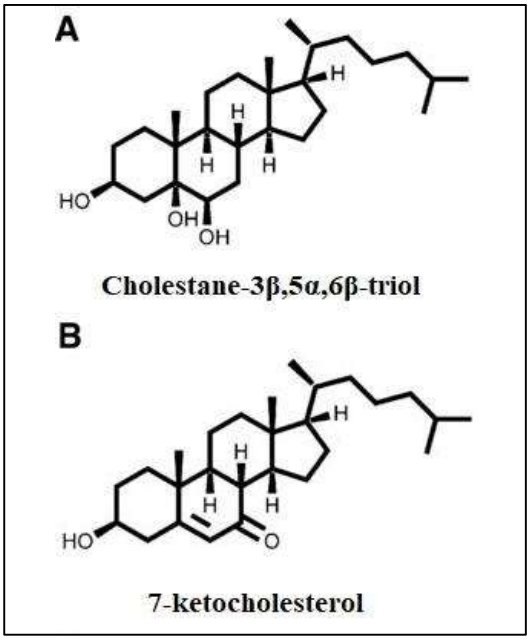

Figure 2.7. Molecular structure of A) C-triol and B) 7-KC (237).

There have only been a few attempts to investigate the role of oxysterols in SCD (238, 239). Even though these studies reported interesting findings, such as increased levels of various oxysterols in the erythrocyte membranes of patients (238), it seems that there has been limited interest for further investigations concerning these results. However, considering the constant oxidative insult in SCD, and the fact that levels of 7-KC and C-triol are identified as accurate measures of circulatory oxidative stress, it is evident that the identification of possible associations concerning oxysterol levels and other laboratory characteristics would contribute to the determination of their effects on the pathophysiology of SCD.

2.6.6. Ceramide Species

Ceramides are bioactive lipids that are comprised of a sphingoid base (most commonly sphingosine, C18-20) and a fatty acid chain (often varying between 2–36 carbons) that are linked with an amide bond (Figure 2.8., adapted and modified) (240, 241).

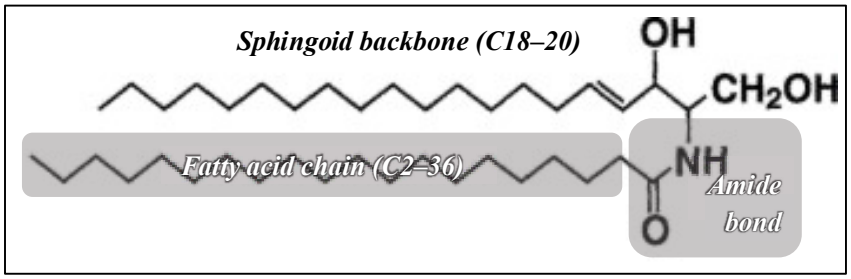

Figure 2.8. Molecular structure of a ceramide (241).

Ceramides are known to have significant influence on cell survival (242), in addition to their roles in other pathways (generally as a second messenger) associated with lipid signaling and sphingolipid metabolism (243). They are very prevalent in cellular membranes due to their presence in the structure of sphingomyelin, and therefore, significantly contribute to the structure and function of these membranes (240). Ceramides are grouped with regard to the length of the fatty acid (acyl chain) that is linked to the sphingosine backbone, which is a justified method of classifying these molecules as it has been shown that their bioactive characteristics (and synthesizing enzymes) are closely linked to the length of these chains (244). The *de*

novo synthesizing enzymes are collectively named ceramide synthases (CerS) 1–6; however, the type of ceramide synthesized is dependent on the specific type of CerS. For instance, C16-ceramide and C18-ceramide are selectively synthesized primarily by CerS5 and CerS1, respectively; whereas, very-long-chain ceramides (C22 and greater) are synthesized by CerS2 and CerS3 (245). However, what is quite noteworthy in terms of ceramide production is that ceramides are also directly released from membranes through the activity of sphingomyelinases (246).

As mentioned before, ceramide species have been associated with the activation of eryptosis (122). However, there are few studies that investigated various ceramides in SCD. A study by Aslan and colleagues (122) showed decreased very-long-chain ceramide concentrations (C22 and C24) in serum samples obtained from SCD patients (247); however, they found no difference in the levels of C16–C20 ceramides which are the ceramide species that have been established to have a major role in apoptosis and eryptosis (122). Despite the limited number of reliable studies that have explored ceramide levels in SCD, several studies seem to agree that the activity of sphingomyelinases (the enzymes that catalyze the hydrolyzation of sphingomyelin to ceramide) are increased in SCD (247, 248), supporting a role for ceramide-related regulation of erythrocyte membrane damage, eryptosis and subsequent hemolysis.

2.7. Treatment

Despite definitive establishment of the biochemical basis SCD and extensive studies, therapeutic options for SCD remain limited. Only a small portion of sickle cell patients benefit from hematopoietic stem cell transplants (249, 250). The great majority of patients are managed with only a few effective medications and blood transfusions (251-253).

Hydroxyurea (hydroxycarbamide) is the primary medication. It induces HbF production and is believed to be a donor of NO (254). Since receiving approval from the FDA (255), it has demonstrated remarkable benefits in patients, including fewer clinical manifestations, lesser pain and better overall prognosis. However,

hydroxyurea does not target the molecular basis of the disease or its pathophysiologic results and therefore does not prevent the progression of disease (256).

Many studies have been exploring various drugs that target the major characteristics of SCD, such as cell adhesion (257-259), inflammatory pathways (260-262), sickling (263, 264), other more reliable forms of HbF induction (265) and coagulation mechanisms (266, 267), in mostly Phase 1 and 2 studies. The results of these studies vary greatly and there is a need for further research to identify reliable treatment options in SCD; because, effective as it is, a large group of patients (13–30%) may not respond to hydroxyurea treatment (termed as non-responders) (268, 269). A new oral drug option, L-glutamine, has recently received FDA approval after it emerged as a safe treatment modality that significantly reduced acute complications according to a phase 3 trial (270). Additionally, recent advances in genetic modification and gene therapy have also proved to be promising, as one patient was reported to have complete clinical remission with lentiviral transfer of a modified ß-globin gene which enabled the encoding of an anti-sickling Hb variant (23).

2.8. Liquid Chromatography and Mass Spectrometry

2.8.1. Liquid Chromatography

Liquid chromatography (LC) is a method by which a liquid solvent mixture (called the mobile phase) flows through a column comprised of specific types of adsorbent material (which is called the solid phase), resulting in differential separation of analytes that were initially dissolved in the mobile phase (271, 272). The differential separation is associated with the complex chemical and physical relationships between the analyte, mobile phase and solid phase (273-275). After separation with LC, the targeted analytes must be detected and measured with precision. This is accomplished through the use of various detector types, such as laser light scatter, evaporative light scatter, fluorescence, infrared/ultraviolet light, refractive index, flame ionization, electron spin resonance, magnetic resonance, and thermal conductivity detectors. Each detector and detection technique has its advantages as well as limitations; thus, the optimal method is largely based on the type of analyte being examined, and in some cases, the specific analyte in question (276).

2.8.2. Mass Spectrometry and its Use in Lipid Quantification

Mass spectrometry (MS) is one of many detection methods that perform quantification after chromatographic separation. However, MS is very accurate because its quantification is reliant on specific mass (m) and charge (z) of analytes in a vaporized sample. It was first coupled with gas chromatography (GC) due to the availability and ease of use with gas-phase solvents in mass and ion detection (277). There are six different mass analyzer types that are currently being used with various advantages and disadvantages: quadrupole, time-of-flight, electrostatic sector, ion-trap, magnetic sector, and ion cyclotron resonance (278).

The reliability and accuracy of MS detection is a crucial advantage that ensures selectivity to mass and ion forms, and also enables the measurement of extremely low concentrations of target analytes (279). Mass spectrometry was later implemented into detection of analytes after LC separation with advances in MS methods, especially following the introduction of the electrospray ionization (ESI) technique which made the coupling of LC separation to MS detection (LC-MS) affordable, reliable and relatively easy to use (280). Shortly after, the use of LC-MS (with ESI) was suggested for lipid quantification (281); thus, a whole new generation of lipid analysis began with developments leading to the simultaneous detection of various types of lipids from a sample –giving birth to a field named 'lipidomics' (282). Since then, LC-MS methods with ESI have become widely adopted methods for the determination of various biological compounds and drugs in analytical chemistry (283-285). Progressive development of these methods resulted in the use of what is called 'tandem MS' (MS/MS), that separated the identification and detection processes of analytes (one MS for each), resulting in further increased accuracy (286).

In lipid detection and measurement, LC separation with tandem MS (referred to as LC-MS/MS hereon) has all but become primary preference for quantification (mostly through ESI) (287, 288). Although some groups still report better accuracy for various compounds with GC-MS/MS (289, 290), and suggest this method as the gold-standard approach, comparative studies of these two methods have revealed near-identical accuracy for many lipids and reviews have also reported similar accuracies with the two methods (291-293). Nevertheless, it must be kept in mind that variations

in these accuracies do exist, and some analyses require the selection of either LC or GC for best accuracy (294-296).

Lipidomics has historically been divided into two approaches: 'shotgun lipidomics' in which MS detections are used to scan all analytes (without chromatographic separation), resulting in the semi-quantitative or qualitative detection of a large number of metabolites, while more selective methods with MS analyzers that are set in specific conditions result in the quantitative analysis of fewer analytes (297). In LC-MS/MS with ESI, the sample obtained through chromatography flow is constantly ionized, separating it into various ions with specific molecular weight and charge. The first MS transfers ions with specific m/z value into a second high-vacuum chamber where collisions occur, leading to the production of product ions. The second MS then detects and further selects these product ions, finally transmitting it to the quantification step, again depending on previously identified m/z values. The result is the selective and accurate quantification of analytes that respond in a specific manner to the settings of the MS detection system. The quantification is extremely reliable and repeatable due to the use of streamlined separation, selection and detection processes made possible by LC and MS coupling.

2.8.3. Sample Preparation for Lipid Quantification

Due to the low molecular weight and small functional-unit and polarity differences between various lipid compounds (even within a subgroup of lipids), the samples obtained for measurement usually have to undergo a preparation step prior to chromatographic separation (298). The simplest and most widely used of these preparation methods are various 'lipid extraction' methods that have been perfected (and compared) according to sample types, target metabolites and various lipids throughout the last 50 years (299-301). The most current and frequently used of these methods can be listed as follows:

- Modified Folch method (302),
- Modified Bligh & Dyer method (303),
- Methyl tert-butyl ether (MTBE) method (304),
- Soxhlet method (305).

However, due to various factors that affect the composition, isolation and characteristics of lipids through the use of each of these methods, usually each LC-MS/MS method employs a fine-tuned method of lipid extraction that is considered best for the target analyte(s) (306).

3. PATIENTS AND METHODS

3.1. Chemicals, Assay Kits and Instruments

Table 3.1. Chemicals list.

Chemical	Supplier and catalog number
3ß-hydroxy-5-cholestene-7-one	Avanti, 700015
3ß-hydroxy-5-cholestenoate-d7	Avanti, LM4103
3ß,5α,6ß-trihydroxycholestane	Toronto Res. Chem., T795100
3ß,5α,6ß-trihydroxycholestane-d7	Toronto Res. Chem., T795102
D-erythro-sphingosine-1-phosphate	Avanti, 860492P
D-erythro-sphingosine-d7-1-phosphate	Avanti, 860659P
N-palmitoyl-D-erythro-sphingosine	Avanti, 860516P
N-stearoyl-D-erythro-sphingosine	Avanti, 860518P
N-arachidoyl-D-erythro-sphingosine	Avanti, 860520P
N-palmitoyl-D-sphingosine	Cambridge Isotope, CLM-9582
Acetonitrile, HPLC Grade	Sigma-Aldrich, 34851-2.5L
Methanol, HPLC Grade	Sigma-Aldrich, 34885-2.5L-R
Isopropanol, HPLC Grade	Sigma-Aldrich, 34863-2.5L
Hexane, HPLC Grade	Sigma-Aldrich, 34859-2.5L
Hydrochloric Acid, 37%	Sigma-Aldrich, 30721-2.5L
Ammonium formate, HPLC Grade	Fluka, 17843-250G
Formic acid, 98%	Sigma-Aldrich, 33015
Chloroform, HPLC Grade	Sigma, 34854
Ammonium acetate, HPLC Grade	Merck, 101115
Methyl tertbutylether, 99.8%	Merck, 101845
Ethyl acetate, 99.8%	Sigma-Aldrich, 270989
4-Methylumbelliferyl βD-N,N,N”-triacetylchitotrioside	Merck, M5639
Sodium citrate dihydrate	Sigma-Aldrich, W302600
Monosodium phosphate	Sigma-Aldrich, S9638
Disodium phosphate	Supelco, 1.06586
Glycine	Sigma-Aldrich, 410225
Sodium hydroxide	Supelco, 106462

Table 3.2. Assay kits list.

Kit	Parameter Measured	Supplier and catalog number
Hemin Assay Kit	Hemin	Abcam, ab65332
FFA Quantification Assay Kit	Free fatty acids	Abcam, ab65341
SAA Human ELISA Kit	Serum amyloid A	Abcam, ab100635
MPO Human ELISA Kit	Myeloperoxidase	Abcam, ab119605
Human LCAT Activity Kit	LCAT Activity	Elabscience, EL168239 (discontinued)

Table 3.3. Instruments list.

Instrument	Supplier
Nitrogen Evaporator	Sigma
Nitrogen Evaporator, automated	VLM
Protein Chemistry Analyzer, IMMAGE 800	Beckman Coulter
Chemistry Analyzer, AU 680	Beckman Coulter
Refrigerated Centrifuge, NF 400R	Nüve
6420 triple quadrupole mass spectrometer	Agilent
1260 Infinity UPLC system	Agilent
LCMS-8040 triple quadrupole mass spectrometer	Shimadzu
LC-20 XR UFLC system	Shimadzu
Xterra MS 18 reverse-phase C18 column	Waters
Symmetry reverse-phase C18 column	Thermo Fisher Scientific
ACE reverse-phase C18 column	ACE

3.2. Study Groups

The first study group was comprised of 35 steady state pediatric SCD patients (27 HbSS, 8 HbSß$^{+}$) and 19 healthy pediatric controls. All patients were enrolled at the Pediatric Hematology Department of Mersin University Hospital, Mersin, Turkey. The samples obtained from this study group were used for the evaluation of lipid profile and its associations with hemolysis, oxidative stress and chronic inflammation. In other words, the first study group focused on the relationships between clinical characteristics, lipid profile, anemia/hemolysis, inflammation and oxidative stress.

The investigations involving oxysterols and ceramides were performed in a second study group comprised of 32 pediatric SCD patients (22 of these patients were diagnosed with HbSS, while 10 were diagnosed with HbSß$^{+}$) and 25 healthy children. The confirmation of subgroup diagnoses was performed via Hb electrophoresis, chromatography methods and/or mutation analyses when needed. There was a 1-year time interval between the collection of samples from the two study groups. Both collection dates were in the early weeks of fall; the fact that samples were collected exactly 1 year apart and in the same season ensured that patient groups were similar with regard to environmental factors that could influence clinical characteristics, laboratory parameters and the frequency of infections, clinical manifestations or disease complications (39).

The control groups were selected from healthy pediatric-age patients from the pediatrics clinic that were undergoing routine check-up, and these were matched with the patients for age and sex. Each control group was enrolled at the same time with their respective SCD patient groups (again, there was a 1-year time interval between the enrollment of the two control groups). In the control groups, we excluded any family that declined participation. We also checked if these patients had applied for any symptoms that could be associated with chronic diseases or acute infections. Patients that had complaints suggestive of acute or chronic diseases, those using any type of medication and those that were prescribed any medications after their routine visit were excluded from the study.

3.2.1. Inclusion and Exclusion Criteria of Patients with SCD

Inclusion of patients was based on the fulfilment of the following criteria (Inclusion criteria):

- Being a long-term patient (at least 3 years) and attending all follow-up investigations during the past year,
- Strictly following physician recommendations and complying to the recommended use of all medications,
- Having HbSS or HbSß$^{+}$,
- Not having suffered from any crises for at least three months,

- Not receiving transfusion(s) for any cause for at least three months,
- Acceptance to provide blood samples and approving participation in the study as a whole.

Approval from children were obtained verbally, while all parents provided written informed consent.

We also excluded patients in the presence of:

- Having a history of, or being diagnosed with any chronic disease,
- Being suspicious for any type of active infection as per the results of medical history and clinical examinations performed on the day of planned blood withdrawal,
- Refusing blood sample withdrawal or participation in the study for any reason,
- Withdrawing from the study at any point.

3.3. Ethics

Ethics approval was provided by the Clinical Research Ethical Committee of Mersin University (Decision date: 28.05.2014, decision number: 2014/115). Written consent was obtained from every parent and included the fact that they were accepting sample withdrawal, study inclusion and use of data in the present study. Each and every study/research step was in conformity with the "Helsinki Declaration" (with its most recent amendments). The Good Clinical Practice guidelines were also followed for the entirety of the study.

3.4. Clinical Characteristics, Disease Manifestations and Complications

In the first study group, all subjects with SCD were recipients of hydruxyurea treatment. Whereas, in the second study group, 4 patients from the HbSS group and 1 patient from the $HbS\beta^{+}$ group did not receive hydroxyurea treatment. The clinical characteristics and complications recorded in the first group were as follows:

- Painful episode (vaso-occlusive event),

- Acute chest syndrome (ACS),
- Cerebrovascular event (CVE),
- Transfusions,
- Renal complications,
- Endocrine complications,
- Cardiac complications.

Since the presence of crises or transfusion within the last 3 months were considered as basis for exclusion, the presence of these two manifestations were recorded until the last 3 months before study inclusion (as none of the patients included had these manifestations within the last 3 months). All remaining manifestations were recorded for the whole year prior to blood withdrawal.

The division of patients according to clinical manifestations and complications were done to compare the HbSS and HbSß$^{+}$ groups, as well as to identify patients' clinical problems. Grouping for painful episodes (crises) were performed with regard to vaso-occlusive episodes suffered within the prior year (a total of three groups: none, 1-5 times, and >5 times), while other disease manifestations and complications were evaluated on a present/absent basis.

Painful episodes were identified pain that necessitated analgesia. The presence of ACS was defined as the identification of conclusive imaging findings for pulmonary infiltration or atelectasis in patients that had presented with respiratory symptoms. Cerebrovascular events (CVEs) were diagnosed with magnetic resonance imaging of the brain.

Renal complications were defined as an increase in creatinine values that indicated renal injury or renal failure, or fulfilling any of the injury types put forth by the Pediatric Risk, Injury, Failure, Loss, and End-stage Kidney (pRIFLE) criteria (307).

Endocrine complications were defined as the development of any of the following metabolic/endocrine abnormalities (308):

- Vitamin D deficiency,

- Insulin resistance,
- Growth hormone deficiency,
- Subclinical hypothyroidism,
- Advance from subclinical hypothyroidism to clinical hypothyroidism,
- Hypogonadism,
- Weight loss resulting in body mass index values lower than the third percentile according to age.

Cardiac complications were defined as the development of any of the following conditions (309):

- Electrocardiogram findings indicative of heart muscle damage,
- Elevated levels of high sensitivity Troponin I,
- Increased systolic pressure in the pulmonary artery,
- Pulmonary hypertension,
- Diastolic heart disease of the left ventricle,
- Arrhythmia.

3.5. Sample Collection

From each patient, three tubes (vacutainer) of blood were collected after 8 hours of fasting. All blood samples obtained from patients and controls via forearm venipuncture were drawn into one serum-separator tube and two EDTA-containing tubes with a volume of 5 mL. One EDTA-containing tube was immediately sent for complete blood count measurements (routine devices, Mersin University Hospital). Notably, S1P concentration may increase while blood samples remain in collection tubes; therefore, tubes were put on ice immediately after collection and were kept in an ice box until centrifugation could be performed (within 25 minutes). Plasma samples were obtained from the second EDTA-containing tube, while serum samples were obtained from serum-separator tubes, by centrifuging at 4000 RPM (10 minutes) in a refrigerated centrifuge (4 °C) (NF 400R, Nüve, Ankara, Turkey). After the completion of centrifugation, aliquots of samples were obtained (3 aliquots with volumes of 0.2–0.5 mL). All aliquots were numbered and coded accordingly, followed

by freezing at -80 °C in a cryobox, and subsequent transfer to Hacettepe University via cold-chain shipping. The aliquots remained in -80 °C storage for a maximum of 2 months (for both sets of study groups), during which all analyses and laboratory investigations were completed. The schematic representation of the sampling protocol, manipulations, acquisition of plasma and serum, transfer, and quantification plans of investigated parameters are depicted in Figure 3.1.

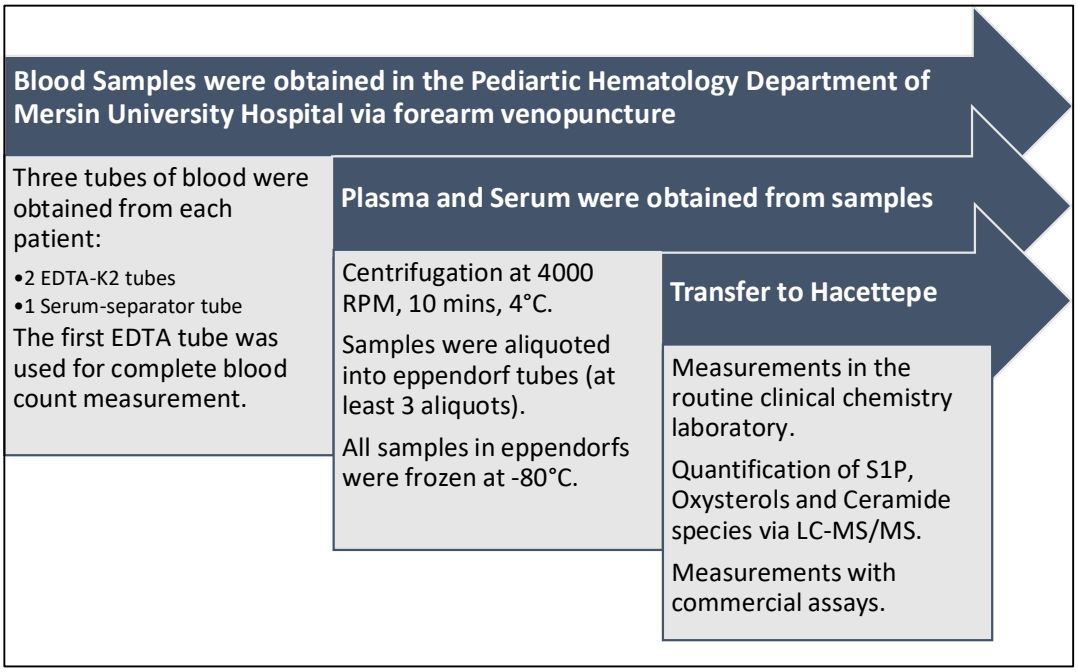

Figure 3.1. Flowchart of the blood sampling and measurement plan.

3.6. Laboratory Measurements

The laboratory measurements performed in the two study groups are comparatively summarized in Figure 3.2.

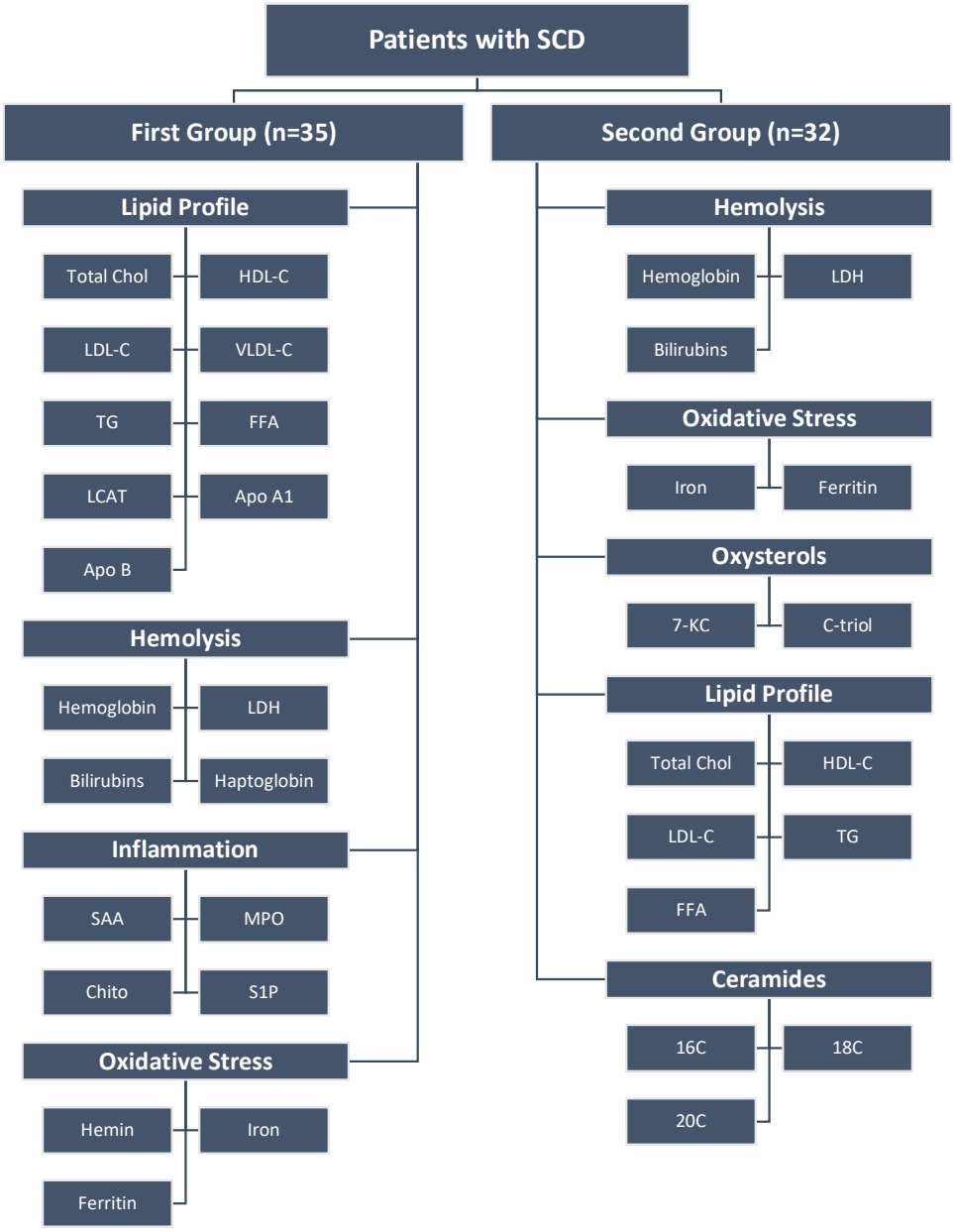

Figure 3.2. Study plan and laboratory analyses performed in each group.

3.6.1. The Measurement Principles of Commercial Assay Kits

Enzyme-Linked Immunosorbent Assay (ELISA)

This method quantitatively determines the level of a specific compound in a sample. It is based on target-specific antibodies that bind the antigen and indirect detection of concentration (310, 311). This detection is achieved by comparative quantification (with a standard graph) of the activity of enzymes that are covalently attached to the antibody, most commonly horse-radish peroxidase; however, kits that employ the enzyme alkaline phosphatase are also frequently found (312, 313). There are various approaches to this assay: direct, indirect, sandwich and competitive ELISA (314). Today, the most reliable and common type is sandwich ELISA which has the advantage of better quantification results due to being relatively more specific. Sandwich ELISAs employ a 2-step binding method: the first being an antibody that is covalently bound to the solid surface of the measurement plate (capture antibody), and binds the antigen. After a washing phase that clears unbound compounds, the second antibody (detection antibody) is pipetted and again binds the targeted antigen (hence the name, sandwich). This latter antibody is conjugated with a molecule or enzyme that enables colorimetric or fluorometric quantification via spectrophotometry (315). All assay kits used in this study used horse-radish peroxidase-conjugated antibodies.

Activity Assays

This type of assay is generally dependent on the activity of a specific enzyme. This enzyme may either be the targeted enzyme for measurement, an approach which is based on the quantification of the reduction in the level of substrate or increase in the concentration of product; or it could be that the enzyme used is affected by the concentration of the target analyte (inhibition or activation); thus, leading to quantification via the determination of activity in the presence and absence of target analyte. In both approaches, a standard calibration graph is used for the determination of the level of the target analyte and usually involves a normalization step (316).

3.6.2. Hematological Parameters

Hematological parameters, including complete blood count and Hb types, were measured in the routine biochemistry laboratory of Mersin University Hospital.

3.6.3. Lipid profile

The routine lipid profiling of all patients were performed with the same autoanalyzer and the same commercial kits at the Clinical Chemistry Laboratory of Hacettepe University Hospital. The AU 680 Chemistry Analyzer (Beckman Coulter, USA) device was used for the quantification of triglycerides (TG) and cholesterol fractions (HDL cholesterol, HDL-C; LDL cholesterol, LDL-C; VLDL cholesterol, VLDL-C) as well as total cholesterol (TC). In addition to routine measurements of TG and cholesterols, we determined the levels of Apo A1 and Apo B with the nephelometric method via the utilization of a different automated analyzer in the same laboratory (Protein Chemistry Analyzer, IMMAGE 800, Beckman Coulter, USA). Other elements of our lipid profile were free fatty acid (FFA) levels and LCAT activity. Commercially available kits for FFA concentration (Abcam, United Kingdom) and LCAT activity (Elabscience, China) were ordered and measurement of these two parameters were performed within the same time period of other lipid profile measurements.

The ratio of TG and HDL-C (TG/HDL-C), which has been associated with endothelial dysfunction in SCD (317), and SAA–Apo A1 ratio (SAA/Apo A1), identified as a ratio that has a relationship with vaso-occlusive events (164), were also calculated.

3.6.4. Hepatic function and others

Although hepatic function was not a primary focus of the present study, we determined the concentrations of alanine aminotransferase (ALT, which is a liver function enzyme) and gamma-glutamyl transferase (GGT, which is a liver function enzyme and also demonstrates increased levels in biliary conditions) in order to be able to evaluate whether important changes had occurred in liver function as a whole (including biliary ducts) (318). Additionally, we measured uric acid levels as this

parameter has been shown to be a trigger of inflammation and also a measure of renal tubular function (319). These three parameters were also quantified in the clinical chemistry laboratory.

3.6.5. Hemolysis

The assessment of hemolysis was based on the results of lactate dehydrogenase (LDH), total bilirubin and direct bilirubin concentrations. In addition to these well-known and readily available markers, we also measured haptoglobin (Hpg) levels with autoanalyzers in our clinical chemistry laboratory with the same devices in order to be able to observe whether the chronic hemolysis was overwhelming to the Hpg defense mechanism in the steady state of the disease.

3.6.6. Oxidative stress

Hemin, iron and ferritin levels were quantified as measures of oxidative stress. A commercially available kit was utilized for the quantification of hemin concentrations (Abcam, United Kingdom), while serum iron levels and ferritin concentrations were measured in the clinical chemistry laboratory with the routine devices. The levels of oxysterols that are associated with oxidative burden were also measured via LC-MS/MS; however, oxysterol quantification is explained in detail below.

3.6.7. Inflammation

Inflammatory activity was determined through the quantification of serum amyloid A (SAA) and myeloperoxidase (MPO) levels, and also through measurement of chitotriosidase activity. Sphingosine 1-phosphate levels were also defined as a measure related to inflammation; however, the measurement of S1P was performed via LC-MS/MS, and is explained later. The quantification of SAA and MPO were done via ELISA kits (Abcam, United Kingdom). Chitotriosidase activity was measured manually, via a method that uses a fluorometric substrate, reported by Hollak et al. (320).

Briefly put, the method for chitotriosidase measurement was as follows:

1. Prepare enough tubes for the number of samples measured.
2. Prepare fresh 100 µl of 0.022 mM 4 MU-chitotrioside (also known as: 4-methylumbelliferyl ß–D-N,N',N''–triacetyl-chitotriose) in 0.1 M citrate / 0.2 M phosphate buffer (pH = 5.2).
3. Add 5 µl of plasma sample and incubate for 15 minutes at 37 °C.
4. Add 2 ml glycine/NaOH buffer (0.3M, pH = 10.6) to quench the reaction.
5. Immediately measure absorbance value at 445 nm wavelength via a spectrophotometer.
6. After obtaining nmol/ml values (for 15 minutes) according to standards and dilution, calculate 1-hour values (multiply by 4).
7. Results are reported as nmol/hour/ml.

3.6.8. Quantification of Sphingosine 1-phosphate

The method we employed for the quantification of S1P was first established during the course of this thesis study (in 2016). This method was later utilized by various other centers and departments as a simple and fast method of S1P detection through LC-MS/MS (321). Briefly, the method was as follows: First, 50 µl plasma from each subject was mixed with 20 µl erythro-sphingosine-d7 (1000 ng/ml) (Avanti, USA) was added. Extraction was performed with the addition of methanol (500 µl) into the mixture, followed by a short vortex and centrifugation at 3000 g for 10 minutes. Taking care not to disturb the pellet, 400 µl's of supernatant was drawn from each sample and 20 µl of this sample was used for the injection in the LC step. Known amounts of S1P were used to obtain the quality control samples of 5 ng/ml and 40 ng/ml (final concentrations).

An Agilent 1260 Infinity UPLC System that included a refrigerated auto-sampler was used for chromatography. The column was an ACE C18 (50 × 4.6 mm) reverse-phase column that consisted of a particle size of 5 µm and its temperature was set to 30 °C. Mobile phases (solvents) were pumped with a binary pump, and were as follows: Solvent A was 0.01% formic acid and 10 mM ammonium acetate in water, solvent B was 0.01% formic acid in methanol. Gradient elution conditions are given in Figure 3.3. (0.5 ml/min flow-rate, 8-minute runtime).

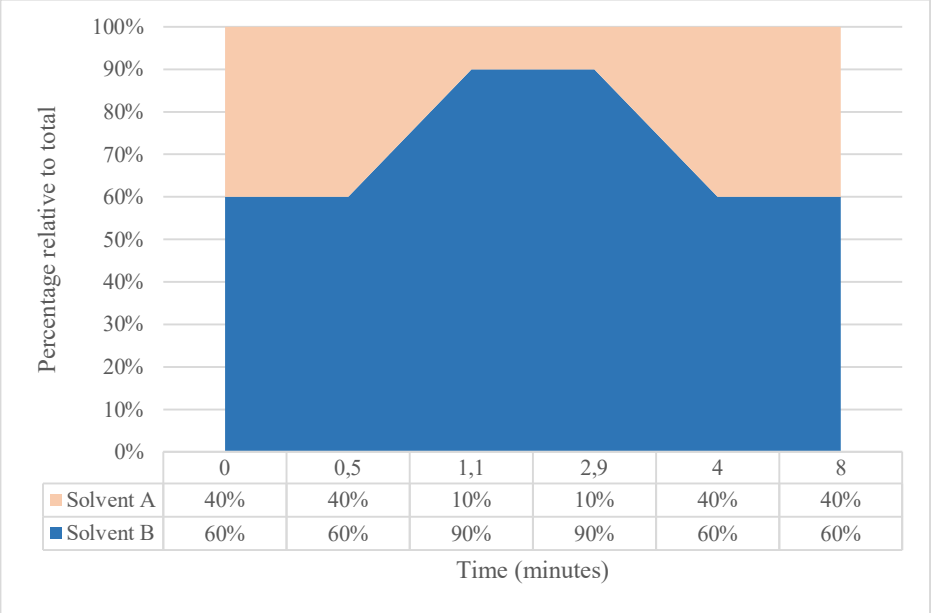

Figure 3.3. Elution conditions for the LC step of S1P quantification.

An Agilent 6420 MS (triple quadrupole) with ESI was used for MS detection and quantification via multiple reaction monitoring (MRM). The gas was at 350 °C, while its flow-rate was 12 liters/min. Nebulizer: 25 psi, capillary: 3500 Volts. Positive ion pairs were used for detection. All MS settings specific to the detection of analytes are given in Table 3.4.

Table 3.4. MS settings for S1P detection.

Analyte	Precursor	Product	Dwell	Frag (V)	CE (V)	Polarity
S1P	380.2	264.3	100	90	12	+
S1P-d7	309.1	164.8	100	60	20	+

Dwel: dwell time, Frag: fragmentation, V: volts, CE: collision energy.

Quantification was performed with the use of an 8-point calibration curve. Calibrators were prepared from a 40 ng/ml stock with serial 2X dilutions until 0.312 ng/ml. The calibration graph with an R^2 value of 0.998 is shown in Figure 3.4.

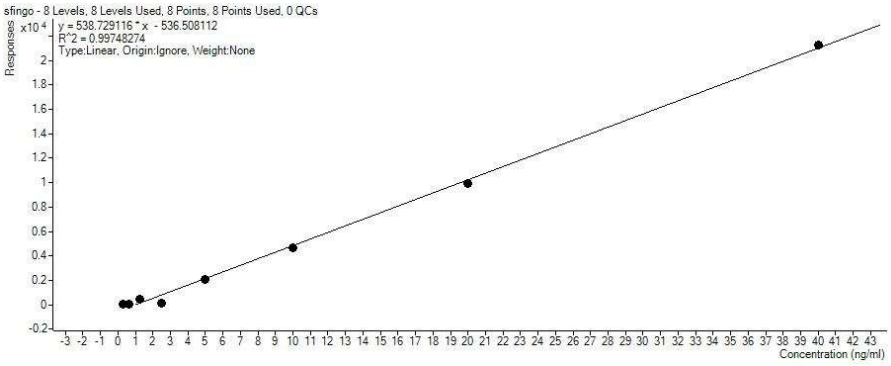

Figure 3.4. Calibration graph used for S1P measurements.

3.6.9. Quantification of Oxysterols

The quantification of the two oxysterols (7-KC and C-triol) through LC-MS/MS was done with a method first put forth by Jiang et al. that had received minor modifications. The initial extraction was done from 50 µl of plasma samples. The preparation phase of samples included a derivatization step with N,N-dimethylglycine, which increases accuracy and reproducibility. The QC samples (quality control) were obtained by preparation of 40 ng/ml and 150 ng/mL (for both 7-KC and C-triol) and also by the use of patient-derived samples (with known levels of both oxysterols) that underwent the same preparation phase. A deuterated (d7) form of 3ß,5α,6ß-trihydroxycholestane (TRC) and a d7 form of 3ß-hydroxy-5-cholestenoate (Avanti) were used and added as internal standards to samples. An 8-point calibration curve (with 2X dilution from 400 ng/ml to 3.12 ng/ml) for both analytes was utilized to quantify the levels through a standard graph that had an R^2 value of 0.996.

Liquid chromatography was undertaken with the use of a reverse-phase column manufactured by Thermo Fisher Scientific (Symmetry C18, 100 × 2.1 mm, 5µm particle size). The solvent used for the mobile phase was singular, and was prepared at a pH of 3 from acetonitrile and water with the addition of ammonium formate at a concentration of 1 mM. Similar to the previous LC-MS/MS methods used in this study, ESI and the MRM mode was used for MS detection with positive ionization. The MS transitions were: 486.0 to 104 for 7-KC and 591.5 to 104 for C-triol, while for the d7 forms, transitions were 493.0 to 104 for 7-KC-d7 and 598.5 to 104 for C-triol-d7 [9].

3.6.10. Quantification of Ceramide Species

From the obtained samples, 100 µl of plasma was diluted to 20 times with ringer lactate and 2 µL internal standard solutions (5 µg/mL) were mixed into the final diluted sample. Internal standard was a labeled C16-ceramide (N-palmitoyl-D-sphingosine-d7, Cambridge Isotope Laboratories, MA, USA). The mixture was vortexed and 375 µL's of 1:2 (v/v) chloroform:methanol was added. The solution was mixed and centrifuged at 2000 RPM (5 minutes). Upper phase was obtained and dried with a nitrogen evaporator (VLM, Bielefeld, Germany). Reconstitution was performed with addition of 100 µL methanol. Calibrator standards were obtained through the same procedure with spiking of known amounts of C16, C18 and C20 ceramides.

After evaporation and drying of samples, calibrators and QC's, chromatographic separation with ultrafast liquid chromatography (LC-20 AD UFLC XR, Shimadzu, Japan) was achieved in a reverse phase C18 column (50 × 2.1 mm) (XTerra 18, MA, USA). Flow-rate: 0.45 ml per minute with the following mobile phases: A: water, acetonitrile and isopropanol (8:1:1, v/v/v) containing 10 mM ammonium formate, and B: acetonitrile and isopropanol (9:1, v/v). The elution gradient used was as follows (A/B): 0–2.0 min: 35/65; 2.0–7.0 min: up to 10/90; 7.0–13.0 min: 10/90; 13.0–30.0 min: 10/90 to 35/65; 30.0–35.0 min: 40/60. Positive electrospray ionization was used in MRM mode for mass spectrometer detection (LCMS-8040, Shimadzu Corporation, Japan). Collision energy of C16-Ceramide was 15 eV and mass transitions were: 538.5–264.40.4 M^+H^+. For C18-Ceramide, collision energy was 15 eV and mass transitions were: 566.3–264.4 M^+H^+. For C20-Ceramide the collision energy was again 15 eV and mass transitions were: 594.6–264.5 M^+H^+. The C16 internal standard had mass transitions of: 554.3–264.3 M^+H^+. 5-point linear calibrators ranged from 39 ng/mL to 625 ng/mL.

3.7. Statistics

The Statistical Package for Social Sciences (SPSS) software for Windows, v25.0 was utilized for analysis of all data obtained throughout the research (IBM, Armonk, NY, USA). P values that were equal to 0.05, or those that were lower than 0.05 were accepted to show significance (2-way). The frequencies (number, n) and

percentage values were used to describe categorical variables, and these were compared with the use of Chi-square tests. For continuous (quantitative) variables, we used the Kolmogorov-Smirnov test (with Lilliefors correction) for the assessment of distribution characteristics. After determining distribution, we provided data with mean ± standard deviation (SD) values or median (min-max) values with regard to distribution normality. The independent samples t-test was utilized to perform 2-group comparisons of normally distributed and parametric continuous variables, and the Mann-Whitney U test was used to compare non-normally distributed and non-parametric continuous variables. Also, the One-way ANOVA or the Kruskal-Wallis tests (depending on distribution normality) were used to perform >2-group comparisons. Post-hoc comparisons, when necessary, were performed according to Bonferroni correction. Evaluation of the relationships between continuous variables were performed with the calculation of coefficients (r) (Pearson or Spearman), with regard to normality of distribution. The effects of highly correlated parameters on primary continuous variables were assessed via linear regression models (stepwise method).

4. RESULTS

4.1. First Patient Group

In this study group, 35 pediatric-aged steady state patients with SCD (27 HbSS, 8 HbSß$^+$) and 19 controls were included in the analysis of the relationships between lipid profile, markers of hemolysis, and parameters associated with oxidative stress and inflammation. The SCD group included 15 girls and 20 boys with a mean age of 13.5 ± 4.1 years. In the healthy children group (controls) there were 9 girls and 10 boys that had a mean age of 13.5 ± 3.5 years. Sex distribution and the mean ages of groups were found to be similar ($p = 0.752$ and $p = 0.642$, respectively). All patients in this group were recipients of hydroxyurea treatment, and none had undergone splenectomy.

4.1.1. Hematological Parameters

We first aimed to identify the statistical differences and similarities between disease subgroups with comparison of the HbSS group and the HbSß$^+$ group in terms of hematological parameters. Mean Hb values were similar in the HbSS and HbSß$^+$ groups (9.3 ± 1.02 g/dl vs. 9.17 ± 1.05 g/dl).

When Hb types (obtained from the most recent patient records, according to results with Hb electrophoresis) were assessed, we found that the distribution of the percentages of Hb types in the HbSS and HbSß$^+$ groups showed significant differences. Hemoglobin S and HbF percentages were significantly higher in those with the HbSS subtype (79.11 ± 7.13 % vs. 66.30 ± 10.67 %, $p = 0.005$; and 14.31 ± 7.45 % vs. 4.93 ± 3.69 %, $p = 0.001$), while those with HbSS had lower HbA_1 proportion (1.87 ± 2.00 % vs. 24.51 ± 11.56 %, $p < 0.001$). Finally, the percentage of HbA_2 levels were similar among the two subtypes ($p = 0.131$).

When the WBC values of the two groups were compared, we found no significant difference between the HbSS (13.3 ± 4.4 x10^3/mm^3) and the HbSß$^+$ (13.2 ± 3.7 x10^3/mm^3) groups. The same was also true for platelet counts. The patients' hematological values and comparison results with regard to disease subgroups are presented in (Table 4.1.).

Table 4.1. Hematological parameters of the HbSS and HbSß$^+$ groups.

Parameters	HbSS (N=27) (Mean ± SD)	HbSß$^+$ (N=8) (Mean ± SD)	P-value
Hemoglobin (g/dl)	9.30 ± 1.02	9.17 ± 1.05	0.815
Hemoglobin S (%)	79.11 ± 7.13	66.30 ± 10.67	**0.005**
Hemoglobin F (%)	14.31 ± 7.45	4.93 ± 3.69	**0.001**
Hemoglobin A_1 (%)	1.87 ± 2.00	24.51 ± 11.56	**<0.001**
Hemoglobin A_2 (%)	4.56 ± 1.67	4.10 ± 2.08	0.131
White Blood Cell count (x10^3/mm^3)	13.3 ± 4.4	13.2 ± 3.7	1.000
Platelet count (x10^3/mm^3)	481 ± 182	616 ± 267	0.349

p-values in bold indicate the presence of statistical significance.

4.1.2. Clinical Characteristics

The HbSS and HbSß$^+$ groups were compared in the context of clinical findings that had been recorded during the last year before blood samples were obtained for this study.

The number of painful crises (vaso-occlusive events) were used to divide each patient subgroup into 3 degrees of severity (none, 1 to 5 crises, and greater than 5 crises). In the HbSS group, there were five patients that did not have any crises, 16 patients that had 1–5 crises, and six patients that had >5 crises. For the HbSß$^+$ group, the same groups were comprised of two, five and one patient(s), respectively. We also determined whether groups were different when compared with regard to the presence/absence of vaso-occlusive events; detailed explanation of this comparison will be given later.

The rest of the clinical manifestations were divided according to presence / absence. With this division, the number of patients positive for manifestations in the HbSS and HbSß$^+$ groups were as follows: ACS (15 vs. 6), CVE (3 vs. 1), transfusion (11 vs. 2), renal complications (7 vs. 2), endocrine complications (5 vs. 1) and cardiac complications (none vs. none). When statistical comparisons with Chi square tests were performed, we found no differences between SCD subtypes in terms of any of the clinical manifestations (Table 4.2.).

Table 4.2. Clinical characteristics of the first group of patients.

Clinical Parameter		HbSS (N=27)	HbSß$^+$ (N=8)	P-value
Number of painful crises	None	5	2	0.806*
	1-5	16	5	
	>5	6	1	
Acute chest syndrome	No	12	2	0.431$^+$
	Yes	15	6	
Cerebrovascular event	No	24	7	1.000$^+$
	Yes	3	1	
Transfusion	No	16	6	0.680$^+$
	Yes	11	2	
Renal complication	No	20	6	1.000$^+$
	Yes	7	2	
Endocrine complication	No	22	7	1.000$^+$
	Yes	5	1	
Cardiac complication	No	27	8	N/A
	Yes	0	0	

N/A: not applicable.
*: Pearson Chi-square.
$^+$: Fisher's exact.

4.1.3. Comparison of Laboratory Measurements in SCD Subtypes

Next, we evaluated laboratory parameters and compared results between the two subtypes. Statistical analyses showed that all laboratory parameters that were measured to determine lipid metabolism, hemolysis, oxidative stress, inflammation and others (including hepatic function) were similar in children with HbSS and HbSß$^+$ (Table 4.3.). Therefore, further analyses and comparisons were performed without subgrouping for genotype.

Table 4.3. Comparison of HbSS and HbSß$^+$ groups, control values as reference.

		Sickle Cell Disease (N=35)		
	Controls (N=19)	**HbSS (N=27)**	**HbSß$^+$ (N=8)**	**HbSS vs. HbSß$^+$**
	Median (min-max)	Median (min-max)	Median (min-max)	**P-value**
Lipid Metabolism				
TG (mg/dL)	89 (54-223)	118 (58-246)	133 (69-160)	0.832
TC (mg/dL)	167 (117-240)	119 (76-170)	108.5 (99-127)	0.073
HDL-C (mg/dL)	50 (32-82)	29 (20-43)	27.5 (21-33)	0.743
LDL-C (mg/dL)	106 (62-143)	72 (44-106)	64 (56-77)	0.080
VLDL-C (mg/dL)	18 (11-45)	24 (12-49)	26.5 (14-32)	0.832
Apo A1 (mg/dL)	198 (136-268)	134 (94.3-378)	144 (123-269)	0.399
Apo B (mg/dL)	82.2 (54.7-107)	73.85 (53.6-202)	72.4 (63.5-77.4)	0.352
FFA (nmol/mL)	68.8 (11.6-167.4)	133.0 (8.0-456.5)	131.8 (8.1-253.5)	0.889
LCAT (U/L)	285 (112-1190)	277 (111-1396)	280 (135-1239)	1.000
Hemolysis				
LDH (U/L)	205 (122-293)	487 (251-1122)	494.5 (331-1088)	0.630
T. Bilirubin (mg/dL)	0.25 (0.08-0.62)	2.03 (0.3-4.4)	1.59 (0.56-3.39)	0.686
D. Bilirubin (mg/dL)	0.05 (0.01-0.13)	0.4 (0.06-0.91)	0.38 (0.14-0.61)	0.832
Oxidative Stress				
Hemin (mg/dL)	0.01 (0-0.27)	2.92 (0.01-22.16)	2.07 (0.66-15.35)	0.286
Ferritin (ng/mL)	12.9 (6-45.8)	112.4 (38.2-1292.5)	80.7 (35.1-581.1)	0.452
Serum Iron (µg/dL)	65 (14-143)	84 (39-237)	73.5 (42-133)	0.618
Inflammation				
SAA (mg/dL)	0.8 (0.27-1.69)	1.19 (0.27-2.37)	1.14 (0.43-2.63)	0.921
MPO (µg/L)	43.4 (29.72-55.67)	76.79 (33.66-188.64)	68 (40.21-100.7)	0.921
Chito (nmol/h/ml)	73.4 (16.3-231)	81.5 (10.8-244.2)	77.3 (28.4-136.8)	0.820
S1P (ng/mL)	149.9 (104.4-378.3)	177.9 (78.4-428.6)	185.9 (88.3-364.3)	0.617
Others				
Uric acid (mg/dL)	3.96 (2.33-6.33)	4.68 (3.45-8.34)	4.84 (3.48-6.44)	0.773
ALT (U/L)	12 (3-36)	19 (10-86)	21 (15-46)	0.773
GGT (U/L)	12 (3-24)	15 (8-180)	12 (9-32)	0.166

Abbreviations: SCD: sickle cell disease, TG: triglycerides, TC: total cholesterol, HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol, VLDL-C: very low density lipoprotein cholesterol, Apo A1: apolipoprotein A1, Apo B: apolipoprotein B, FFA: free fatty acids, LCAT: lecithin acyl cholesterol transferase, LDH: lactate dehydrogenase, SAA: serum amyloid A, MPO: myeloperoxidase, Chito: chitotriosidase, ALT: alanine transaminase, GGT: gamma-glutamyl transferase.

4.1.4. Lipid Profile

Hypocholesterolemia was present in all patient samples from the first group as demonstrated by significantly lower levels compared to controls: TC [116 (76-170) mg/dl vs. 167 (117-240) mg/dl, $p < 0.001$], HDL-C [28 (20-43) mg/dl vs. 50 (32-82) mg/dl, $p < 0.001$] and LDL-C [70 (44-106) mg/dl vs. 106 (62-143) mg/dl, $p < 0.001$].

Patients also demonstrated decreased ApoA1 and ApoB when compared to controls ($p < 0.001$ and $p = 0.016$, respectively). However, the concentrations of TG and FFA were both elevated in patients at a statistically significant level ($p = 0.042$ for TG and $p = 0.008$ for FFA) (Table 4.4.) (Figure 4.1.).

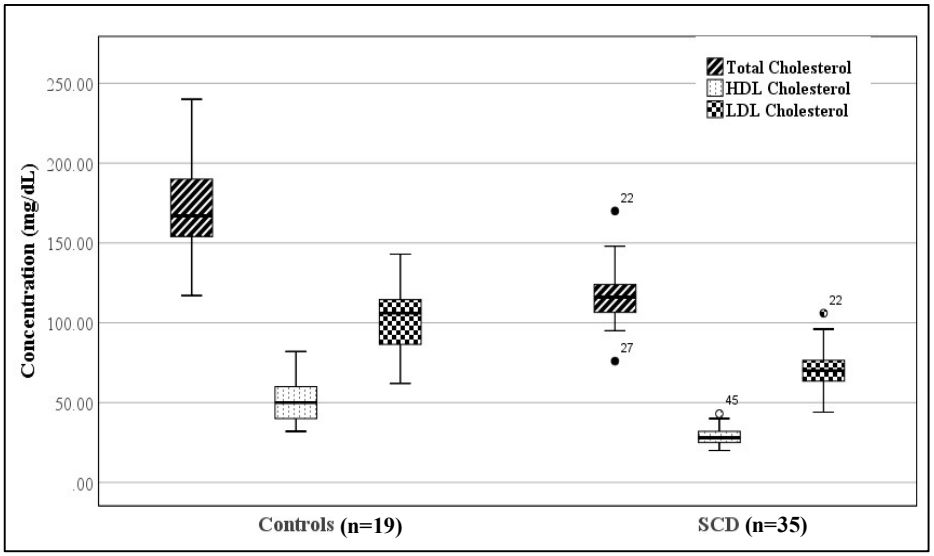

Figure 4.1. Comparison of cholesterol fractions in the first study group.

4.1.5. Hemolysis, Oxidative Stress and Inflammation

Levels of LDH ($p < 0.001$), T. bilirubin ($p < 0.001$) and D. bilirubin ($p < 0.001$) in patients, as measures of hemolysis, were significantly higher than that of controls (Figure 4.2. and Figure 4.3.).

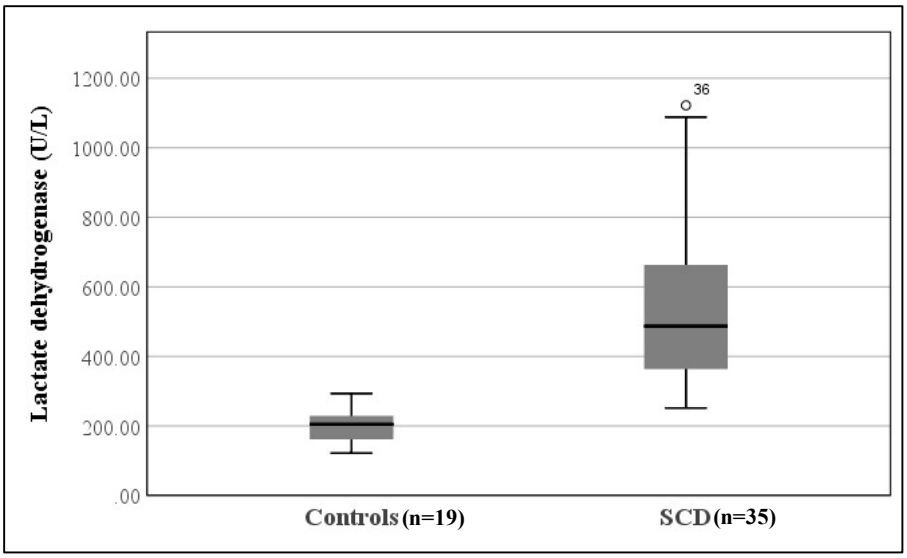

Figure 4.2. Comparison of LDH levels in the first study group.

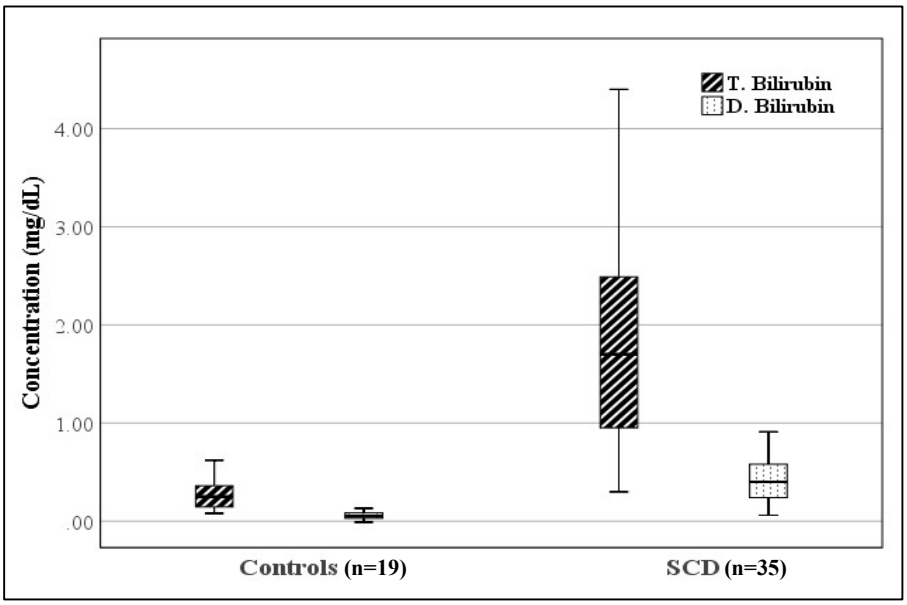

Figure 4.3. Comparison of bilirubin levels in the first study group.

Median haptoglobin concentration was 83.4 mg/dL in controls. However, in the SCD group, haptoglobin concentrations were undetectably low in patients with SCD. Only three patients had haptoglobin concentrations that could be detected. The values obtained in these patients were as follows: 6.2, 32.4, and 88.2 mg/dl.

The oxidation of heme was apparently elevated in SCD, as determined by hemin concentrations in patients versus controls [2.8 (0.01-22.16) mg/dl versus 0.01 (0-0.27) mg/dl; $p < 0.001$]. The majority of controls had hemin levels that were undetectably low. Ferritin levels had a median value of 112 (35–1293) ng/ml in patients, which was extremely high compared to the median value of the control group 13 (6–46) ng/ml ($p<0.001$).

In terms of inflammation, we had measured the levels of markers such as SAA, MPO, chitotriosidase and S1P. The patient versus control comparisons of the concentrations of SAA [1.14 (0.27-2.63) mg/dl versus 0.8 (0.27-1.69) mg/dl, $p < 0.001$] (Figure 4.4.) and MPO [76 (34-189) µg/L versus 43 (29-56) µg/L, $p < 0.001$] (Figure 4.5.) demonstrated significantly higher values in patients. However, the remaining two parameters associated with inflammation (chitotriosidase activity and S1P concentrations) were found to be similar in the patient and control groups.

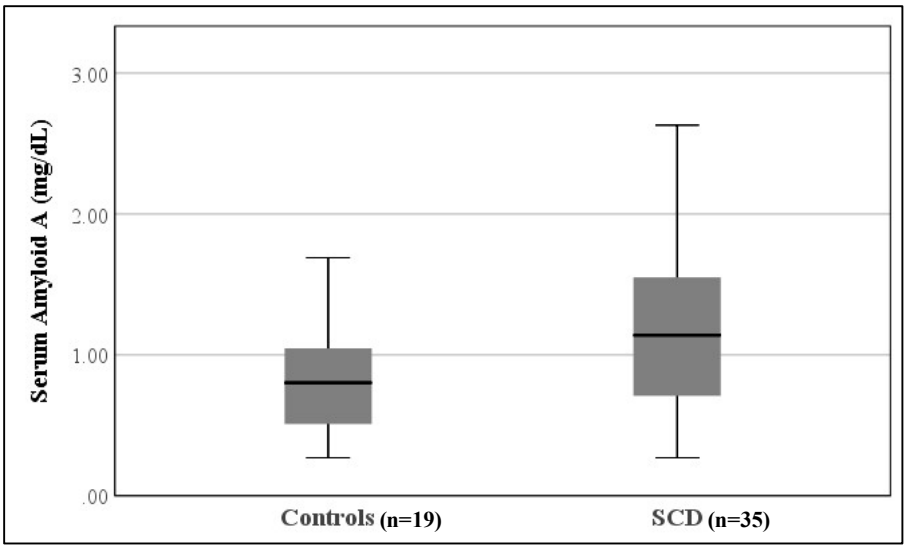

Figure 4.4. Comparison of SAA levels in the first study group.

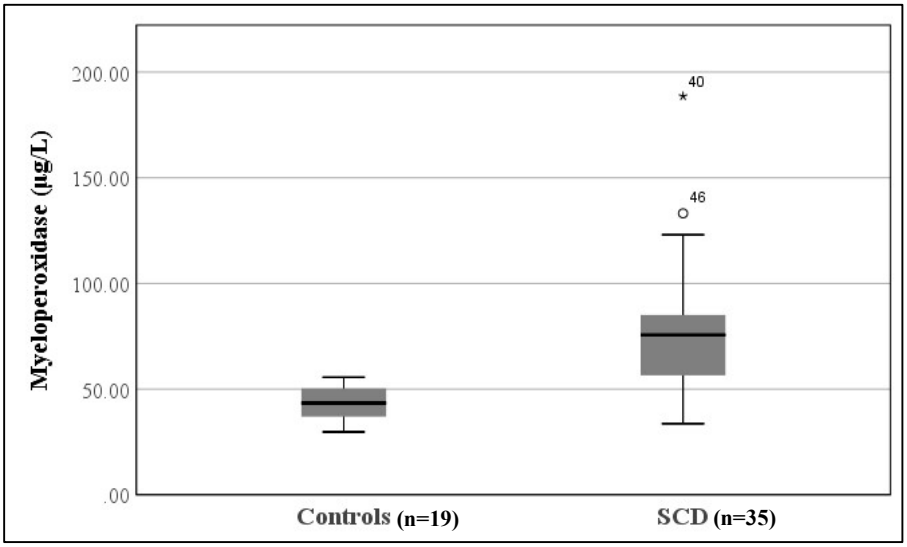

Figure 4.5. Comparison of MPO levels in the first study group.

Although liver functions were only evaluated according to ALT and GGT levels (which may increase due to other causes), compared to controls, we found both markers to be slightly increased in SCD. However, the levels of both parameters were within physiologically-acceptable ranges in the great majority of patients (ALT: 21 (10-86) U/L, GGT: 14 (8-180) U/L).

Finally, uric acid concentrations were significantly increased among patients with SCD compared to controls [4.68 (3.45-8.34) mg/dl vs. 3.96 (2.33-6.33) mg/dl, p = 0.025]. Uric acid was measured as a parameter that is associated with both renal tubular function (322) and inflammation (323) (Table 4.4.).

Table 4.4. Comparison of laboratory characteristics in the first study group.

	SCD Patients (N=35) Median (min-max)	**Controls (N=19)** Median (min-max)	**P-value**
Lipid Metabolism			
TG (mg/dL)	127 (58-246)	89 (54-223)	**0.042**
TC (mg/dL)	116 (76-170)	167 (117-240)	**<0.001**
HDL-C (mg/dL)	28 (20-43)	50 (32-82)	**<0.001**
LDL-C (mg/dL)	70 (44-106)	106 (62-143)	**<0.001**
VLDL-C (mg/dL)	25 (12-49)	18 (11-45)	**0.035**
Apo A1 (mg/dL)	134 (94 -378)	198 (136-268)	**<0.001**
Apo B (mg/dL)	73 (54-202)	82 (55-107)	**0.016**
FFA (nmol/mL)	133 (8-456)	69 (12-167)	**0.008**
LCAT (U/L)	133 (8-457)	285 (113-1190)	0.441
Hemolysis			
LDH (U/L)	487 (251-1122)	205 (122-293)	**<0.001**
T. Bilirubin (mg/dL)	1.7 (0.3-4.4)	0.25 (0.08-0.62)	**<0.001**
D. Bilirubin (mg/dL)	0.4 (0.06-0.91)	0.05 (0.01-0.13)	**<0.001**
Oxidative Stress			
Hemin (mg/dL)	2.80 (0.01-22.16)	0.01 (0-0.27)	**<0.001**
Ferritin (ng/mL)	112 (35-1293)	13 (6-46)	**<0.001**
Serum Iron (µg/dL)	81 (39-237)	65 (14-143)	0.385
Inflammation			
SAA (mg/dL)	1.14 (0.27-2.63)	0.8 (0.27-1.69)	**0.041**
MPO (µg/L)	76 (34-189)	43 (29-56)	**<0.001**
Chito (nmol/h/ml)	82 (11-244)	73 (16-231)	0.894
S1P (ng/mL)	178.2 (78.4-428.6)	149.9 (104.4-378.3)	0.394
Others			
Uric acid (mg/dL)	4.68 (3.45-8.34)	3.96 (2.33-6.33)	**0.025**
ALT (U/L)	21 (10-86)	12 (3-36)	**<0.001**
GGT (U/L)	14 (8-180)	12 (3-24)	**0.023**

Abbreviations: SCD: sickle cell disease, TG: triglycerides, TC: total cholesterol, HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol, VLDL-C: very low density lipoprotein cholesterol, Apo A1: apolipoprotein A1, Apo B: apolipoprotein B, FFA: free fatty acids, LCAT: lecithin acyl cholesterol transferase, LDH: lactate dehydrogenase, SAA: serum amyloid A, MPO: myeloperoxidase, Chito: chitotriosidase, S1P: sphingosine 1-phosphate, ALT: alanine transaminase, GGT: gamma-glutamyl transferase.
p-values in bold indicate the presence of statistical significance.

4.1.6. Relationships between Laboratory Parameters

Next, we assessed the results of correlations in order to be able to determine whether relationships existed between laboratory markers of anemia, lipid profile, inflammation and oxidative stress.

Regarding the relationships between lipid profile and anemia parameters, we found that LDH was inversely correlated with HDL-C level ($r = -0.590$, $p < 0.001$). When the relationships between markers of lipid profile and oxidative stress were assessed, an inverse correlation between Apo B and serum iron levels ($r = -0.360$, $p = 0.050$) was identified. In terms of lipid profile and inflammation parameters, LCAT activity was found to be negatively correlated with chitotriosidase activity ($r = -0.499$, $p = 0.005$). Assessment of the relationships between oxidative stress and inflammation only showed a moderate relationship, between the levels of hemin and MPO ($r = 0.383$, $p = 0.040$). These and other important correlations determined in our patient group are shown in (Table 4.5.) with corresponding correlation coefficients (r) and p-values.

Table 4.5. Significant correlations between parameters in the first set of patients.

Parameter	Correlating Parameter	r value	p-value
HDL Cholesterol	Hemoglobin	0.454	**0.012**
	LDH	-0.590	**<0.001**
Apolipoprotein B	Serum Iron	-0.360	**0.050**
	Ferritin	0.438	**0.025**
Serum Amyloid A	Apolipoprotein A1	-0.482	**0.008**
	Free fatty acids	-0.425	**0.012**
Chitotriosidase	Hemoglobin	-0.471	**0.012**
	Myeloperoxidase	0.436	**0.020**
	GGT	-0.571	**0.002**
	LCAT	-0.499	**0.005**
Hemin	Myeloperoxidase	0.383	**0.040**

HDL: high density lipoprotein, GGT: gamma-glutamyl transferase, LCAT: lecithin acyl cholesterol transferase.
p-values in bold indicate the presence of statistical significance.

4.1.7. Relationships between Laboratory and Clinical Findings

The comparison of groups formed in accordance with the presence/absence of painful episodes (vaso-occlusive events) in the past year (n=27 and n=8, respectively), showed that median [min–max] FFA concentrations were significantly increased in patients who had had painful episodes compared to those who had not (155 [19–456] nmol/mL vs. 59 [8–260] nmol/mL, p=0.010), while HDL-C levels were similar. However, 3-group painful episode comparison (none, 1 to 5, and >5 times), the median [min–max] HDL-C concentration of subjects who had suffered >5 painful episodes in

the last year was 32 (28–43) mg/dL –significantly elevated compared to the HDL-C levels of those without any episodes (27 [23–32] mg/dL) and those with 1–5 episodes (26 [20–34] mg/dL) (p=0.039) (Table 4.6.).

Table 4.6. Comparison of various parameters according to painful crisis groups.

	None (N=7)	**1-5 (N=21)**	**>5 (N=7)**	
	Median (min-max)	Median (min-max)	Median (min-max)	**P-value**
TG/HDL-C ratio	5.4 (1.8-5.8)	4.6 (2.1-10.4)	3.3 (1.45-5)	**0.035**
FFA (nmol/mL)	58.8 (8-260.2)	178.9 (19.2-456.6)	80.9 (30.6-266.2)	**0.005**
HDL-C (mg/dL)	27 (23-32)	26 (20-34)	32 (28-43)	**0.039**

Abbreviations: TG: triglycerides, FFA: free fatty acids, HDL-C: high density lipoprotein cholesterol
p-values in bold indicate the presence of statistical significance.

In those with renal complications (n = 8), GGT levels were found to be significantly higher compared to those who did not have renal complications (32 U/L, min-max: 9-180 vs. 13 U/L, min-max: 8-32) (p = 0.018).

Comparisons with regard to endocrine complications only showed that uric acid levels were significantly higher in those with complications (6.37 mg/dL, min-max: 4.49-8.34) compared to those without (4.61 mg/dL, min-max: 3.45-7.64) (p = 0.031).

None of the parameters demonstrated significant differences in the comparisons of ACS and transfusion groups. Also, since none of the patients suffered from cardiac complications, evaluations could not be performed in this regard.

4.2. Second Patient Group

The relationships between oxysterols, ceramide species and parameters of anemia/hemolysis, lipid profile and oxidative stress were assessed in a second set of subjects. The second group of patients was comprised of 32 subjects. Of these, 22 were diagnosed with HbSS and 10 were diagnosed with HbSß$^{+}$. There were 25 children in the control group. The HbSS group included 14 boys, 8 girls (mean age: 13.2 ± 4.3 years), the HbSß$^{+}$ group included 6 boys, 4 girls (mean age: 16.1 ± 3.6 years). Finally, the healthy control group had 13 boys, 12 girls (mean age: 13.7 ± 3.6 years). When compared with regard to age and sex, groups were similar (p = 0.161 and p = 0.715,

respectively). While the majority of patients were receiving hydroxyurea treatment, 4 from the HbSS, and 1 from the HbSß$^+$ group did not receive hydroxyurea. The two disease subgroups were similar in terms of hydroxyurea use (p = 0.555) and the presence of splenectomy (p = 0.325) (Table 4.7.).

Table 4.7. Age, sex and treatment comparison of the second set of subjects.

		Control group (n=25)	**HbSS group (n=22)**	**HbSß$^+$ group (n=10)**	**P-value**
Age	Mean ± SD	13.7 ± 3.6	13.2 ± 4.3	16.1 ± 3.6	0.161
		N (%)	**N (%)**	**N (%)**	
Sex distribution	Boy	13 (52%)	14 (63.6%)	6 (60%)	0.715
	Girl	12 (48%)	8 (36.4%)	4 (40%)	
Hydroxyurea	Receiving	-	18 (81.8%)	9 (90%)	0.555*
	Not receiving	25 (100%)	4 (18.2%)	1 (10%)	
Splenectomy	Yes	-	2 (9.1%)	0 (0%)	0.325*
	No	25 (100%)	20 (90.9%)	10 (100%)	

*Hydroxurea and splenectomy p-values calculated for the comparison of HbSS and HbSß$^+$.

4.2.1. Hemolysis, Oxidative Stress and Lipid Profile

The control and SCD groups were significantly different in terms of all parameters of lipid profile except for TG and LCAT activity, and when the HbSS and HbSß$^+$ groups were compared with each other, the levels of all parameters were similar except for TC (p = 0.025) (Table 4.8.). Graph comparing the SCD patients and control subjects in terms of cholesterol fractions is given in Figure 4.6.

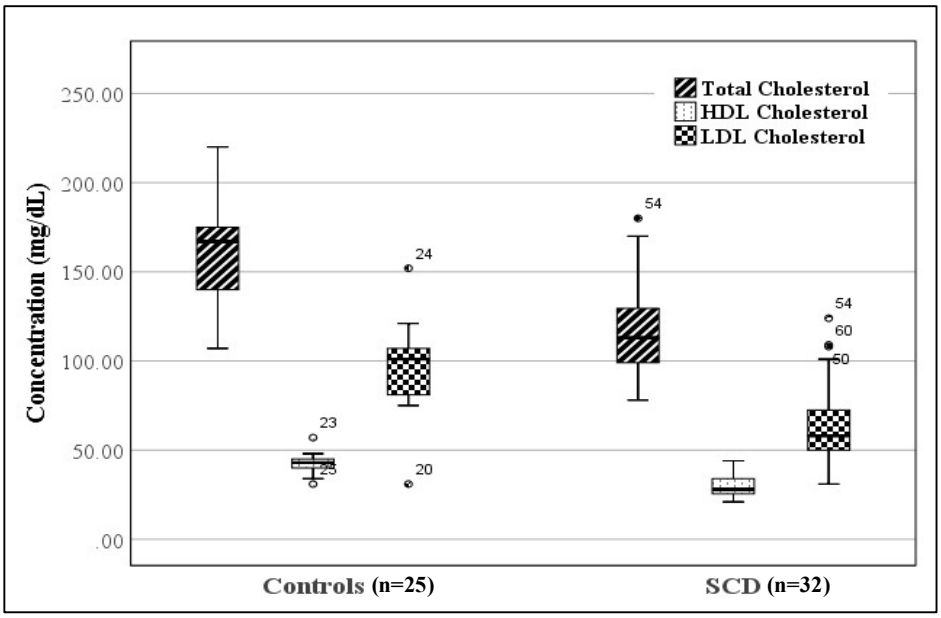

Figure 4.6. Comparison of cholesterol fractions in the second study group.

Of note, mean TG levels in those with HbSS was somewhat elevated with respect to the levels in the HbSß$^{+}$ group; however, statistical significance was not present (p = 0.897). Finally, LCAT activity levels also seemed to be somewhat increased in patients with HbSS; however, results were similar in the SCD and control groups (p = 0.121), as well as the HbSS and HbSß$^{+}$ groups (p = 0.316) (Figure 4.7.).

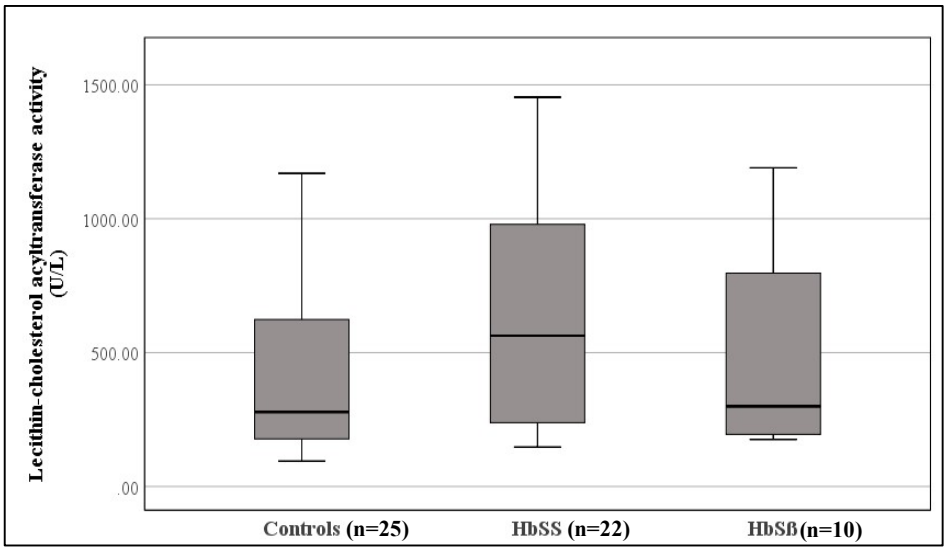

Figure 4.7. Comparison of LCAT activity in the second study group.

Furthermore, we must note that the levels of LDH, bilirubins, serum iron and ferritin could not be measured in the healthy control group due to the lack of sufficient samples. However, it is well-known that these parameters are altered in the steady state of SCD. Moreover, our results with the first patient group are also in support of these changes. Finally, we also observed that mean serum iron concentration in the HbSS group (93.3 ± 43.6 µg/dL) seemed to be higher than that of subjects with HbSß$^{+}$ (69.3 ± 26.3 µg/dL); however, statistical significance was absent in the comparison of these two subgroups (p = 0.092).

When chitotriosidase levels were compared as a measure of inflammatory activity, we found that SCD patients (overall mean value: 128.8 ± 97.4 nmol/h/ml) had significantly higher values when compared with the healthy controls (68.7 ± 37.6 nmol/h/ml, p = 0.014). Furthermore, although we observed a marginally increased value in HbSS patients (132.2 ± 109.1 nmol/h/ml) compared to those with HbSß$^{+}$ (121.9 ± 73.1 nmol/h/ml), the two groups had statistically similar values (p = 0.791) (Figure 4.8.).

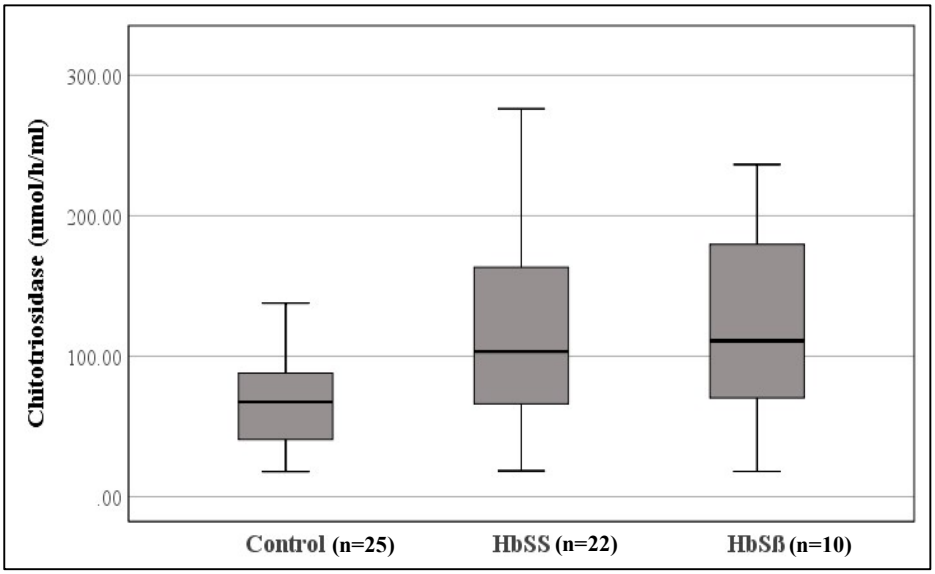

Figure 4.8. Comparison of chitotriosidase levels in the second study group.

Table 4.8. Laboratory results comparison of the second set of subjects.

		Sickle Cell Disease (n=32)		P-values	
	Control (n=25) Mean ± SD	**HbSS (n=22)** Mean ± SD	**HbSß$^+$ (n=10)** Mean ± SD	**SCD vs. Control**	**HbSS vs. HbSß$^+$**
Lipid Profile					
TC (mg/dL)	161.8 ± 25.4	124.7 ± 27.6	102.3 ± 12.9	**<0.001**	**0.025**
HDL-C (mg/dL)	42.5 ± 5.7	30.7 ± 6.2	28.1 ± 7.6	**<0.001**	0.238
LDL-C (mg/dL)	95.9 ± 25.8	69.0 ± 26.2	50.4 ± 11.1	**<0.001**	0.084
TG (mg/dL)	117.2 ± 40.9	124.8 ± 46.1	118.9 ± 35.0	0.563	0.897
LCAT (U/L)	421 ± 323	533 ± 447	469 ± 361	0.121	0.316
FFA (nmol/mL)	51.7 ± 96.4	89.9 ± 63.6	119.2 ± 75.4	**<0.001**	0.219
Hemolysis					
Hb (g/dL)	13.8 ± 0.9	8.9 ± 1	8.9 ± 1.2	**<0.001**	0.726
LDH (U/L)	-	491 ± 222	528 ± 206	N/A	0.562
T. Bilirubin (mg/dL)	-	3.05 ± 1.74	3.29 ± 1.57	N/A	0.764
D. Bilirubin (mg/dL)	-	0.55 ± 0.26	0.54 ± 0.14	N/A	0.795
Oxidative Stress					
Serum Iron (µg/dL)	-	93.3 ± 43.6	69.3 ± 26.3	N/A	0.092
Ferritin (ng/mL)	-	245.6 ± 303.7	177.8 ± 168.1	N/A	0.458
Oxysterol Species					
7-KC (µg/L)	27.86 ± 11.61	45.98 ± 3.67	44.41 ± 4.96	**<0.001**	0.434
C-triol (µg/L)	13.86 ± 9.23	20.47 ± 1.79	19.90 ± 1.73	**0.003**	0.366
Ceramide Species					
C16 (ng/mL)	1156 ± 97	1246 ± 230	1356 ± 209	**0.021**	0.260
C18 (ng/mL)	495 ± 124	573 ± 136	662 ± 213	**0.023**	0.302
C20 (ng/mL)	379 ± 59	474 ± 118	493 ± 176	**0.001**	0.781
Other					
Chito (nmol/h/ml)	68.7 ± 37.6	132.2 ± 109.1	121.9 ± 73.1	**0.014**	0.791

Abbreviations: TC: total cholesterol, HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol, TG: triglycerides, FFA: free fatty acids, Hb: hemoglobin, LDH: lactate dehydrogenase, 7-KC: 7-ketocholesterol, C-triol: cholestane-3β,5α,6β-triol, C16: C16-ceramide, C18: C18-ceramide, C20: C20-ceramide, Chito: chitotriosidase.

N/A: not applicable due to lack of results in the control group.

p-values in bold indicate the presence of statistical significance.

4.2.2. Oxysterol Levels

Mean oxysterol levels were higher in those with SCD; however, the ttwo disease subgroups were similar for both 7-KC (p=0.434) and C-triol (p=0.366) (see Table 4.8.) (Figure 4.9.).

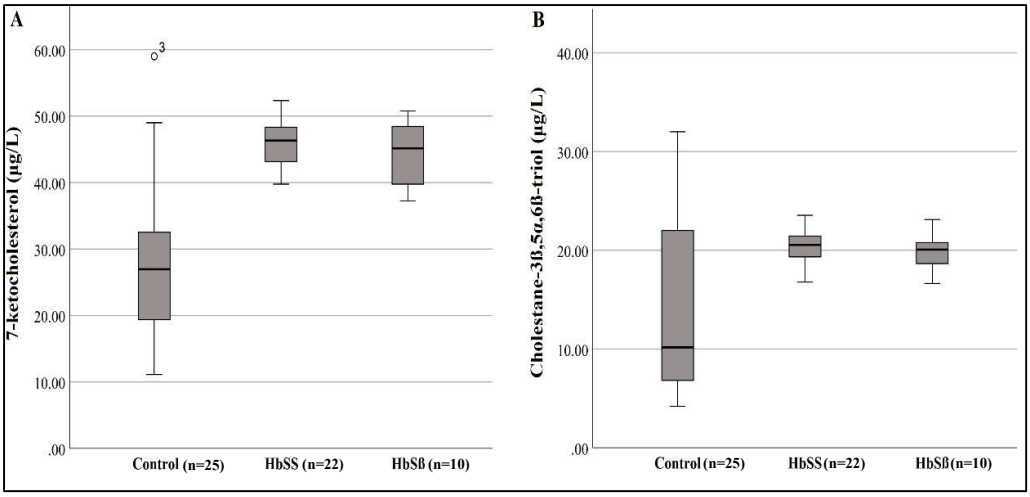

Figure 4.9. The levels of A) 7-KC and B) C-triol in groups.

4.2.3. Relationships Between Oxysterols and Other Parameters

Since the HbSS and the HbSß$^{+}$ subgroups were similar in terms of lipid profile, hemolysis and oxidative stress, no subgrouping was performed in the correlation analyses and all patients (n = 32) were included as a single group. Results revealed that 7-KC concentration was negatively correlated with Hb concentration (r = –0.539, p = 0.007) (Figure 4.10.), whereas it was positively correlated with LDH concentration (r = 0.518, p = 0.002) (Figure 4.11.). These correlations were not present in the control group.

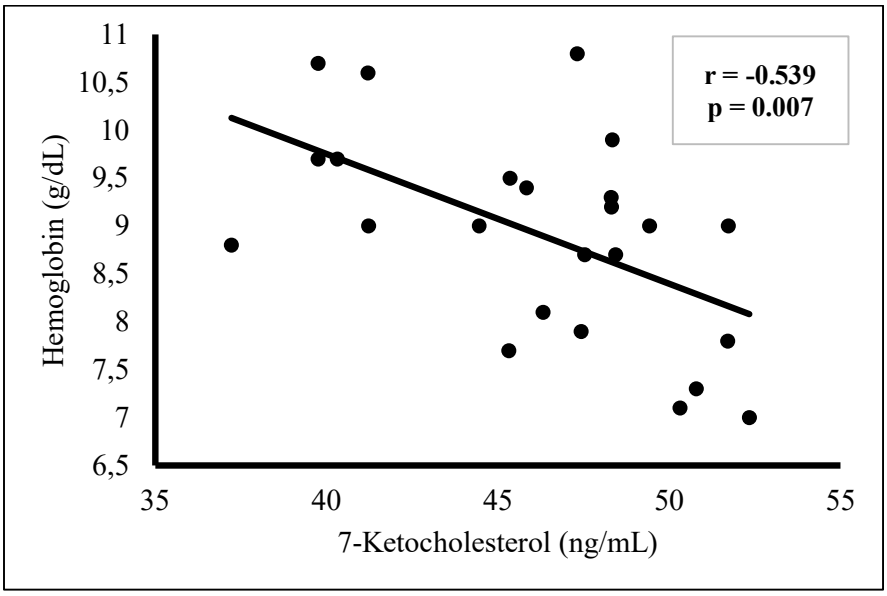

Figure 4.10. Correlation graph between Hb and 7-KC in patients with SCD.

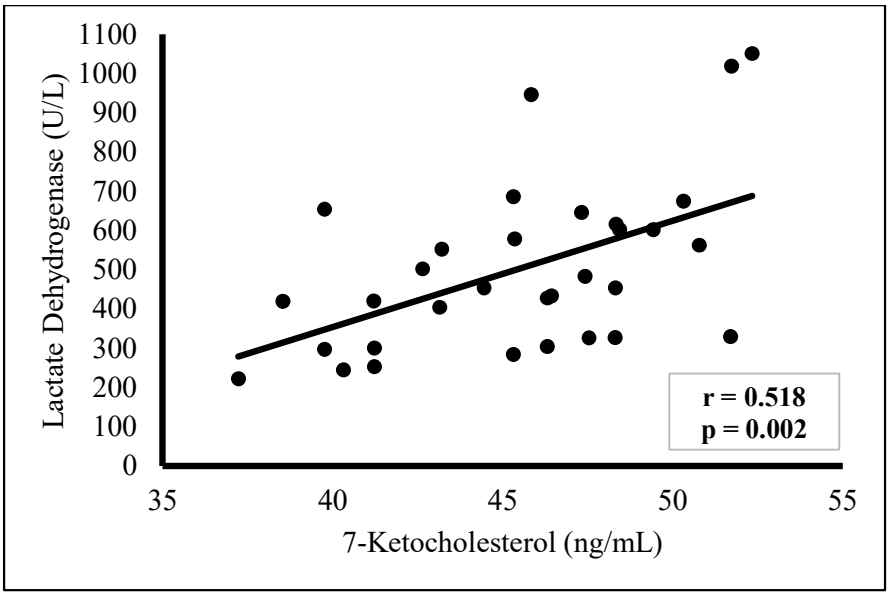

Figure 4.11. Correlation graph of LDH and 7-KC in patients with SCD.

Furthermore, the concentration of C-triol showed a correlation that was inverse to HDL-C level (r = -0.439, p = 0.022) (Figure 4.12.), again in the SCD group. When other relationships were examined within the SCD group, we found that oxysterol concentrations were not correlated with any of the other parameters analyzed, including T. bilirubin and D. bilirubin which were utilized as measures of hemolysis.

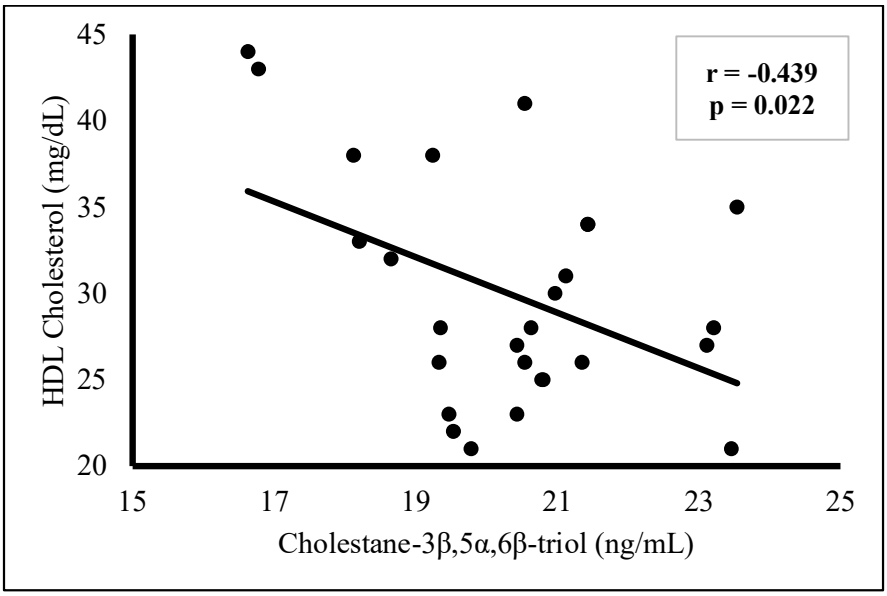

Figure 4.12. Correlation graph of HDL-C and C-triol in patients with SCD.

However, another important finding associated with correlation values was that the levels of 7-KC and C-triol in the SCD group were not correlated with each other ($p = 0.278$) (Figure 4.13.), even though, in the control group, the concentrations of these two oxysterols were strongly correlated ($r = 0.620$, $p = 0.001$) (Figure 4.14.).

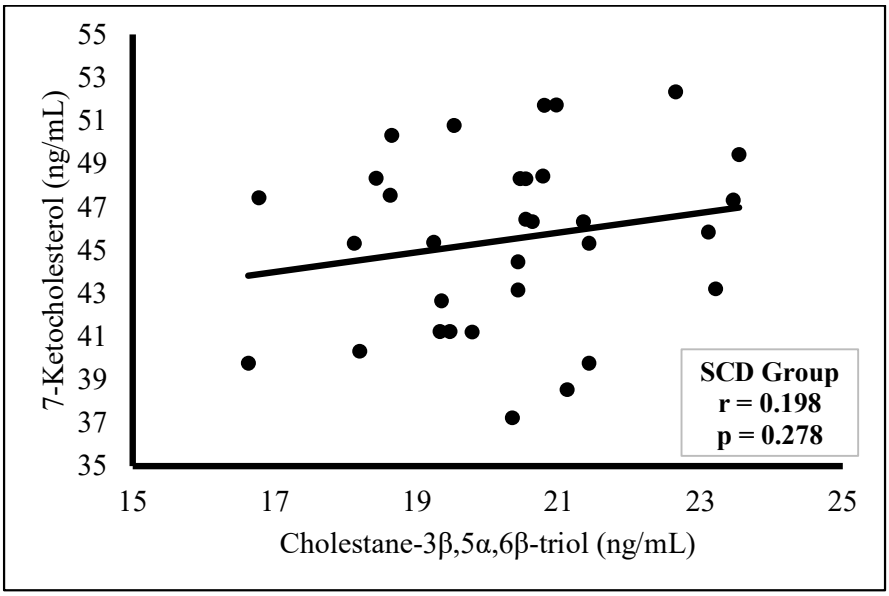

Figure 4.13. Lack of 7-KC and C-triol correlation in patients.

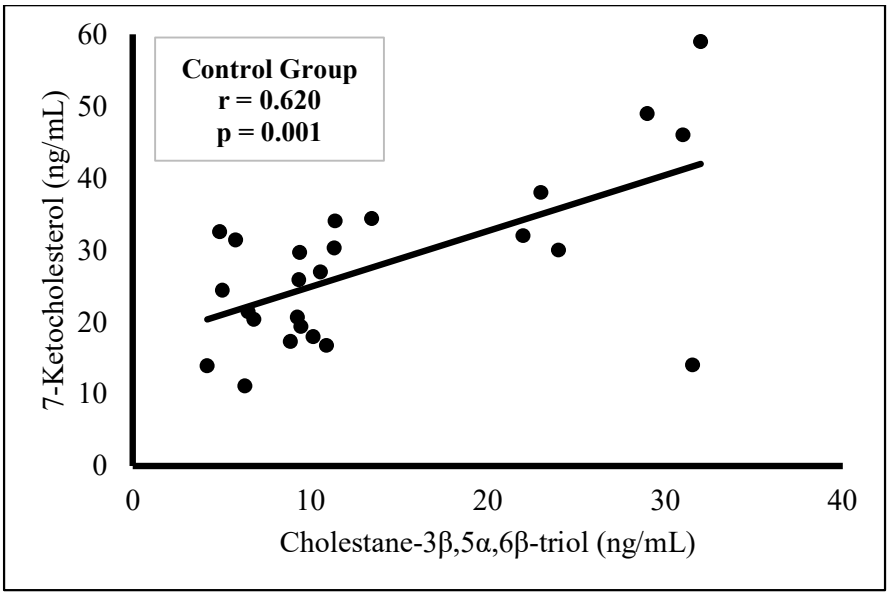

Figure 4.14. 7-KC and C-triol correlation in controls.

4.2.4. Ceramide Levels

The levels of ceramide species (C16, C18 and C20) in the plasma of patients with HbSS and HbSß$^{+}$ were similar ($p = 0.260$, $p = 0.302$ and $p = 0.781$, respectively); however, there were significant differences between the controls and the SCD group

in terms of C16, C18 and C20 levels ($p = 0.021$, $p = 0.023$ and $p = 0.001$, respectively) (see Table 4.8.) (Figure 4.15.).

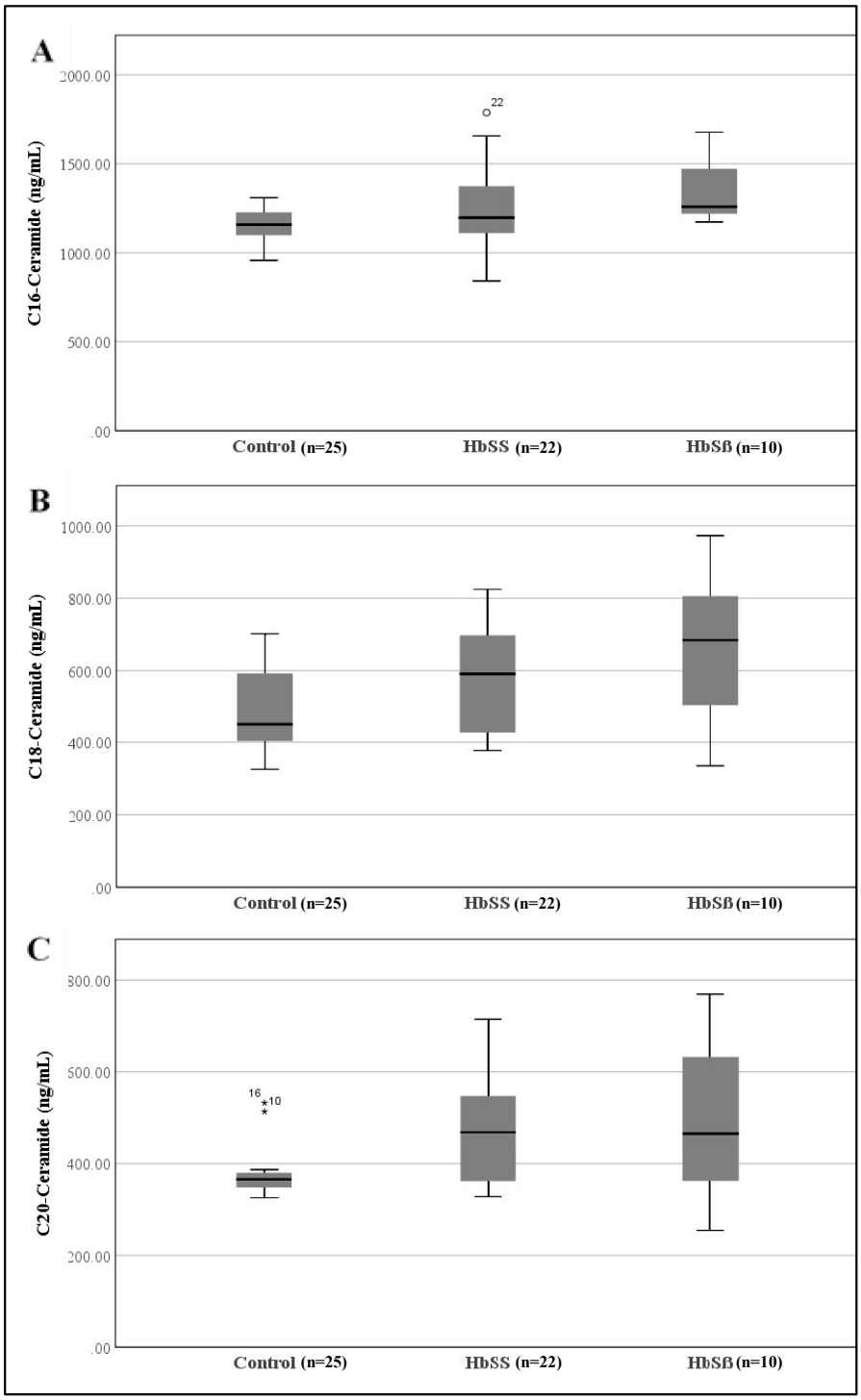

Figure 4.15. The levels of A) C16, B) C18 and C) C20 ceramides in groups.

4.2.5. Relationships Between Ceramides and Other Parameters

When the correlations between different ceramide species and parameters of lipid profile, hemolysis, oxidative stress and oxysterol levels were investigated in patients with SCD, we found that C16-ceramide had no significant correlations with any of the parameters apart from other ceramide species (C18 and C20 ceramide); however, C18-ceramide and C20-ceramide levels were positively correlated with various parameters. It is also critical to note that the concentrations of these ceramide species could only be determined in 31 patients and 18 controls (5 samples were excluded due to extreme hemolysis and 6 samples could only be received after the measurement of ceramides). Therefore, the degrees of freedom (dF) statistic for these correlations (in the SCD group) is a maximum of 31.

The concentrations of C18-ceramide had moderate positive correlations with the levels of direct bilirubin ($r = 0.442$, $p = 0.016$), total bilirubin ($r = 0.522$, $p = 0.004$), LDH ($r = 0.462$, $p = 0.012$) and C-triol ($r = 0.597$, $p = 0.001$). C20-ceramide concentrations were also correlated positively with direct bilirubin ($r = 0.480$, $p = 0.008$), total bilirubin ($r = 0.555$, $p = 0.002$) and C-triol; with the latter relationship being particularly strong compared to other correlations ($r = 0.646$, $p < 0.001$). Additionally, C20-ceramide concentrations were correlated inversely with the HDL-C levels in the SCD group ($r = -0.465$, $p = 0.019$) (Table 4.9.).

Table 4.9. Correlations between ceramide species and other parameters.

Ceramide Species	Correlating Parameter	r-value	p-value
C18-Ceramide	Direct Bilirubin	0.442	**0.016**
	Total Bilirubin	0.522	**0.004**
	LDH	0.462	**0.012**
	C-triol	0.597	**0.001**
C20-Ceramide	Direct Bilirubin	0.480	**0.008**
	Total Bilirubin	0.555	**0.002**
	C-triol	0.646	**<0.001**
	HDL-C	-0.465	**0.019**

Abbreviations: LDH: lactate dehydrogenase, HDL-C: high density lipoprotein cholesterol, C-triol: cholestane-3β,5α,6β-triol

p-values in bold indicate the presence of statistical significance.

To assess the relationships between C18 and C20 ceramides and the correlating parameters, we performed linear regression with the stepwise method. For C18-ceramide, we included direct bilirubin, total bilirubin, LDH and C-triol in the model. The results at the 2nd step demonstrated that C-triol and total bilirubin levels were independently associated with C18-ceramide concentration (Table 4.10.).

Table 4.10. Linear regression results for factors affecting C18-ceramide.

						95% confidence interval	
	Uns. β	**SE**	**β**	**t**	**p**	**Lower**	**Upper**
(Constant)	-424.542	248.308		-1.710	0.099	-934.946	85.863
C-triol	44.443	12.473	0.502	3.563	0.001	18.804	70.082
T. Bilirubin	37.460	13.046	0.405	2.871	0.008	10.642	64.277

Dependent variable: C18-ceramide; F: 13.609; R^2:0.511

For C20-ceramide, linear regression performed with the inclusion of direct bilirubin, total bilirubin, C-triol and HDL-C levels revealed that C-triol and total bilirubin levels were independently influential on the concentration of C20-ceramide (Table 4.11.).

Table 4.11. Linear regression results for factors affecting C20-ceramide.

						95% confidence interval	
	Uns. β	**SE**	**β**	**t**	**p**	**Lower**	**Upper**
(Constant)	-416.397	187.365		-2.222	0.035	-801.532	-31.262
C-triol	39.143	9.491	0.541	4.124	0.000	19.633	58.653
T. Bilirubin	33.201	10.389	0.419	3.196	0.004	11.847	54.555

Dependent variable: C20-ceramide; F: 18.060; R^2:0.581

5. DISCUSSION

Sickle cell disease is a severe, inherited disease which leads to various complications that are frequently life-threatening without appropriate supportive care (324-327). Even though available treatments have reduced complications in recipients, the risks associated with complications remain, and the exact pathophysiology of the various effects of the disease are relatively unknown (326). Therefore, the assessment of the relationships between hemolysis, chronic inflammation, oxidative stress and lipid abnormalities (and any other characteristics associated with the disease) is crucial to our understanding of SCD and its consequences. As such, this study was planned and conducted to determine whether any of the assessed relationships could contribute to the data concerning the pathophysiological characteristics of the disease, particularly lipid abnormalities such as hypocholesterolemia.

We investigated the association between lipid abnormalities and pathophysiological alterations such as anemia, inflammation and oxidation. The primary findings of the first patient group were those concerning the relationships between laboratory results. The comparison of these results with controls showed that patients with SCD had significant alterations from controls in terms of almost all parameters measured to determine hemolysis, inflammation, oxidative stress and lipid profile. These differences were shown in the well-defined steady state of the disease, indicating that, even when patients did not have clinical manifestations, they were under the continuous underlying effects of this chronic disease. Hemolysis and anemia were evidently shown by measurements of Hb, LDH and bilirubins. Furthermore, haptoglobin was diminished in all but three patients, suggesting that chronic hemolysis even at steady state was leading to extreme levels of erythrocyte loss –exceeding the production of (and thus the protective capability of) haptoglobin.

In terms of lipid profile, patients had reduced cholesterol in all fractions except for VLDL-C. These are common findings throughout the world as reported by various researchers; however, when the decreased levels of Apo A1 and Apo B are considered, it seems that there may be an underlying process other than loss of erythrocytes. Previous studies had suggested that the decreased levels of cholesterol in SCD were associated with extreme erythrocyte loss that caused increased erythropoietic activity

(328). Other lipid profile parameters, such as FFA and TG, were significantly increased in SCD.

The oxidative stress of patients with SCD was assessed by the measurement of hemin, serum iron and ferritin levels (329, 330). All three parameters were observed to be increased in SCD; however, statistical significance (in comparison to controls) was only observed with hemin and ferritin. Of note, we also found that hemin levels were undetectable in controls, indicating that the presence of detectable levels of hemin in the circulation was an important characteristic in SCD due to the chronic hemolysis in patients. As hemin is oxidized heme, this result demonstrated how severe the oxidative insult is in the circulation of SCD patients.

Underlying inflammatory activity in the steady state of SCD was demonstrated by the increased levels of SAA and MPO levels in patients (331). However, in the first group of patients, chitotriosidase levels did not show differences; whereas, in the second study group, patients with SCD had significant elevation from controls, indicating the presence of some variations regarding chitotriosidase activity (and possibly inflammation). Even so, it is apparent that chronic activation of inflammation, can be shown in subjects with SCD through various markers. Finally, the initial comparisons between the HbSS and HbSß$^{+}$ subtypes revealed no differences in any of the investigated parameters. Therefore, it is feasible to suggest that the alterations in laboratory parameters are similar in patients with either subtype of SCD.

In the second patient group, comparison of the SCD and controls showed similar features, with only the levels of TC showing significant differences between disease subgroups. Parameters of lipid profile, hemolysis, oxidative stress, oxysterols and ceramide species demonstrated significant differences between patients and controls. The most notable results were perhaps significantly increased 7-KC, C-triol, and C16, C18 and C20 ceramide levels in patients. Furthermore, correlations of laboratory variables in the SCD group showed that 7-KC was positively associated with LDH and negatively associated with Hb levels, while C-triol was negatively associated with HDL-C. These relationships were absent in the control group. The lack of correlations among the two oxysterols in the patient group was also interesting, as the levels of 7-KC and C-triol were strongly correlated in healthy subjects. These

findings may suggest that these two oxysterols are relevant to the level of hemolysis and cholesterol levels in SCD. The levels of ceramide species (C18 and C20) also demonstrated significant correlations with various parameters of lipid profile, hemolysis and oxysterols.

5.1. Lipid Alterations and Their Relationships with Other Parameters

The altered lipid profiles observed in SCD, especially those concerning cholesterol levels, have been documented by numerous studies (317, 332, 333). Although some of the lipid alterations may be more prominent during vaso-occlusive episodes (333, 334) or may be associated with development of manifestations (317), it is well-known from studies throughout the world that they are present in the steady state of disease. Furthermore, despite the interest in identifying lipid-related markers for clinical assessment in SCD, it must be taken into account that there is little evidence to support causation in studies focusing on such correlations. That is, since the basis of lipid changes in SCD remain unknown (335), it is impossible to put forth any convincing role for lipid alterations in SCD through clinical studies comprised of limited number of patients.

Contrary to the renowned presence of hypocholesterolemia in SCD, relatively recent studies (317, 336) in this field seem to have surfaced a less-evident, but increasingly frequent finding: elevated TG levels compared to healthy controls (317, 336, 337). In the earliest study published in 2010, triglyceride concentrations were reported to be associated with endothelial (dys)function (as measured by flow-mediated vasodilation) in a community-based study of 4887 healthy subjects (338). The results of this study showed an inverse correlation between flow-mediated vasodilation values and TG levels, indicating the emergence of endothelial dysfunction with increased levels of TG. The authors concluded that TG level was an independent contributor to endothelial dysfunction (338). In studies focused on TG levels in patients with SCD, it was reported that patients had significant elevations compared to healthy controls (334, 339). Our results with TG measurements also showed increased TG levels in patients compared to controls. Additionally, we also found that TG/HDL-C (suggested as another biomarker of endothelial dysfunction) was correlated with LDH which is a reliable hemolysis measure in patients; thus, pointing to a link between

hemolysis and elevated TG levels –possibly through increased impairment of endothelial function. Although there were no relationships between clinical manifestations and TG levels in the current study, we did not evaluate pulmonary hypertension as a separate entity (we included it as a cardiac complication); however, this complication has been associated with TG levels, even though TG values alone were suggested to be a better marker than TG/HDL-C ratio (317). Nevertheless, considering these results and previous studies, it is advisable to pursue the possible role of this marker with regard to SCD characteristics and clinical findings. In the light of our findings and the results of previous studies, it seems that TG levels may have a role in SCD; however, further studies are required to determine whether a causal relationship exists.

After digestion of food in the intestine, fatty acids are taken into intestinal cells, packed into chylomicrons, and delivered to the liver via circulation. The liver redistributes these fatty acids in the VLDL and LDL structures that are sent to other tissues via circulation. However, they also exist freely in the circulation (hence the name, FFA) primarily after being released from adipocytes during lipolysis (340). In the context of SCD, an early study had identified alterations in phospholipid fatty acids; however, the authors did not report any explanation to the nature of these changes (341). A relatively recent research concluded that this increase could cause significant adverse effects on erythrocyte membrane fluidity (342). Membrane fluidity and structural stability have been suggested to be associated with disease pathophysiology in hemoglobinopathies (335). In normal physiology, the majority of FFAs lipolyzed from adipocytes are captured by lipoproteins in the circulation (343). However, considering the chronic baseline inflammatory activity, oxidative burden and constant hemolysis of erythrocytes (releasing membranous lipid as well as their contents), it is apparent that these lipoproteins will suffer extreme conditions while trying to perform their duty of collecting FFAs.

Despite the low levels of interest for the evaluation of FFA in the circulation of patients with SCD, published results seem to demonstrate important features of the lipid profile and the altered lipid metabolism in SCD. For instance, a preliminary study reported increased FFA and decreased esterified fatty acid levels in the circulation of

patients with SCD (344). However, we must note that this study also found lower TG in the sera of SCD compared to controls, which is seemingly in contrast with the overwhelming majority of SCD studies (344). In another study, similar to our results, SCD patient were found to have significantly increased TG and FFA levels when blood was drawn after fasting; however, after food consumption, FFA and TG decreased to reference levels (345). This result is in agreement with our findings and supports the notion that there is a functional change in lipid metabolism with SCD, particularly in response to hunger. Taking this a step further, we may feasibly assume that nutritional state could have a direct influence on the erythrocyte membrane and its deformability in SCD.

We also report that SAA and FFA levels demonstrated inverse correlation at a significant level. This is very interesting, especially considering the fact that patients were found to have increased FFA levels and decreased HDL-C levels (The HDL particle carries SAA). When data from previous studies are evaluated together with our results, it is a given that inflammation is active at a marked level, FFA levels are increased, and HDL-C is decreased in SCD. So the increase in SAA levels is expected, but its negative correlation with FFA levels suggests indirect relationships between lipoprotein particles in the circulation or possible alterations in their function due to the increase in SAA concentration in the HDL particle. Furthermore, interestingly, higher FFA levels were found among patients who had suffered from painful crises within the past year, a puzzling result when it is apparent that those with painful crises would have had heightened levels of inflammation (albeit for short periods) within the last year when compared to patients who had not suffered from painful episodes. However, considering the presence of pro-inflammatory HDL in relation with chronic inflammation (201), it is possible to suggest that the negative correlation between FFA and SAA levels are a result of this phenomenon. Nevertheless, it is evident that detailed basic science research is necessary to elucidate nature of the relationships among FFA levels, inflammation and lipoproteins in the context of SCD pathophysiology. We believe that simultaneous determination of FFA content in the circulation and lipoproteins may contribute to our understanding of these alterations, and could elucidate the lipid uptake of lipoproteins in patients with SCD. Finally, it is apparent

that our results concerning the relationship between inflammation and FFA levels require confirmation from future studies.

5.1.1. HDL Dysfunction

The function of the LCAT enzyme and its activity are critical for the characteristic role of the HDL particle, as this enzyme is carried by HDL and is the primary component of the whole reverse cholesterol transport process (346). In a relatively recent study, which is one of the very few studies we could find on this topic, it was reported that protein levels of LCAT were decreased in SCD (347); furthermore, another study concisely showed that SCD patients had decreased activity of the LCAT enzyme in comparison to healthy patients (348), indicating that LCAT activity was inhibited in addition to lower protein levels. In the current study, LCAT activity levels were not altered in patients, which was an unexpected finding; however, Apo A1 levels were significantly decreased in patients. When it is considered that Apo A1 is the cofactor of the LCAT enzyme, the significance of decreased Apo A1 levels becomes apparent and may indicate that the unfavorable circulatory environment could lead to changes in the function of LCAT. In agreement with our results, several studies have observed significantly decreased ApoA1 levels at the steady state of SCD and also in vaso-occlusive events (164, 198). Therefore, although LCAT activity levels were found to be similar in patients and controls in the current study, *in vivo* reverse cholesterol transport may be adversely affected by factors that damage Apo A1 or influence its levels, such as SAA, MPO and hemin.

Serum amyloid A replaces Apo A1 and becomes the major apolipoprotein of the HDL particle during the acute phase response (349). Elevated SAA in SCD has been previously demonstrated (163, 164, 350); a result which is confirmed by our findings. Moreover, our laboratory results demonstrated that SAA and HDL-C were inversely correlated, demonstrating an important link between inflammatory activation and reduced cholesterol in the HDL particle. Considering that SAA is carried in the HDL structure, it is very likely that this structural alteration of HDL is significantly associated with dysfunctions in normal HDL function.

Myeloperoxidase is an enzyme that is critical for the antimicrobial activity of leukocytes, specifically neutrophils. It is responsible for the production of HOCl that plays a primary role in oxidative burst –an inflammatory mechanism that destroys pathogens through excessive oxidative insult. Nevertheless, MPO activity could also lead to increased oxidative stress, a finding supported by its reported oxidative effects on Apo A1 (351). In mice models of SCD, inhibition of MPO was suggested to be beneficial and led to vasodilation and reduced oxidative stress (157). Therefore, it was not surprising to observe substantially increased MPO levels in the SCD subgroups, with statistically significant differences from controls. Furthermore, hemin levels (oxidative stress) and chitotriosidase activity (as a measure of inflammatory activity) were also correlated (both positively) with MPO levels in patients with SCD. These two correlations are easily explained by the dual role of MPO –with its activation during inflammation and its subsequent production of oxidative products.

Hemin is another parameter that may contribute to HDL dysfunction through damage to the ApoA1. The production of hemin transpires from the oxidation of free heme in the circulation (329). Since the level of free heme is reportedly a disease severity marker in SCD (352), it is plausible that hemin may as well be related to disease severity. This suggestion is particularly noteworthy when it is considered that hemin itself is a cause of oxidative stress with significant oxidative effects on LDL and HDL particles (353). Our results showed that hemin concentration was increased in patients, while it was barely detectable in the great majority of controls. Hemin may also activate inflammation (due to its relationship with MPO levels shown in this study), which could increase HDL dysfunction. These results show that oxidative insult may oxidize Apo A1 and disrupt reverse cholesterol transport which is directly associated with HDL function.

Apart from the dysfunction in cholesterol transport, the modification of the HDL particle into a pro-inflammatory state may be an important contributor to SCD pathogenesis (201). In the current study, patients that had suffered more painful crises had higher HDL-C and, in relation, lower TG/HDL-C ratio. Although these results may initially be considered contradictory, sickle cell patients suffer from constant oxidative stress and chronic inflammation –demonstrated by our results and numerous

studies in the literature (354-357). Therefore, the HDL particle in SCD may be damaged to such a degree that its increase may translate to an elevation in inflammatory activity and worse prognosis. Considering the results of a particularly interesting (and surprising) study, which suggested that statins could contribute to SCD treatment through their cholesterol-reducing effects (358), it is apparent that lipid parameters, especially the HDL and LDL particles, require extensive research in order to pinpoint their roles in SCD pathophysiology.

S1P is a bioactive lipid with important cellular effects and signaling properties, and it has also been associated with inflammation (359, 360). A few studies have reported increased S1P in SCD mice, proposing that this relationship is associated with sickling (167, 361). In the current study, S1P was relatively increased in SCD; however, the difference from controls was insignificant. An explanation of this may be the fact that –contrary to SCD mice– our patients were receiving hydroxyurea treatment for SCD, which may have caused changes in S1P levels due to the fact that both S1P and hydroxyurea have been associated with NO bioavailability. Conversely, a very recent study reported increased S1P in pediatric SCD patients (247). However, in their study, S1P measurements were performed with serum. However, it is known that S1P levels increase during platelet activation in serum separator tubes (362), and considering the significantly increased platelet count in SCD circulation (363), the serum concentration of S1P in SCD patients would be expected to increase relatively more than control samples during clotting. Therefore, it is absolutely crucial that S1P is measured in the plasma of SCD patients in order to come to an accurate conclusion of its true *in vivo* concentration (364). Lastly, in addition to being stored in platelets, S1P is carried inside erythrocytes; making them a critical contributor to circulatory levels of S1P (365). Therefore, the erythrocyte counts in patients with SCD (also other factors) may be an important parameter to consider when measuring S1P levels as put forth by prior studies (366).

5.2. Associations Between Clinical and Laboratory Findings

We also evaluated the relationships between clinical manifestations and laboratory results obtained from our study group. The most notable of these results were those obtained from the comparison of the painful crises and CVE groups.

We found that painful crisis frequency related to lower TG/HDL-C ratio and higher HDL-C and FFA. The former result is in dramatic conflict with the study by Teixeira et al. (334). The authors of said study found that TG/HDL-C ratio was higher in those that had suffered from ACS and vaso-occlusive episodes. However, almost half of their patients were not receiving hydroxyurea treatment, exclusion from the study was not performed when patients had suffered vaso-occlusions within the past 3 months, and they also reported that the success of hydroxyurea treatment was not sufficient in those that had been receiving the drug (patients were undertreated) (334). Furthermore, even though their results showed that TG/HDL-C ratios were similar among patients with and without treatment, those with treatment had a mean ratio value that was 20% lower than patients not receiving treatment; thus, it is apparent that the groups formed in their study were not representative of a strictly steady state SCD population that had been receiving appropriate treatment. In another study, by Zorca et al. (317), higher TG/HDL-C levels were reported in patients with pulmonary hypertension; it was suggested that this ratio might measure endothelial function in SCD. However, in the discussion of their findings, Zorca and colleagues (317) reported that TG levels were significantly better as a marker of endothelial function, and thus, pulmonary hypertension. Even though it seems that previous studies suggesting TG-HDL-C ratio as a biomarker for various different complications of SCD are seriously limited, it is apparent that our results showing the exact opposite are also confusing. Therefore, evidently, much research must be done before drawing conclusions regarding the possibility of lipid biomarkers in patients with SCD (especially those in association with TG levels).

With regard to the elevated FFA levels in crises, we must note that the significant increase was only evident in the 1–5 painful crises group. It was interesting to observe that post-hoc comparisons demonstrated no difference between those without any painful crises and those that had suffered from >5 episodes. We found that FFA levels were higher in those with crises when present / absent groups were compared. This may indicate that, rather than being a result of the pathophysiology of SCD or being associated with the overall severity of disease, FFA modifications could be caused by underlying differences in lipid metabolism. The majority of available literature on this topic is not directly comparable with our findings in relation to

clinical characteristics; therefore, we are unable to compare these results (please see the prior discussion regarding FFA levels).

We also found that the frequency of CVEs was associated with higher uric acid and –surprisingly– lower hemin levels; however, there were only four patients in the CVE group. Therefore, the relevance of these results must be evaluated and confirmed in future studies.

We also report higher GGT values in those with renal complications, and higher uric acid levels in patients with endocrine complications. Finally, all parameters were similar when children with / without ACS or transfusions were compared within their respective groups.

5.3. Oxysterol Levels and Relationships with Anemia and Lipid Profile

Very few studies have investigated oxysterols and their relationships with other parameters in patients with SCD; however, these studies have reported some interesting findings (238, 239). Although oxysterols contribute to various conditions and diseases (367), many questions remain unanswered regarding the molecular mechanisms of these relationships and their direct effects on different pathways. Considering the fact that 7-KC and C-triol are primarily produced by oxidative stress (in contrast to various other oxysterols formed enzymatically), they are not natural elements in physiological mechanisms; rather, it is feasible to suggest that they influence these mechanisms pathologically. However, they do have established roles in various pathophysiological processes, mostly in relation to the fact that they can cross physiological membranes quite easily due to their (mostly) lipophilic characteristics. For instance, 7-KC has been shown to inhibit HMG-CoA reductase and reduce cholesterol (228). The majority of other proven effects of 7-KC seem to be related with oxidative effects, including inflammatory activation (368), cytotoxic effects on endothelial cells (369, 370), induction of ROS production (230), and atherosclerosis development and progression (371). Similarly, C-triol also has various effects that may be associated with its oxidative nature, including elevations in ROS production (372), dysfunctions in calcium and sodium channels (373, 374), and direct damages to endothelial cells (229). Considering that SCD is a cause of significant

anemia and the disease is known to progress with hypocholesterolemia, constant inflammatory activation and oxidative stress (66), the listed properties of these non-enzymatically produced oxysterols seem to be closely associated with the pathophysiology of SCD.

In a preliminary study, our study group had shown that pediatric SCD patients had similar C-triol levels with controls, while they had significantly increased 7-KC levels (239). This finding indicated an alteration in the oxidative stress-related (nonenzymatic) formation of oxysterols in patients; by proxy, this also suggested that the means of oxysterol formation was altered. Although the current study did not show the C-triol similarity in groups, the fact that both 7-KC and C-triol were increased in SCD does not negate the possibility that there was an underlying difference of oxysterol formation in SCD. In a study performed almost 30 years ago, Kucuk et al. (238) had demonstrated increased oxysterol concentrations in the erythrocyte membranes of patients with SCD. These oxysterols included 7-KC and C-triol, as well as 5α,6α-epoxycholesterol and 19-hydroxycholesterol (238). Although oxysterol plasma levels were not determined in their study, they concluded that these oxysterols were possibly being exchanged to and from the membranes of erythrocytes rather easily, due to the lipophilic and hydrophilic characteristics demonstrated by oxysterols (238). In relation with their crucial involvement in the structure of erythrocyte membranes, it has also been shown that externalization of PS, thus leading to eryptosis, was seen in healthy erythrocytes that were subject to oxysterol addition (C-triol and 7-KC) (119). Moreover, this increased degree of PS externalization in erythrocyte membranes with the administration of oxysterols and the subsequent activation of eryptosis was also seen among patients with SCD (120, 375). When evaluated in a holistic manner, our findings and the suggestions/conclusions of prior studies seem to point to an altered state of oxysterol formation in SCD and also a relationship between oxysterol levels and the hemolysis/eryptosis of patients with SCD.

The correlations between 7-KC and parameters that are associated with hemolysis (Hb and LDH levels) indicate that this particular oxysterol may be associated with the hemolysis in SCD. Although our literature review did not yield any studies evaluating these oxysterols in SCD, we found a remarkable study by Kulig and

coworkers that demonstrated oxysterols caused disruption in the structure of lipid membranes. The highlight of this analysis was that the disruption of lipid membranes was found to be especially increased with oxysterols that were oxidized on their sterol ring (such as both oxysterols measured in the present study) (376). Moreover, the results from a study that investigated these characteristics in both normal and sickle erythrocytes, reported that oxidation of the 7^{th} carbon in the cholesterol structure severely altered membrane fluidity (and thus, deformability). Even though they reported that the alterations in membrane fluidity were more pronounced in healthy erythrocytes compared to SCD, this can easily be explained by the inherent rigidity of SCD erythrocytes (377); thus, even though the degree of change from normal equilibrium might be lesser in SCD, it is highly likely that increased 7-KC concentrations translate to further adversities in the structure of SCD erythrocytes. Therefore, it is plausible to conclude that 7-KC levels contribute to the hemolysis and anemia of patients with SCD. We believe oxidative changes on the 7^{th} carbon of the cholesterol ring may adversely affect erythrocyte membrane rheology, which, in turn, may increase membrane fragility, leading to hemolysis.

The other correlation associated with oxysterol levels was the one between C-triol and HDL-C concentrations, suggesting a relationship between cholesterol levels and oxysterol production. Even though the correlation level was mild-to-moderate, we believe this association demonstrates a role for C-triol in the production of cholesterol in SCD. We cannot suggest such an effect in healthy subjects because C-triol has not been shown to have any effect on cholesterol producing enzymes. Nevertheless, 5,6 (α and ß) epoxycholesterols, which are formed very easily in the presence of oxidative stress (and are then metabolized into C-triol), are suggested to influence various pathways and mechanisms, including oxidative stress / antioxidant mechanisms (378), cholesterol metabolism (379), the levels of other oxysterols (380). On the other hand, the cytotoxicity of oxysterols has been determined in previous studies, with reports suggesting that C-triol was one of the strongest oxysterols in terms of cytotoxic effects (even at low concentrations); whereas, 5,6 epoxycholesterols did not show comparable levels of cytotoxicity (381). Therefore, the previously proven effects of oxysterols, such as their influence on cholesterol synthesis/metabolism and the activation of eryptosis after administration of oxysterols, seem to support the hypothesis that SCD

pathophysiology is associated –at least in part– with the formation of oxysterols in the presence of constant oxidative stress that is characteristic of SCD.

As mentioned before, our preliminary study with oxysterol measurements demonstrated that patients and controls were similar with regard to C-triol levels, although 7-KC levels were significantly different. Despite the lack of such a finding in the current results, it seems that the disproportionate change in the levels of oxysterols might be exemplified by the current results due to the strong correlation between 7-KC and C-triol in controls and the absence of correlation in the those with SCD. Both these oxysterols are produced/elevated as a result of increased non-enzymatic oxysterol formation due to oxidative stress (382). However, in patients with SCD, it seems that the heightened oxidative stress level was disproportionately effective on the levels of these oxysterols; inevitably breaking the balance (that is, the correlation) between these compounds which was present in the healthy state. With a detailed literature review, we found that oxysterol formation is associated with the actual type of oxidative compound causing the formation –also with some relation to any other compounds at the oxidation site (383). Considering the significant changes in the blood and vasculature of SCD patients and the different forms of oxidative stress, the formation of these oxysterols might have been disproportionately affected. In the context of altered oxidative burden and localized differences, it is likely that the constant oxidative insult on membrane cholesterol could lead to specific non-enzymatically formed oxysterols which may disrupt the membrane and lead to hemolysis, increasing similar type of oxidative burden, and therefore, the plasma levels of these oxysterols. However, this hypothesis requires confirmation in future studies investigating oxysterol levels in erythrocyte membranes.

5.4. Ceramide Levels and Relationships with Various Parameters

Quantification of ceramides has shown marginal but significant differences between patients and controls. Furthermore, we also found that C18 and C20-ceramides were correlated with a number of parameters measured in this study. For instance, C18-ceramide was positively correlated with parameters of hemolysis and C-triol levels. C20-ceramide levels were also positively correlated with parameters of

hemolysis and C-triol. These relationships demonstrate that the hemolysis seen in SCD may be partially caused by elevated ceramide levels and their contribution to eryptosis.

Ceramides have various roles in cellular physiology which are very closely associated with their acyl chain length (244, 384). They trigger apoptosis in numerous types of cells and they are well-known to stimulate eryptosis (385). Various studies have also demonstrated that ceramide levels were associated with cholesterol levels in the circulation (386) and lipid rafts of membranes (387). In addition to *de novo* synthesis via CerS1–6, the activities of sphingomyelinases cause the hydrolysis of sphingomyelin, producing free ceramide (246). To date, only one previous study that has investigated the levels of ceramide species in SCD exists. This research shows no similar levels of C16, C18 and C20 levels in SCD and controls; however, they found patients had lower C22 and C24 ceramide concentrations (247). However, interestingly, the same study reported a significant increase in the activity of neutral sphingomyelinase, with patients demonstrating a 2-fold elevation in activity levels; thus, suggesting an increase in the release of ceramides (247). In other studies of SCD patients, sphingomyelinase activity was also found to be elevated (247, 248), and further, its inhibition has been demonstrated to prevent hemolysis (388). In our study, subjects with SCD had marginally but significantly increased levels of C16, C18 and C20 ceramides. Furthermore, C18-ceramide was positively correlated with total/direct bilirubin, LDH levels and C-triol concentrations, while C20-ceramide was correlated positively with total/direct bilirubin and C-triol levels, and negatively with HDL-C levels. Of note, we also found that both C18-ceramide and C20-ceramide demonstrated their strongest correlations with C-triol levels, suggesting that ceramide release from the erythrocyte membranes could significantly influence the production of oxysterols via the possible activation of eryptosis that would lead to increased levels of heme and Hb in the circulation. Linear regression revealed that total bilirubin and C-triol levels were the two factors that were independently associated with both C18 and C20 ceramide concentrations. However, C16-ceramide, the ceramide that has been shown to trigger eryptosis (in addition to C6-ceramide) (385), was not associated with any of the parameters of hemolysis.

5.5. Limitations

This research is among the few studies to exhaustively assess the relationships between the major characteristics of SCD, markers of hemolysis, lipid profile, inflammation, oxidative stress, oxysterols and ceramides. However, there are several limitations to be mentioned. Firstly, in addition to the rarity of SCD, the process of patient inclusion limited group size; thus leading to a separation of groups. If all studies could have been performed in a larger group of patients, subgroups according to clinical and laboratory characteristics could have been formed, and detailed assessments could be performed. However, the importance of rigidly adhering to exclusion criteria was absolute, due to the fact that our study aimed to perform investigations in steady state patients. In relation, there were very few patients that had had clinical manifestations and complications in the current study; thus, the effects of these factors could not be reliably investigated. Secondly, as the HbSS and HbSß$^{+}$ groups were very similar in terms of all clinical and laboratory parameters, we evaluated these groups as an overall SCD group in the majority of analyses, which enabled better assessment of relationships between parameters. Even though this was not an entirely erroneous approach, these subtypes may have critical underlying differences with regard to pathophysiology. Thirdly, as this was a study of the pediatric age group, development of various characteristics and the alterations in laboratory parameters and clinical features may have been associated with the duration of disease (due to patients' ages) and treatment. Also, the effects of the use of various medications, such as analgesics and antibiotics, may have caused differences between groups, especially with regard to the SCD groups. In association with this limitation, it is also evident that not all patients respond to hydroxyurea treatment equally; thus, the effects of treatment may have varied among patients and could have led to clinical differences that could translate to altered levels of laboratory findings.

6. CONCLUSIONS AND SUGGESTIONS

6.1. Conclusions

The most widely reported lipid abnormality in SCD, hypocholesterolemia, was reflected with the significantly reduced TC, LDL-C and HDL-C levels in this study. We also found various mild-to-moderate correlations between parameters of lipid profile, hemolysis, inflammation and oxidative stress, which could partially explain the intertwined nature of disease pathophysiology in SCD.

Decreased Apo A1 levels suggest that the *in vivo* activity of LCAT may be reduced in SCD. Also, SAA, MPO and hemin levels may be impactful on HDL dysfunction and its pro-inflammatory transformation, leading to reduced reverse cholesterol transport.

An association between hypocholesterolemia and cholesterol oxidation (oxysterols) were found for the first time in this study. Furthermore, hemolysis was also associated with oxysterol levels, suggesting a vicious cycle between oxysterol formation due to oxidative stress, hemolysis, and the subsequent increase in inflammatory response causing further oxidative stress.

Also, while 7-KC and C-triol demonstrated a strong correlation in the control group, patient values were not related, suggesting significant alterations in the microenvironment of oxysterol formation in SCD. These findings indicate that the production of specific oxysterols due to oxidizing effects may lead to further hemolysis and formation of these particular oxysterols, ultimately leading to a vicious cycle.

Ceramide species were elevated (albeit marginally) in SCD. While C18 and C20 ceramides were correlated with a number of parameters, we did not find any relationships between C16-ceramide and any of the variables. These findings could be the result of eryptosis activation by ceramides, which may, in turn, lead to elevated levels of oxidative compounds in the circulation; thus increasing inflammatory activity and hemolysis.

6.2. Suggestions and Future Perspectives

The current relationships demonstrated between the major characteristics of SCD show that these pathological aspects of the disease are closely related to each other. Future studies may benefit from assessing the biochemical mechanisms of these relationships in order to be able to come to accurate conclusions.

Oxysterols were associated with hemolysis and cholesterol levels, and it may be feasible to suggest that the non-enzymatic production of oxysterol was severely altered in SCD. We believe that determining the possibly-altered *in vivo* production of these compounds could shed light into the changes and relationships we have found in oxysterols.

We did not determine oxysterol levels in the erythrocyte. Further studies investigating the production of 7-KC and C-triol levels in the erythrocytes of patients could provide a chance to compare the levels of specific oxysterols in sickle erythrocytes, and may also elucidate their contribution to the plasma pool of oxysterols.

There are other oxysterols that may be important in SCD pathophysiology such as enzymatically produced oxysterols (7α- and 7ß-hydroxycholesterol, 24-, 25- and 27-hydroxycholesterol) and their measurement will contribute to illuminating this issue.

Determining the role of ceramides in SCD may require in-depth analysis of their contribution to eryptosis. The fact that there are very few studies investigating ceramides in SCD remains as an important problem, especially considering the attention these bioactive lipids have received in studies focused on cellular survival and cancer. Therefore, there is a very real possibility of a wide range of studies on this topic, from the determination of molecular mechanisms of eryptosis to the effects of sphingomyelinase inhibitors in animal models of SCD.

To conclude, we believe that our results represent an important characterization of the relationships between lipid metabolism and other critical features of the disease. Especially considering the relationships shown in the first group of patients and also

the novel associations determined in this study concerning oxysterols and ceramides, we suggest that further lipidomics studies aimed at elucidating the biochemical mechanisms of these relationships should be performed.

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8. SUPPLEMENTS

SUPPLEMENT-1: Ethical committee approval for this thesis study.

T.C.
MERSİN ÜNİVERSİTESİ REKTÖRLÜĞÜ
KLİNİK ARAŞTIRMALAR ETİK KURULU

Karar Tarihi	Toplantı Sayısı	Karar Sayısı
28/05/2014	10	2014/115

Mersin Üniversitesi Tıp Fakültesi Dahili Tıp Bilimleri Bölümü Çocuk Sağlığı ve Hastalıkları Ana Bilim Dalı Öğretim Üyesi Prof. Dr. Selma ÜNAL'ın ve Hacettepe Üniversitesi Tıp Fakültesi Biyokimya Ana Bilim Dalı Öğretim Üyesi Doç.Dr. Yeşim ERÖZTAŞ'ın sorumluluğunda yapılması tasarlanan "Orak Hücre Hastalığında Lipit Metabolizmasındaki Değişimlerin Araştırılması ve Hastalık Patogeneziyle İlişkilendirilmesi" adlı araştırma için hazırlanmış olan ve 16/05/2014 tarihinde sunulan Girişimsel Olmayan Klinik Araştırmalar İçin Başvuru Formu ile ilgili belgeler araştırmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş, araştırmanın yürürlükte olan ilgili yasal düzenlemelere uyularak yürütülmesi ve sonuçlandırılması koşulu ile gerçekleştirilmesinde etik sakınca bulunmadığına toplantıya katılanların oy birliği ile karar verilmiştir.

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Başkan

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Prof. Dr. Olgu HALLIOĞLU KILINÇ
Başkan Yrd.

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Yrd. Doç. Dr. Nimet KARAGÜLLE
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Üye

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Üye

(Katılamadı)
Yrd. Doç. Dr. Oya ÖGENLER
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Üye

(Katılamadı)
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Üye

(Katılamadı)
Hürrem Betül LEVENT ERDAL
Üye

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Lale DAĞLI
Üye

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SUPPLEMENT-2: Turnitin digital receipt.

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HACETTEPE UNIVERSITY
GRADUATE SCHOOL OF HEALTH SCIENCES

DETERMINATION OF LIPID METABOLISM ALTERATIONS AND THEIR ASSOCIATION WITH DISEASE PATHOGENESIS IN SICKLE CELL DISEASE

Dr. Ahmet YALÇINKAYA

Program of Biochemistry
DOCTOR OF PHILOSOPHY THESIS

ANKARA
2020

SUPPLEMENT-3: Turnitin originality report.

TEZİN TAM BAŞLIĞI: DETERMINATION OF LIPID METABOLISM ALTERATIONS AND THEIR ASSOCIATION WITH DISEASE PATHOGENESIS IN SICKLE CELL DISEASE

ÖĞRENCİNİN ADI SOYADI: AHMET YALÇINKAYA

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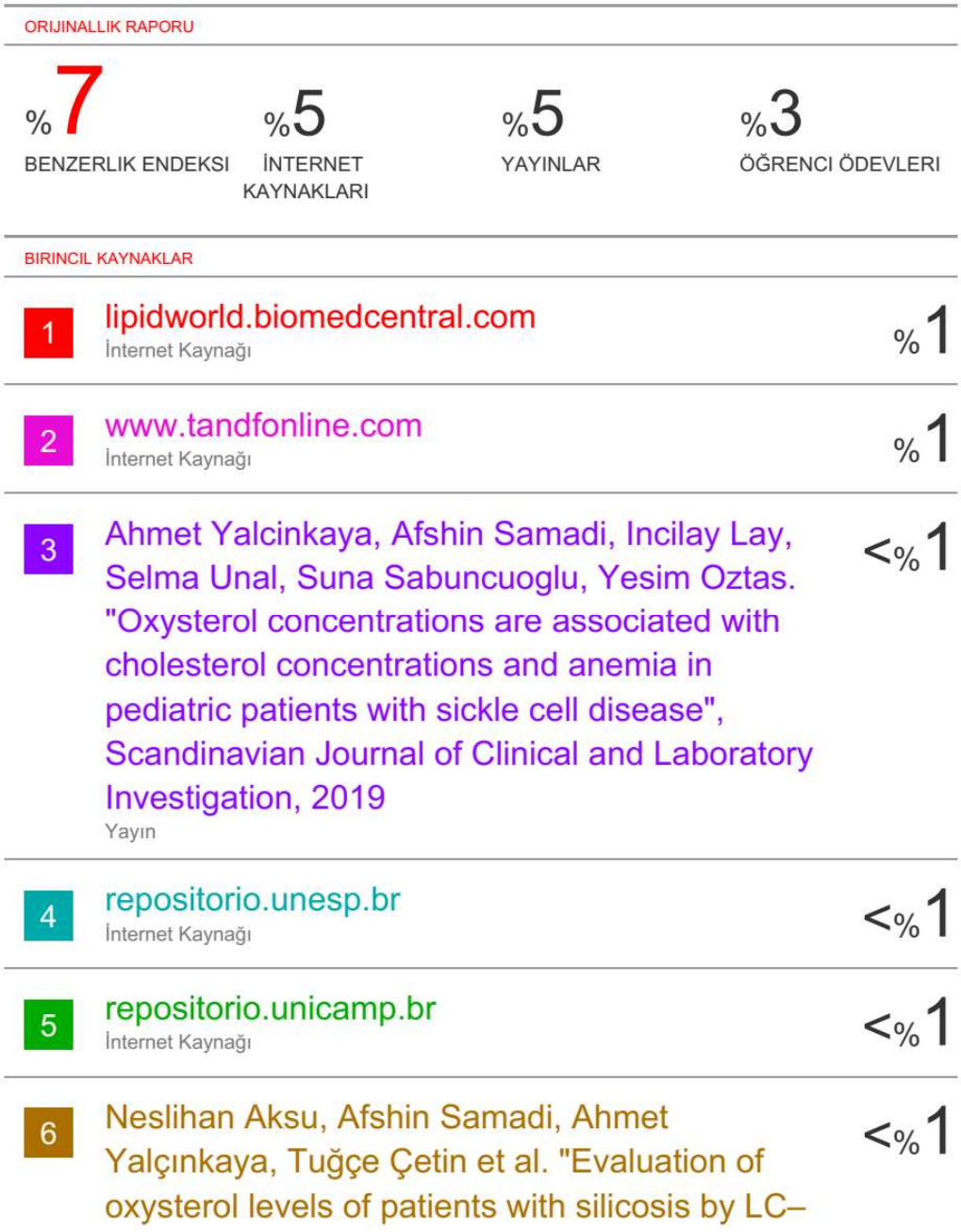

ORIJINALLIK RAPORU

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BENZERLIK ENDEKSI	İNTERNET KAYNAKLARI	YAYINLAR	ÖĞRENCI ÖDEVLERI

BIRINCIL KAYNAKLAR

1	lipidworld.biomedcentral.com İnternet Kaynağı	%1
2	www.tandfonline.com İnternet Kaynağı	%1
3	Ahmet Yalcinkaya, Afshin Samadi, Incilay Lay, Selma Unal, Suna Sabuncuoglu, Yesim Oztas. "Oxysterol concentrations are associated with cholesterol concentrations and anemia in pediatric patients with sickle cell disease", Scandinavian Journal of Clinical and Laboratory Investigation, 2019 Yayın	<%1
4	repositorio.unesp.br İnternet Kaynağı	<%1
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6	Neslihan Aksu, Afshin Samadi, Ahmet Yalçınkaya, Tuğçe Çetin et al. "Evaluation of oxysterol levels of patients with silicosis by LC–	<%1

SUPPLEMENT-4: Journal articles published from this thesis.

1. Sabuncuoğlu S, Öztaş Y, Yalcinkaya A, Ünal S, Baydar T, Girgin G. The increased neopterin content in turkish pediatric patients with sickle cell anemia. Annals of Hematology. 2020;99(1):41-7.

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Article 1: The increased neopterin content in turkish pediatric patients with sickle cell anemia.

Annals of Hematology
https://doi.org/10.1007/s00277-019-03817-5

ORIGINAL ARTICLE

The increased neopterin content in turkish pediatric patients with sickle cell anemia

Suna Sabuncuoğlu[1] · Yeşim Öztaş[2] · Ahmet Yalcinkaya[2] · Selma Ünal[3] · Terken Baydar[1] · Gözde Girgin[1]

Received: 27 March 2019 / Accepted: 5 October 2019

Abstract
In the present study, the possible activation of cellular immunity in SCD patients was investigated. As immune activation parameters, neopterin concentrations and kynurenine/tryptophan ratio for tryptophan degradation in 35 pediatric patients with sickle cell disease (31 HbSS and 4 HbSß) were determined. Our results have shown that neopterin levels (both urinary and serum) are increased in pediatric patients with sickle cell disease. The increase in neopterin concentration was accompanied by significantly increased biopterin, kynurenine concentration and kynurenine/tryptophan ratio. The mechanism of immune activation and the effects of inflammatory mediators in sickle cell disease are poorly understood, especially in terms of cell-mediated immunity. Further in-vivo and in-vitro studies are required to illuminate the association between neopterin levels and neutrophil activation in sickle cell disease.

Keywords Neopterin · Tryptophan · Kynurenine · Biopterin · Sickle cell Anemia

Introduction

Sickle cell disease (SCD) is a genetic disorder that involves a single point mutation in the beta globin gene which encodes the beta subunit of hemoglobin (Hb). This mutation translates to a single amino acid substitution of glutamic acid to valine which causes the sickle hemoglobin (HbS) [1, 2]. It is estimated that over 300,000 children are born with SCD every year in the world and the condition is especially frequent in Africa (180,000 births per year). The case number is expected to increase to up to 400,000 individuals by 2050 [3, 4]. According to the national statistics, there are about 1200 SCD patients in Turkey and the prevalence of HbS is 0.03%. [5]

The main pathophysiological basis of SCD is the polymerization of HbS in the cytoplasm of erythrocytes [1, 2]. In addition to its physical effects on cellular structure, the polymerization of HbS causes several biochemical, cellular and physiological pathologies, which are related to a variety of clinical complications including reticulocytosis, painful vaso-occlusive crises (VOCs), and end organ damage due to persistent tissue hypoxia. Red blood cells (RBC) containing HbS become rigid due to polymerized HbS fibers and are unable to pass through small arteries and capillaries, especially in hypoxic conditions. These vasoocclusions prevent blood flow in the vessels and may be exacerbated by the adhesive characteristics of various blood cells, which contribute to the occurrence of VOCs [6–8]. These patients are also subject to oxidative stress due to the constant state of inflammation in the circulation. It has been suggested that the abnormalities in SCD result from oxidative stress of the blood cells, including RBC, white blood cells (WBC) and endothelial cells, as well as activation and adhesion of platelets. The hypoferremia in SCD patients are explained by various mechanisms, such as increased iron use during the proliferation and differentiation of immune cells and the stimulation of ferritin production by TNF-α, IL-1, and IL-6, which lead to a reduction in the level of circulating iron [9, 10].

Neopterin (NP) is produced by monocytes/macrophages upon stimulation by the pro-inflammatory cytokine interferon γ (IFN-γ), as well as IFN-α, endotoxins and other cytokines [11]. Neopterin –a metabolite of guanosine triphosphate, is a biomarker of cell-mediated immunity [12]. It has been shown that NP levels increase in several inflammatory pathological

✉ Gözde Girgin
ggirgin@hacettepe.edu.tr; gzdgirgin@gmail.com

[1] Faculty of Pharmacy, Department of Toxicology, Hacettepe University, 06100 Sihhiye, Ankara, Turkey

[2] Faculty of Medicine, Department of Biochemistry, Hacettepe University, Sihhiye, Ankara, Turkey

[3] Faculty of Medicine, Department of Pediatrics, Mersin University, Mersin, Turkey

Published online: 23 November 2019

conditions including viral, bacterial, and parasitic infections [13].

In mammals, another unconjugated pteridine derivative, tetrahydrobiopterin (BH4) serves as an intracellular antioxidant to scavenge reactive oxygen species (ROS). BH4 also has cofactor functions for different enzyme systems such as aromatic amino acid hydroxylases, nitric oxide synthase (NOS), and alkyl-glycerol monooxygenase [14]. Deficiency in BH4 may result in hyperphenylalaninaemia and decreased synthesis of the neurotransmitters dopamine, norepinephrine, epinephrine and serotonin [15]. BH4 can be found in biological fluids in different forms as BH4, dihydrobiopterin (BH2), and biopterin (BP). The full oxidized form, BP is fluorescent and can be detected easily in biological fluids by HPLC [16].

Besides neopterin, tryptophan (Trp) degradation has also been shown to contribute to the modulation of cellular immunity. Tryptophan has an important role in protein biosynthesis as a nutritional and essential amino acid. Kynurenine is the main metabolite of Trp degradation. The rate-limiting step in the kynurenine pathway is catalyzed by indoleamine 2,3-dioxygenases (IDO1 and IDO2) and tryptophan 2,3-dioxygenase (TDO) [17]. TDO is known to be induced by Trp and steroids, whereas IDO is induced by proinflammatory stimuli and cytokines like IFN-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-6. Hence, IDO is responsible for the immune mediated Trp degradation [18].

Cytokine release may occur due to pathological conditions including inflammatory diseases. Hematopoiesis can be affected by the enhancement or inhibition of cellular differentiation and proliferation. IFN-γ has several important effects, including the inhibition of erythropoiesis, reduction of transferrin receptor synthesis, stimulation of iron capture from the circulation, and enhancement of the expression of ferritin mRNA. In SCD, this condition may impair hemoglobin formation and anemia [19, 20].

Patients with SCD demonstrate chronic inflammation even during the steady state of the disease [21]. In the present study, the possible activation of cellular immunity was investigated via determination of neopterin concentration and tryptophan degradation in patients with SCD.

Materials and methods

Subjects and samples

The experimental protocol was approved by the Ethics Committee of Hacettepe University (GO-17/942–04).

A total of 35 (20 males, 15 females) steady-state SCD patients (31 HbSS and 4 HbSß) followed by the Pediatric Hematology Department of Mersin University Hospital (Mersin, Turkey) and 28 healthy children (14 males, 14 females) were included in the study. The patients' SCD diagnoses were confirmed via Hb electrophoresis and β-globin gene mutation analysis. All the patients were receiving treatment with hydroxyurea. Mean age of the patient group was 13.7 ± 3.79 years (range: 5–18 years), while the mean age of the control control group was 12.7 ± 3.1 years (range: 4–17 years). The groups were similar in terms of age and sex.

Blood samples were drawn via forearm venous puncture and collected into vacutainer tubes, urine samples were collected and kept in sterile containers. Serum samples were obtained through 10 min of centrifugation at 3000 rpm. Urine samples were collected when patients consulted by the physician, preferable in the morning. All serum and urine samples were aliquoted into 2 ml eppendorf tubes and stored at −80 °C until analysis.

In the SCD group, mean (±SD) hemoglobin concentration was 9.18 ± 1.069 g/dl with a mean HbS percentage of 73.49 ± 10.05. The iron concentrations of the patients amounted to an average of 87.68 ± 41.49 μg/dL while their mean white blood count was 13,249 ± 4127 per μl and mean platelet count was 517,941 ± 212,862 per μl.

Determination of urinary neopterin, biopterin and creatinine concentrations

Before analysis, all urine samples were centrifuged and diluted with deionized water. Urinary neopterin, biopterin and creatinine concentrations were analyzed by high- performance liquid chromatography (HPLC, HP Agilent 1100, Vienna, Austria) and an isocratic, reversed-phase liquid chromatographical technique was used as described before. The coefficient of variance were 7.44 and 4.45%, respectively for μmol neopterin/mol creatinine and μmol biopterin/ mol creatinine ($n = 6$). Briefly, urine samples were 1/10 diluted with mobile phase and passed with a phosphate buffer through the octadecylsilyl (ODS) column (250 × 4.6 mm, 5 μ) which is filled with a packing of octadecylsilyl groups (ODS groups or C18 groups) chemically bonded to a silica gel carrier. This columns are very convenient for reverse-phase chromatography. The determination of neopterin was performed by a fluorescence detector (HP Agilent 1100, Vienna, Austria; excitation 353 nm, emission 438 nm). Creatinine was detected simultaneously with a UV detector (HP Agilent 1100, Vienna, Austria) at 235 nm. The results were expressed as micromoles of neopterin per mole of creatinine [22].

Determination of serum neopterin

Serum neopterin concentrations were determined by commercial enzyme-linked immunosorbent assay (ELISA) kits obtained from IBL (Hamburg, Germany) which were used according to the manufacturer's instructions. Serum samples were applied directly to ELISA kit. Optical density was

measured with a Spectra Max M2 microplate reader at 450 nm. Serum neopterin levels were expressed as nmol/L.

Determination of serum Trp and Kyn

Serum Trp and Kyn concentrations were determined by HPLC (HP Agilent 1100, Vienna, Austria), as described in another work with minor modifications [23]. Samples were passed through an ODS column (250 × 4.6 mm, 5 μ) with a phosphate buffer. Trp was detected with a fluorescence detector (excitation 286 nm, emission 366 nm). Kyn was detected simultaneously with a UV detector at 360 nm. The Trp and Kyn concentrations were both expressed in μmol/L. The coefficient of variance were 1.25 and 1.38%, respectively for Trp and Kyn. The Kyn/Trp ratio was calculated to estimate IDO activity in terms of Trp degradation, the result was expressed as micromole Kyn per millimole Trp [23, 24].

Statistical analysis

Statistical analyses were performed using the SPSS version 20 software (IBM, Armonk, NY). All results were expressed as mean ± standard error of the mean (SEM). Statistical comparisons between groups were performed using the non-parametric Mann–Whitney U-Test. Correlations among continuous variables were evaluated with the Spearman's rank correlation analysis. *p*-values less than 0.05 were considered to indicate statistical significance.

Results

Demographic and hematologic features of the patients

The general demographic and hematologic characteristics of the patients are given in Table 1.

Table 1 Demographic and hematologic features of the pediatric patients ($n = 35$)

Characteristics	Results
Age	13.7 ± 3.79 years
Sex	20 males, 15 females
Iron level	87.68 ± 41.49 μg/dL
Hb level	9.18 ± 1.069 g/dl
HbS	73.49 ± 10.05%
WBC	13,249 ± 4127/ μl
PLT	517,941 ± 212,862/μL

Hb: Hemoglobin; HbS: Hemoglobin S; WBC: White blood cell; PLT: Platelet.

Neopterin and biopterin concentrations

In the present study, serum and urine neopterin, urinary biopterin, kynurenine and tryptophan levels were measured in pediatric SCD patients and healthy controls.

Our results showed that urinary neopterin levels were significantly increased in SCD patients compared to controls ($p < 0.05$). (Fig. 1) Urinary neopterin levels were measured as 554.3 ± 93.8 and 345.5 ± 65.9 μmol neopterin/mol creatinine in patients and controls, respectively. Additionally, biopterin levels are also determined as 79.6 ± 15.2 and 155.8 ± 26.4 μmol biopterin/mol creatinine in control and patients, respectively.

As it is shown in Fig. 2, serum neopterin concentrations were also significantly increased in the patients (9.6 ± 1.6 nmol/L) compared to control group (6.4 ± 1.2 nmol/L) ($p < 0.05$).

Serum concentrations of tryptophan, kynurenine and Kyn/Trp ratio

The concentrations of Trp and Kyn were measured and the Kyn/Trp ratio was calculated in order to estimate IDO activity. The Kyn/Trp ratio and Kyn concentrations were found to be significantly higher in SCD patients ($p < 0.05$), while Trp levels were similar ($p > 0.05$). (Fig. 3). The Kyn/Trp ratio was calculated as 32.77 ± 6.19 in control group and as 45.14 ± 5.54 in SCD group.

Correlations among parameters

Urinary neopterin concentrations were positively correlated with serum neopterin level and Kyn/Trp ratio in SCD patients. (Table 2).

Discussion

SCD is a genetic RBC disorder, affects individuals in many different region in the world including African, Mediterranean, and Asian descent. Patients with SCD have various symptoms and complications which reduce quality of life. It has been reported that especially in high resource countries most of the children with milder forms of SCD have higher lifespan. Thus, SCD patients may require comprehensive, life-long management in adulthood and therefore, new studies are necessary in order to understand the mechanism and management of SCD [25].

The chronic inflammatory state in SCD has been associated with various manifestations of the disease and is suggested to play a critical role in its pathophysiology [26]. However, the mechanism of immune activation and the effects of inflammatory mediators in SCD are poorly understood, especially in

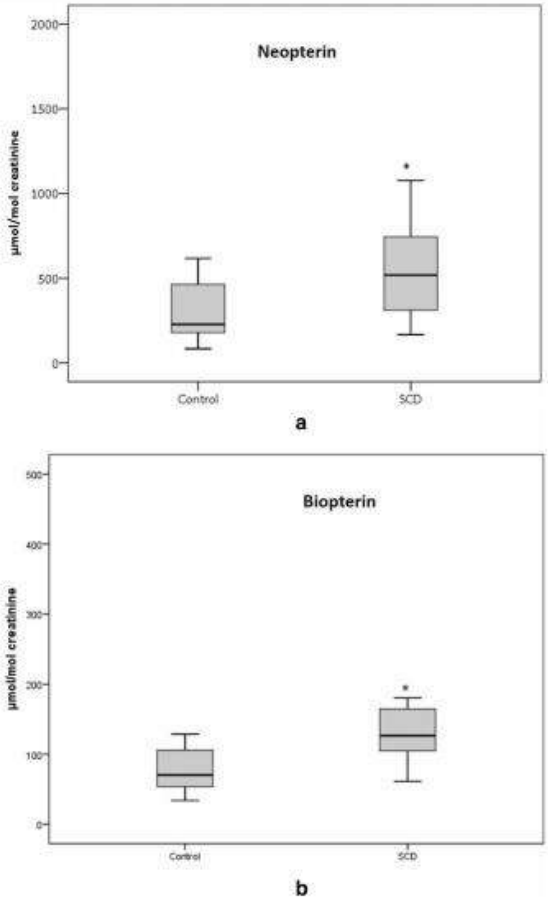

Fig. 1 Urinary neopterin (a) and biopterin (b) levels in SCD patients compared to control group, SCD: sickle cell disease, (*$p < 0.05$)

terms of cell-mediated immunity. An earlier study by Sanhadji et al. reported that, while natural killer cell activation was unremarkable, there were functional abnormalities of cell-mediated immunity in SCD patients, demonstrated by SS lymphocyte activation in culture with application of autologous sera [27]. Various other studies have also reported changes in the cell-mediated immunity of SCD in terms of T and B cells. While B cell changes were only found in a few studies, many authors have reported decreased CD4+ and CD8+ T cell proportions among patients who had normal CD4+ and CD8+ counts [28,29]. Pro-inflammatory cytokines are also found to increase significantly in SCD patients, particularly during VOCs [30–32]. Keikhaei and colleagues reported that patients who receive hydroxyurea treatment had decreased levels of TNF-α, IL-1β and IL-17; while a study by Rodrigues et al. showed increased IL-3 levels in hydroxyurea-treated patients with SCD. This data may suggest that hydroxyurea therapy also improves inflammatory problems in SCD [30].

With the aforementioned studies in perspective and given the fact that NP is considered an accurate marker of cell-

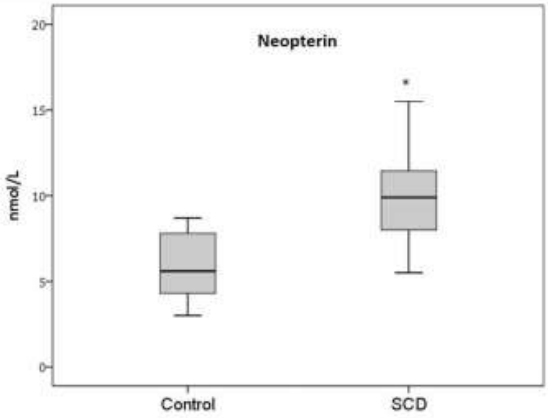

Fig. 2 Serum neopterin levels in SCD patients compared to control group. SCD: sickle cell disease, (*$p < 0.05$)

mediated immunity [33], we hypothesized that NP levels may increase in patients with SCD. Our study reports significantly elevated levels of NP in both urine and serum samples of SCD patients. To our knowledge, there are only a few studies on this topic. A study by Rodrigues et al., reported significantly increased NP levels among SS and SC patients compared to controls. The same study did not find any difference between patients and controls regarding IFN-γ concentration [34]. However, a study comprised of 76 adult steady-state SCD patients authored by Lanaro et al. found that, while circulating IFN-γ levels were similar to healthy controls, the gene expression of IFN-γ was increased in neutrophils [35].

BH4 acts as a cofactor in various processes of every cell or tissue of higher organisms and plays an important role in monoamine neurotransmitters metabolism [14,16]. Defects in the metabolism of BH4 resulting to BH4 deficiency are associated with many neurological, inflammatory and progressive diseases [16]. Many different studies showed that increased intracellular BH_4 has a role in protection of the cells from reactive oxygen radicals due to the scavenging activity

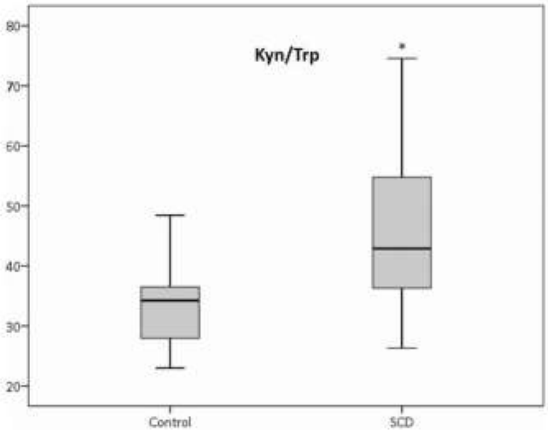

Fig. 3 Serum concentrations of Kyn/Trp ratio, SCD: sickle cell disease, (*$p < 0.05$)

Table 2 Correlation ratios between the parameters in SCD and control groups

	Control			SCD		
	Urinary biopterin	Kyn/Trp	Serum neopterin	Urinary biopterin	Kyn/Trp	Serum neopterin
Serum neopterin		$r = -0{,}087$			$r = 0.067$	
Urinary neopterin	$^{*}r = 0.577$	$r = -0.164$	$^{*}r = 0.535$	$r = 0.124$	$^{*}r = 0.586$	$^{*}r = 0.711$
Urinary biopterin		$r = 0.064$	$r = 0.061$		$r = -0.080$	$r = -0.144$

$^{*}p < 0.05$

[36]. Higher levels of BH4 in rat reticulocytes compared to mature erythrocytes were interpreted as BH4 might have a role in erythrocyte maturation. Moreover BH4 has been suggested to be have neuroprotective effects for NO toxicity, with generation of superoxide in NO-producing neurons. BPs are pteridin derivatives which are oxidized products of BH4. They have important biological functions as endogenous enzyme cofactors in many species. [16, 37].

In the current study, cell-mediated immunity in SCD was investigated by the measurement of NP, Trp and Kyn concentrations. Our findings have shown that NP levels (both urinary and serum) are increased in pediatric patients with SCD. The increase in NP concentration was accompanied by significantly increased BP, Kyn concentration and Kyn/Trp ratio. Whereas Trp concentration was found to be similar among groups. Elevation of BP and NP levels might also be related to increased oxidative stress in these patients.

As it is known from the previous studies that inflammation and immune activation are also related to the increased Trp degradation. [23]. Tryptophan forms kynurenine mediated by IDO, found ubiquitously in all tissues, and TDO which is mainly localized in the liver [38]. IDO is induced during inflammation by IFN-γ [39]. According to our results, Kyn/Tryp ratio, which is used as an estimation of IDO activity, are increased in patients with SCD compared to controls.

Although there is limited data on cytokine production in SCD patients, the result of a study indicate that a significant percentage of SCD patients in both the steady state and infectious state associated with crisis, have impaired IFN-γ production. It is suggested that immunomodulatory functions of IFN-γ, this apparent defect may be another factor to explain the increased frequency and severity of infections in SCD [40].

Taken together, these results may suggest that, IFN-γ may be specifically increased in sites where VOCs occur (due to activation of neutrophils), resulting in an alleviation of local ROS and inflammatory response which may, in turn, contribute to VOCs and the chronic inflammatory state in SCD. Furthermore, as NP is produced by monocytes and macrophages after stimulation with IFN-γ, the increase in NP levels shown in our study may be a direct result of the chronic immune activation in SCD patients. However, further in-vivo and in-vitro studies are required to illuminate the association between NP levels and neutrophil activation in SCD.

Compliance with ethical standards

Conflict of interest No conflict of interest was declared by the authors.

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Article 2: Oxysterol concentrations are associated with cholesterol concentrations and anemia in pediatric patients with sickle cell disease.

SCANDINAVIAN JOURNAL OF CLINICAL AND LABORATORY INVESTIGATION
https://doi.org/10.1080/00365513.2019.1627578

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ORIGINAL ARTICLE

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Oxysterol concentrations are associated with cholesterol concentrations and anemia in pediatric patients with sickle cell disease

Ahmet Yalcinkaya[a], Afshin Samadi[a], Incilay Lay[a], Selma Unal[b], Suna Sabuncuoglu[c] and Yesim Oztas[a]

[a]Department of Medical Biochemistry, Faculty of Medicine, Hacettepe University, Ankara, Turkey; [b]Department of Pediatric Hematology, Faculty of Medicine, Mersin University, Mersin, Turkey; [c]Department of Toxicology, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey

ABSTRACT

Sickle cell disease (SCD) causes anemia, oxidative stress, chronic inflammation, and lipid abnormalities. Oxysterols are oxidized derivatives of cholesterol and affect cholesterol metabolism and eryptosis. Our aim was to determine whether the plasma concentrations of 7-ketocholesterol (7-KC) and cholestane-3β,5α,6β-triol (C-triol) were associated with hemolysis and lipid profile in patients with SCD. A total of 32 steady-state pediatric patients with SCD (22 HbSS and 10 HbSß$^+$) and 25 healthy controls were included in the study. Hemolysis parameters, ferritin, serum iron, lipids, 7-KC and C-triol concentrations of all subjects were measured. Oxysterols were quantified with N,N-dimethylglycine derivatization via LC-MS/MS. 7-KC and C-triol concentrations were found to be increased in SCD patients, while there was no difference between the HbSS and HbSß$^+$ subgroups. 7-KC concentrations s were correlated negatively with hemoglobin and positively with lactate dehydrogenase concentrations, while C-triol concentrations were negatively correlated with HDL cholesterol. Furthermore, while 7-KC and C-triol concentrations were highly correlated among controls, there was no correlation in patients. The findings of our study suggest that 7-KC and C-triol may have a role in SCD pathophysiology. The lack of correlation in patients' 7-KC and C-triol concentrations suggest alterations in oxysterol production in patients with SCD.

ARTICLE HISTORY
Received 22 March 2019
Revised 14 May 2019
Accepted 1 June 2019

KEYWORDS
Sickle cell disease; lipid profile; oxysterol; 7-ketocholesterol; cholestane-3β,5α,6β-triol

Introduction

Sickle cell disease (SCD) identifies a group of hemoglobinopathies in which a single nucleotide mutation in the beta globin gene results in the production of hemoglobin S, a type of hemoglobin that forms polymers in hypoxic conditions which distort the characteristic shape of the erythrocyte [1]. These sickle-shaped erythrocytes are prone to hemolysis which manifests as severe anemia, especially in patients who have inherited two defective beta-globin genes from their parents – a homozygote condition (HbSS) conveniently named as sickle cell anemia (SCA) [2]. Patients who have inherited one copy of the defective gene may also have other beta-globin variants which cause compound hemoglobinopathies such as ß-thalassemia (HbSß), hemoglobin C (HbSC), hemoglobin D disease (HbSD), among others [3]. The most common types of SCD in Turkey are the HbSS and HbSß$^+$ genotypes.

The major features of SCD are anemia, oxidative stress, chronic inflammation, and lipid abnormalities including hypocholesterolemia [4]. Hypocholesterolemia in SCD was suggested to be related to increased oxidative stress in the erythrocyte by a previous study [5]. Oxysterols are oxidized derivatives of cholesterol that are produced enzymatically or by autoxidation via free and non-free radical-mediated pathways. They are known to contribute to cholesterol homeostasis by their influence on production [6]. Among oxysterols, quantification of 7-ketocholesterol (7-KC) and cholestane-3β,5α,6β-triol (C-triol) are sufficient to monitor free radical-mediated cholesterol oxidation. Furthermore, 7-KC and C-triol have been shown to increase eryptosis in red blood cells [7].

Considering these findings, we hypothesized that 7-KC and C-triol may affect hypocholesterolemia, lipid concentrations, anemia, and, thus, the pathogenesis of SCD. Therefore, our aim was to determine the relationships between oxysterol concentrations, markers of anemia, and lipid profile in the plasma of pediatric patients with SCD.

Materials and methods

Study group

A total of 32 pediatric patients with SCD who were diagnosed with HbSS ($n = 22$) or HbSß$^+$ ($n = 10$) via hemoglobin electrophoresis and/or beta globin gene mutation analysis, and 25 healthy pediatric controls were included in the study. Patients attended regular follow-up at the

CONTACT Yesim Oztas yesimeroztas@gmail.com Department of Medical Biochemistry, Faculty of Medicine, Hacettepe University, Ankara 06100, Turkey

Pediatric Hematology Clinic of Mersin University Hospital, Mersin, Turkey, and were in the steady-state of the disease at time of inclusion. Patients who had suffered vaso-occlusive crises and those who had received transfusions within the last 3 months were excluded from the study. All patients except five were receiving hydroxyurea treatment (one from the HbSß$^+$ and four from the HbSS group). Controls were selected from pediatric patients who had applied for normal pediatric follow-up and did not have any acute or chronic illnesses.

The study protocol adhered to the Declaration of Helsinki and good clinical practice guidelines, and was approved by the Ethics Committee of Mersin University (2014/115). All parents of the patients and controls provided written informed consent.

Measurements

Blood samples were drawn into ethylenediaminetetraacetic acid dipotassium (EDTA-K_2) containing tubes and serum separator tubes (Becton Dickinson, Franklin Lakes, NJ) via forearm puncture. Plasma and serum samples were obtained by centrifugation, immediately frozen at −80 °C and transferred to Hacettepe University, Department of Medical Biochemistry. Plasma samples were used for oxysterol and free fatty acid quantification, while serum samples were used for the measurement of lactate dehydrogenase (LDH), total and direct bilirubin, serum iron, ferritin, total cholesterol, HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), and triglycerides (AU 680 Chemistry Analyzer and Protein Chemistry Analyzer IMMAGE 800, Beckman Coulter, Brea, CA). Free fatty acids were measured with a fluorometric commercial kit (Abcam, Cambridge, UK).

Oxysterols

Quantification of 7-KC and C-triol were performed with a modified version of the LC-MS/MS method developed by Jiang et al. [8]. Plasma samples (50 µl) were used for detection. Sample preparation included derivatization with N,N-dimethylglycine (DMG), and a cleaning phase via LC. Internal standards were 3ß,5α,6ß-trihydroxycholestane D7 (Toronto Research Chemicals) and 3ß-hydroxy-5-cholestenoate D7 (Avanti Polar Lipids). Plasma quality control samples were prepared via addition of known amounts of 7-KC and C-triol to yield a concentration of 40 and 150 ng/mL for both oxysterols. Quantification was performed with an eight-point calibration curve (3.12–400 ng/mL). LC was performed with a Symmetry C18 column (100 mm × 2.1 mm, 5 µm) (Thermo Fisher Scientific, Waltham, MA). Mobile phase was a linear gradient of water and acetonitrile (1 mM ammonium formate, pH = 3). Mass spectrometry analysis was performed in the positive ionization mode using electrospray ionization. The multiple reaction monitoring transitions used for the detection of oxysterols were as follows: M/z of 7-KC and C-triol in M^+H^+ were 486.0 and 591.5, while the *m/z* of 7-KC-D7 and C-triol-D7 in M^+H^+ were 493.0 and 598.5, respectively [9].

Statistical analysis

Statistical analyses were performed with SPSS version 25 (IBM, Armonk, NY). Continuous variables were given as mean ± standard deviation (SD) and categorical variables were given as frequency (*n*) and percentage (%). 2-group comparisons of continuous variables were performed with the Students *t*-test and the Mann–Whitney *U* test, depending on normality of distribution which was checked with the Shapiro–Wilk test. Three-group comparisons were also performed according to normality of distribution with the Kruskal–Wallis or the one-way ANOVA tests. For correlation analyses, the Pearson significance was calculated for normally distributed variables, while the non-parametric Spearman's rho was calculated for non-normally distributed variables. A *p* value of .05 and lower was accepted to show statistical significance.

Results

The HbSS and HbSß$^+$ groups were comprised of 22 patients (14 boys and 8 girls) with a mean age of 13.2 ± 4.3 years, and 10 patients (6 boys and 4 girls) with a mean age of 16.1 ± 3.6 years, respectively. The control group was comprised of 25 healthy pediatric controls (13 boys and 12 girls) with a mean age of 13.7 ± 3.6 years. All groups were statistically similar in terms of age and sex. Additionally, there were no differences between the HbSS and HbSß$^+$ groups in regard to hydroxyurea treatment and splenectomy (Table 1).

All compared parameters except for TG were significantly different between SCD patients and controls (Table 2). Total cholesterol was significantly higher in the HbSS group compared to the HbSß$^+$ group. Significantly higher concentrations of both oxysterols were observed in SCD patients compared to controls. However, there was no difference between the HbSS and HbSß$^+$ groups in terms of 7-KC ($p = .434$) and C-triol ($p = .366$) concentrations (Figure 1).

Correlation analyses were performed without subgrouping ($n = 32$), since no significant differences were observed in the concentrations of oxysterols, iron, ferritin and hemolysis parameters between the HbSS and HbSß$^+$ subgroups. 7-KC concentrations were correlated negatively with hemoglobin ($r = -0.539$, $p = .007$) and positively with LDH concentrations ($r = 0.518$, $p = .002$) in the SCD group, whereas C-triol concentrations were negatively correlated with HDL concentrations ($r = -0.439$, $p = .022$) (Figure 2). No other significant correlations were determined between oxysterols and the remaining parameters including total and direct bilirubin concentrations. Another finding was the lack of correlation between 7-KC and C-triol concentrations in the SCD group ($p = .278$), even though there was a strong correlation between the concentrations of these oxysterols in the control group ($r = 0.620$, $p = .001$).

Table 1. Comparison of the age, sex and treatment characteristics of groups.

		Control ($n = 25$)	HbSS ($n = 22$)	HbSß$^+$ ($n = 10$)	p Value
Age (years)	Mean ± SD	13.7 ± 3.6	13.2 ± 4.3	16.10 ± 3.6	.161
		N (%)	*N* (%)	*N* (%)	
Sex	Male	13 (52%)	14 (63.6%)	6 (60%)	.715
	Female	12 (48%)	8 (36.4%)	4 (40%)	
Hydroxyurea treatment	Yes	–	18 (81.8%)	9 (90%)	.555[a]
	No	25 (100%)	4 (18.2%)	1 (10%)	
Splenectomy	Yes	–	2 (9.1%)	0 (0%)	.325[a]
	No	25 (100%)	20 (90.9%)	10 (100%)	

[a]Only HbSS and HbSß$^+$ are compared for hydroxyurea and splenectomy.

Table 2. Comparison of groups in terms of laboratory results.

		Sickle cell disease ($n = 32$)			
	Control ($n = 25$) Mean ± SD	HbSS ($n = 22$) Mean ± SD	HbSß$^+$ ($n = 10$) Mean ± SD	SCD versus control	HbSS versus HbSß$^+$
Hemolysis					
Hemoglobin (g/dL)	13.82 ± 0.91	8.91 ± 1.02	8.92 ± 1.24	**<0.001**	0.726
Lactate dehydrogenase (U/L)	–	491 ± 222	528 ± 206	N/A[a]	0.562
Direct bilirubin (µmol/L)	–	9.41 ± 4.45	9.41 ± 2.39	N/A[a]	0.795
Total bilirubin (µmol/L)	–	52.3 ± 29.9	56.4 ± 27	N/A[a]	0.764
Oxidative stress					
Serum iron (µmol/L)	–	16.7 ± 7.8	12.4 ± 4.7	N/A[a]	0.092
Ferritin (µg/L)	–	245.6 ± 303.7	177.8 ± 168.1	N/A[a]	0.458
Oxysterols					
7-ketocholesterol (µg/L)	27.86 ± 11.61	45.98 ± 3.67	44.41 ± 4.96	**<0.001**	0.434
Cholestane-3β,5α,6β-triol (µg/L)	13.86 ± 9.23	20.47 ± 1.79	19.90 ± 1.73	**0.003**	0.366
Lipid profile					
Total cholesterol (mmol/L)	4.18 ± 0.66	3.22 ± 0.71	2.65 ± 0.34	**<0.001**	**0.025**
HDL cholesterol (mmol/L)	1.1 ± 0.15	0.8 ± 0.16	0.73 ± 0.2	**<0.001**	0.238
LDL cholesterol (mmol/L)	2.48 ± 0.67	1.78 ± 0.68	1.3 ± 0.29	**<0.001**	0.084
Triglycerides (mmol/L)	1.32 ± 0.46	1.41 ± 0.52	1.34 ± 0.4	0.563	0.897
Free fatty acids (mmol/mL)	51.7 ± 96.4	89.9 ± 63.6	119.2 ± 75.4	**<0.001**	0.219

[a]The concentrations of these parameters were not measured in the control group.
p-values in bold indicate statistical significance.

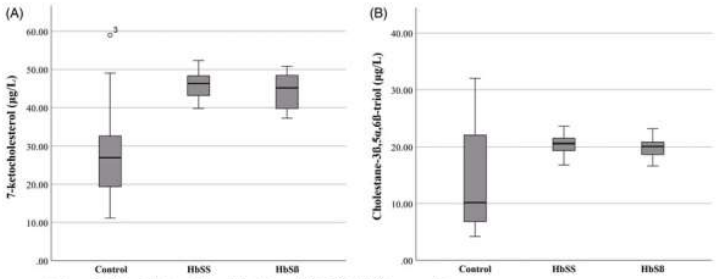

Figure 1. The concentrations of 7-ketocholesterol (A) and cholestane-3β,5α,6β-triol (B) in regard to groups.

Discussion

In the current study, we found that plasma 7-KC and C-triol concentrations are increased among patients with SCD compared to controls. Moreover, our correlation analyses revealed that, in patients with SCD, 7-KC concentrations were correlated negatively with hemoglobin concentrations and positively with LDH concentrations, while C-triol concentrations were found to be negatively correlated with HDL cholesterol. These findings indicate that oxysterols may have a role in the hypocholesterolemia and anemia observed in patients with SCD; a role which may be similar in nature to the vicious cycle of hemolysis, inflammation and endothelial dysfunction in SCD. Thus, oxysterols that are produced non-enzymatically as a consequence of oxidative stress may be a cause of further hemolysis (and/or eryptosis), inflammation and subsequent oxidative stress; forming another vicious cycle that may contribute to disease pathophysiology, as well as being a result of the condition.

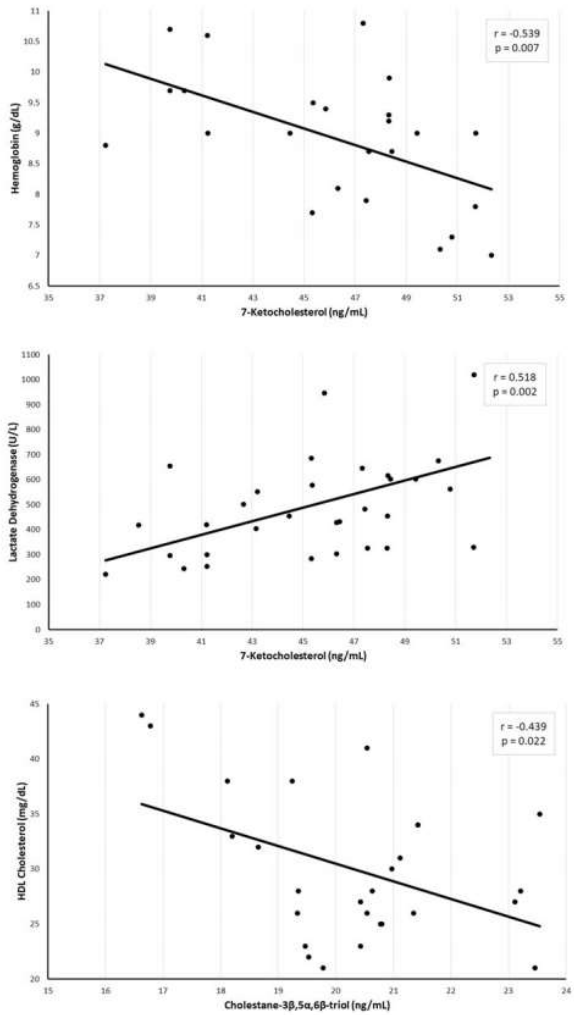

Figure 2. Significant correlations between oxysterols and other parameters.

There are only a few studies which have focused on oxysterol concentrations in SCD [10,11]. However, the effects of oxysterols on cholesterol production and other pathways have been investigated and reported in a number of studies. In particular, 7-KC is known to: decrease cholesterol synthesis by inhibition of HMG-CoA reductase [12], activate inflammation [13], induce cytotoxic effects on endothelial cells [14], upregulate the production of reactive oxygen species (ROS) [15], and it has also been implicated in atherosclerosis development [16]. In terms of C-triol, it has been shown to promote the production of ROS [17,18], affect the function of calcium and sodium channels [19,20], and

damage endothelial cells [21]. It is remarkable that all of the listed effects may have a role in the pathophysiology of SCD, as the disease causes severe anemia, influences lipid concentrations, induces chronic inflammation, disrupts endothelial function and increases oxidative stress [22].

In a previous study, we showed that plasma 7-KC concentrations were increased in SCD while C-triol concentrations were similar to controls; however, in subgroups, we had found that the C-triol concentrations of the HbSß$^+$ group were higher than that of the HbSS group [11]. In this study, both oxysterols were found to be significantly increased in the plasma of patients with SCD, regardless of subgroups. Similar to our findings, Kucuk and colleagues, in an earlier study, reported significantly increased concentrations of 7-KC, C-triol, 5α,6α-epoxycholesterol, and 19-hydroxycholesterol in the erythrocyte membrane of patients with SCD. Although they did not report plasma concentrations, they suggested that oxysterols would be dynamically exchanged between the plasma and RBC membrane [10]. We believe that, in addition to this supposed dynamic exchange, the oxysterols may cause eryptosis and/or hemolysis which could contribute to the increase in these oxysterols in the bloodstream. In a more recent study, Tesoriere et al. showed that the addition of 7-KC and C-triol to whole blood promoted eryptosis in normal RBCs via phosphatidylserine externalization [7]. Increased phosphatidylserine externalization and eryptosis were also shown in the erythrocytes of patients with SCD [23,24]. Therefore, it can be implicated that increased cholesterol autoxidation as a consequence of oxidative stress may also have a role in the eryptosis observed in SCD; thereby contributing to the anemia observed in SCD. The relationships between hypoxia and oxidative stress in the pathology of SCD has been discussed in detail previously; however, one particularly interesting association is one that was shown by Wu et al. in their study which found that the Lands' cycle was impaired in SCD and was a contributor to SCD pathology. Considering that the Lands' cycle is the only mechanism by which erythrocytes are able to perform membrane maintenance, it is rather evident that the hypoxia-mediated disruption of this mechanism will lead to problems in membrane packing and reduced defense against oxidative damage. This is another very vital aspect of SCD pathology, which indicates that the effects of hypoxia and oxidative stress are intertwined in regard to their effects on membrane structure. As such, oxidative stress (via formation of oxysterols and their adverse influence on membrane structure) and hypoxia (via impairment of the Lands' cycle) would be expected to contribute to membrane disruption in SCD; further aggravating hemolysis and/or eryptosis.

The results of the correlation analyses in our study show that plasma 7-KC is negatively correlated with hemoglobin concentrations and positively correlated with LDH concentrations. Even though bilirubin concentrations were not found to be correlated with any of the oxysterols, we believe these results demonstrate an association between oxysterol formation and anemia in SCD, especially considering the pro-eryptotic effects of 7-KC and C-triol. In support of this finding, a study by Kulig et al. reported that oxysterols, especially ring-oxidized sterols (such as 7-KC and C-triol), significantly disrupt the structure of lipid membranes. They showed that sterols with ring oxidation would be more prone to tilting within the lipid bi-layer, leading to disruption of membrane structure [25]. As such, the increase in 7-KC concentration may exhibit adverse effects on the erythrocyte membrane (in addition to causing eryptosis) which could explain the correlations found between 7-KC and the concentrations of hemoglobin and LDH in our study. We also found a moderate negative correlation between C-triol and HDL cholesterol concentrations which implies that the hypocholesterolemia seen in SCD may be associated with C-triol concentrations and/or the pathological consequences leading to its production. However, to our knowledge, there is currently no conclusive evidence that C-triol or 5,6 epoxides (formed before transformation to C-triol) contribute to cholesterol synthesis. Therefore, with current data, it is impossible to conclude that the increase in C-triol concentrations are indeed associated with the reduction in HDL cholesterol [18].

In the light of our results and considering the fact that oxysterols are highly influential on cholesterol metabolism and eryptosis, we can feasibly conclude that these compounds may have a role in the pathophysiology of SCD. Although we did not analyze the erythrocyte membrane in this study, we believe it is likely that the relationship between hemolysis and oxysterol concentrations are primarily due to the adverse effects of oxysterols on the plasma membrane. However, in order to confirm this assumption, further data from studies focused on the erythrocyte membrane are required.

It was also quite interesting to observe the lack of correlation between 7-KC and C-triol in the patient group, even though they were highly correlated in controls. However, while both 7-KC and C-triol are known to be mainly produced by ROS interaction [26], it has been shown that the type of ROS or other compounds present at the site of oxidative stress may influence the type of oxysterol formed [27]. Therefore, it is reasonable to assume that the microenvironment of oxysterol formation in SCD is quite different from those without SCD. Increased oxidative stress in the sickle erythrocyte is due to unstable HbS that leads to the production of free radicals [28]. Therefore, it is possible that membrane-bound cholesterol is oxidized locally, which may result in the production of a specific set of oxysterols (for instance, 7-KC); thus causing the lack of correlation between 7-KC and C-triol concentrations among patients in the current study. We did not measure oxysterol concentrations in patient erythrocytes. Future studies that measure the changes in the oxysterol content of the erythrocyte membrane may increase our understanding of the relationship between oxysterol formation and hemolysis (or eryptosis) in SCD.

Our study has several limitations. First, our study group may be considered small which could limit the generalization of our results; however, SCD is a rare disease and the inclusion criteria of our study further limited the number of

patients that could be included in the study. Second, we could not measure the concentrations of LDH, total and direct bilirubin, serum iron and ferritin in controls; however, the changes in the concentrations of these parameters have been documented in numerous studies. Besides, the lack of these values did not affect correlation analyses that were performed within the SCD group. Thirdly, disease duration (in relation with patient age), treatment characteristics and other recent interventions or medications (such as the use of antibiotics or pain medications) could have affected results. However, all patients were steady-state at time of blood withdrawal and all but five patients were receiving treatment with hydroxyurea; furthermore, patients who had received transfusion(s) and those who had suffered vaso-occlusive crises in the last 3 months were excluded from the study. Finally, the fact that we have not measured oxysterol concentrations in the erythrocyte membranes is another limitation of our study. However, this study for the first time suggested a link between increased plasma oxysterol concentrations, namely 7-ketocholesterol and C-triol, and SCD pathogenesis.

Conclusion

In this study we have found that plasma 7-KC and C-triol concentrations are increased in patients with SCD. Additionally, we have shown significant relationships between 7-KC concentration and the concentrations of hemoglobin and LDH, while C-triol concentrations were correlated with HDL cholesterol concentrations. We believe that these findings indicate an association between oxysterols and important characteristics of the disease such as anemia and hypocholesterolemia. Further studies with larger patient groups must be performed in order to confirm our findings and elucidate the roles of oxysterols in SCD. Future studies would also benefit from comparing oxysterol results with other parameters that have been shown to have a role in the pathophysiology of SCD.

Disclosure statement

The authors declare there are no conflicts of interest.

Funding

This work was supported by the Scientific Research Projects Coordination Unit of Hacettepe Üniversitesi under Grant number TSA-2016-7276.

ORCID

Ahmet Yalcinkaya http://orcid.org/0000-0002-1172-3330
Afshin Samadi http://orcid.org/0000-0001-7702-2273
Incilay Lay http://orcid.org/0000-0002-1466-5746
Selma Unal http://orcid.org/0000-0002-9951-0291
Suna Sabuncuoglu http://orcid.org/0000-0002-9702-4214
Yesim Oztas http://orcid.org/0000-0002-8638-9744

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Article 3: Altered HDL particle in sickle cell disease: decreased cholesterol content is associated with hemolysis, whereas decreased Apolipoprotein A1 is linked to inflammation.

Yalcinkaya *et al. Lipids in Health and Disease* (2019) 18:225
https://doi.org/10.1186/s12944-019-1174-5

Lipids in Health and Disease

RESEARCH Open Access

Altered HDL particle in sickle cell disease: decreased cholesterol content is associated with hemolysis, whereas decreased Apolipoprotein A1 is linked to inflammation

Ahmet Yalcinkaya[1], Selma Unal[2] and Yesim Oztas[1*]

Abstract

Background: Hypocholesterolemia is the most frequently encountered lipid abnormality in sickle cell disease (SCD). We enrolled pediatric patients to determine the relationships between lipid profile and parameters of hemolysis, oxidative stress and chronic inflammation in SCD.

Methods: The study involved 35 pediatric SCD patients and 19 healthy controls. Patients were crisis-free and had not received transfusions for the last 3 months. Total cholesterol, triglyceride, HDL-C, LDL-C, VLDL-C, apolipoprotein A1, apolipoprotein B, LCAT, LDH, bilirubin, haptoglobin, iron, ferritin, hemin, serum amyloid A (SAA), myeloperoxidase (MPO), uric acid, ALT and GGT levels were evaluated in patients' blood.

Results: Patients had hypocholesterolemia depicted by lower levels of total cholesterol, HDL-C, LDL-C, as well as Apolipoprotein A1 and Apolipoprotein B compared to controls. The chronic hemolysis of SCD was evident in patients by higher LDH and bilirubin and almost undetectable haptoglobin levels. Hemin levels (as a measure of oxidized heme) were significantly increased in patients with SCD. Inflammation markers, SAA and MPO, were significantly increased in the patients as well. There were negative correlations between HDL-C and LDH, and Apo A1 and SAA. Hemin was positively correlated to MPO.

Conclusion: Hemolysis was associated with decreased HDL –C, and Inflammation was linked to decreased apolipoprotein A1 levels in our SCD patients. Therefore, we suggest that the HDL particle is altered during the course of the disease. The altered HDL in SCD may become dysfunctional and result with a slowing down of the reverse cholesterol transport.

Keywords: Sickle cell disease, Hypocholesterolemia, Anemia, Inflammation, Oxidative stress

Introduction

Sickle cell disease (SCD) is an inherited hemoglobinopathy, caused by a point mutation in the beta globin gene that induces abnormal hemoglobin production (Hemoglobin S, HbS) [1]. The mutant HbS monomers polymerize under hypoxia, acidosis and dehydration, and the erythrocyte gains a sickled shape. Sickle erythrocytes become prone to hemolysis, and patients with SCD present with anemia. Sickle erythrocytes have increased adhesion to each other and vascular endothelium, resulting with vaso-occlusions. Vaso-occlusions lead to ischemia-reperfusion injury and inflammation. Besides acute clinical conditions (painful episodes, crises), severe systemic complications, such as stroke and acute chest syndrome, have been observed in SCD patients, leading to significant morbidity and mortality [2, 3].

SCD is a chronic condition and patients have increased hemolytic, oxidant and inflammatory stress, even at the steady state of the disease [4]. Anemia is a result of chronic hemolysis, induced by continuous sickling of

* Correspondence: yoztas@hacettepe.edu.tr
This study was presented as a poster abstract in ASH, 59th Annual Meeting and Exposition, December 9-12, 2017 Atlanta, Georgia, USA.
[1]Department of Medical Biochemistry, Faculty of Medicine, Hacettepe University, Ankara, Turkey
Full list of author information is available at the end of the article

the erythrocyte. This may also induce Hb denaturation and lead to increased oxidative stress through various mechanisms. Of these mechanisms, higher methemoglobin levels, heme and iron release into the circulation, and hemin formation from free heme are the most prominent. Ischemia-reperfusion injury, consumption of endothelial nitric oxide by plasma free hemoglobin and generation of free radicals by heme, hemin and free iron are all involved in the generation of a systemic inflammatory response [4]. Clinically, almost all patients with SCD suffer from various complications at very early ages, including acute chest syndrome, painful crises, cerebrovascular events and endocrine and renal problems.

Hypocholesterolemia is the most frequently encountered lipid abnormality in SCD, with decreases in all lipoprotein cholesterol fractions except VLDL [5, 6]. It occurs as a result of consumption of plasma cholesterol pool, in response to increased erythropoietic activity due to chronic anemia of various etiologies [7]. Erythrocyte lipid peroxidation product, malonyl dialdehyde, was reported to be inversely associated with plasma cholesterol levels by our group [8]. Besides increased oxidative stress and free radical production in SCD may end up with increased oxidation of plasma cholesterol producing oxysterols, such as 7-ketocholesterol. We found increased 7-ketocholesterol levels in the plasma of SCD patients compared to healthy controls [9, 10].

Lipoprotein particles undergo continuous remodeling in the plasma as they have a dynamic equilibrium [11]. Low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) levels were reported to decrease in SCD, in addition to some alterations in their composition [12]. Oxidative damage to the components of these lipoprotein particles may also lead to the increased removal of these particles from the circulation by the macrophages [13]. Lipid peroxidation products may affect the function of lipoprotein particles, such as decreased cholesterol uptake by HDL particle (reverse cholesterol transport) and increased plasma elimination of LDL [14].

During acute hemolysis, membrane remnants, particularly cholesterol, are carried to the spleen by the reticuloendothelial system. An acute phase protein, serum amyloid A (SAA) was known to contribute to this process [15]. There is one study that suggested increased SAA levels as an acute phase marker in SCD [16]. However, SAA was not investigated in SCD in line with its mentioned effects on lipid metabolism.

We hypothesized that hypocholesterolemia is associated with chronic hemolysis, oxidative stress and inflammation in the course of SCD. We enrolled pediatric SCD patients (i) to determine the lipid profile and parameters of hemolysis, oxidative stress and chronic inflammation and (ii) to evaluate the relationships between lipid profile and all of these parameters in SCD.

Material and methods

Study group

The study group involved pediatric patients aged between 5 and 19 years. The diagnoses of all patients with SCD had been confirmed via hemoglobin electrophoresis, high performance liquid chromatography and mutation analyses. Inclusion criteria were: attending regular follow-up at the pediatric hematology clinic of Mersin University Hospital, having a diagnosis of homozygous SCD (HbSS) or sickle beta thalassemia (HbSß$^{+}$), being crisis-free for at least 3 months, and not having received transfusion(s) during the last 3 months. Exclusion criteria were: suffering from any other chronic disease, having an active infection or an inflammatory condition, not accepting to participate in the study or withdrawing at any point. All patients had been receiving treatment with hydroxyurea.

The control group was comprised of age- and sex-matched healthy children who had applied to the hospital for routine pediatric check-up. Those who declined to participate, those with any acute or chronic illnesses and those using any medications were excluded from the study.

The following clinical characteristics (during the year before their blood withdrawal) of all patients were obtained from patient records: painful episode (crisis), acute chest syndrome, cerebrovascular event, transfusion, and renal, endocrine and cardiac complications. Patients were categorized into 3 groups, according to the number of painful episodes (none, 1–5 times, and > 5 times). The other characteristics were evaluated on a present/absent basis. The presence or absence of renal, endocrine and cardiac complications was defined as a new development of complication or significant worsening of patients' ongoing problems as reported by a pediatric hematologist (S.U). Acute chest syndrome was defined with the emergence of radiological findings in the presence of respiratory and/or systemic symptoms related to an infection (cough, fever, shortness of breath). Cerebrovascular events were defined according to magnetic resonance imaging that was ordered in the event of clinical suspicion. Renal, endocrine and cardiac complications were defined according their respective imaging/clinical findings (if applicable) and laboratory results (creatinine levels, thyroid/liver/adrenal test results and troponin levels, respectively).

Ethical approval was obtained from Mersin University Clinical Research Ethical Committee (2014/115). All patients and controls (and their caretakers) provided written informed consent. All steps of the study conformed to the Helsinki Declaration and Good Clinical Practice guidelines.

Sample collection

Blood samples were collected in serum separator and EDTA containing tubes. Serum and plasma from these samples were obtained via centrifugation (4000 RPM, 10 min) at Mersin University Hospital, Department of Pediatric Hematology. The samples were aliquoted, immediately frozen at − 80 °C and transferred to Hacettepe University Faculty of Medicine, Department of Medical Biochemistry, where all measurements were performed.

Laboratory investigations

Lipid profile was determined by measuring total cholesterol (TC), HDL cholesterol (HDL-C), LDL cholesterol (LDL-C), VLDL-C and triglycerides (TG) in the serum by an autoanalyser (AU 680 Chemistry Analyser, Beckman Coulter, USA), using commercial kits in the clinical chemistry laboratory of Hacettepe University Hospital. Additionally, apolipoprotein A1 (Apo A1), apolipoprotein B (Apo B), free fatty acids (FFA) and lecithin cholesterol acyl transferase (LCAT) activity were measured. Commercial ELISA and EIA kits were used to measure FFA concentration (Abcam, United Kingdom) and LCAT activity (Elabscience, China), while Apo A1 and Apo B were measured via a nephelometric method in the clinical chemistry laboratory (Protein Chemistry Analyser, IMMAGE 800, Beckman Coulter, USA).

Hemolysis was evaluated by measuring lactate dehydrogenase (LDH), total bilirubin, direct bilirubin (AU 680 Chemistry Analyser, Beckman Coulter, USA) and haptoglobin (Hpg) in the clinical chemistry laboratory (Protein Chemistry Analyser IMMAGE 800, Beckman Coulter, USA).

Oxidative stress was evaluated by measuring hemin, iron and ferritin levels. Hemin was measured via enzyme immunoassay kit (Abcam, United Kingdom). Iron and ferritin were measured in the clinical chemistry laboratory (AU 680 Chemistry Analyser, Beckman Coulter, USA).

Inflammation was assessed by the determination of the levels of serum amyloid A (SAA), myeloperoxidase (MPO) and chitotriosidase activity. SAA and MPO were measured with commercially available ELISA kits (Abcam, United Kingdom).

Levels of alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT) as measures of hepatic function and uric acid levels as endogenous antioxidant were determined in the clinical chemistry laboratory of Hacettepe University Hospital (AU 680 Chemistry Analyzer, Beckman Coulter, USA).

Statistical analysis

All results and data were obtained during the study were analyzed by SPSS v20 software (IBM, Armonk, NY, USA). Categorical variables were given as frequency (N) and percentage (%). Continuous variables were given as mean ± standard deviation (mean ± SD) or median (min-max) with regard to normality of distribution and statistical results. Normality of distribution was tested with the Shapiro-Wilk test. Categorical variables were compared with chi-square tests. 2-group comparisons of normally distributed continuous variables were performed with the independent samples t-test, while the Mann-Whitney U test was used to compare non-normally distributed continuous variables. > 2-group comparisons were performed with the Kruskal-Wallis test for continuous variables. The non-parametric Spearman correlation and the parametric Pearson correlation coefficients were calculated to evaluate relationships between continuous variables with regard to normality of distribution. *P* values that are lower than or equal to 0.05 were accepted as statistical significance.

Results

We recruited 54 individuals of pediatric age group, 35 patients with SCD and 19 healthy controls. The patient group was comprised of 20 boys and 15 girls with a mean age of 13.5 ± 4.1 years, while the control group was comprised of 10 boys and 9 girls with a mean age of 13.5 ± 3.5 years. There were no differences between the groups in terms of age and sex.

There were no statistically significant differences in the clinical and laboratory findings between the patients with HbSS and HbSß$^+$ genotypes. Hematological parameters of the patients and clinical history of the patients in the last year were summarized in Tables 1 and 2, respectively. When patients were compared in terms of laboratory parameters with regard to the presence or absence of complications, there were no differences in any of the laboratory parameters in any of the complications.

Patients with SCD had hypocholesterolemia depicted by lower levels of TC ($p < 0.001$), HDL-C ($p < 0.001$) and LDL-C ($p < 0.001$), compared to controls. Apo A1 and Apo B levels were also decreased in SCD patients

Table 1 Hematological parameters of the SCD patients

	SCD Patients (*N* = 35)
Hb (g/dL) (mean ± SD)	9.2 ± 1.1
Hb S (%)	79.8 ± 7.0
Hb F (%)	13.6 ± 7.6
Hb A_1	1.0 ± 1.7
Hb A_2	4.5 ± 1.7
White Blood Cell count (× 10^3/mm^3) (mean ± SD)	13.1 ± 4.4
Platelet count (× 10^3/mm^3) (mean ± SD)	464 ± 171

SCD: Sickle cell disease
Hb: Hemoglobin

Table 2 Number of SCD patients having disease related complications in the last year

		SCD patients (N = 35)
Number of Painful crises	1–5	21
	> 5	7
Acute chest syndrome		21
Cerebrovascular event		4
Transfusion		13
Renal complication		9
Endocrine complication		6
Cardiac complication		0

SCD: sickle cell disease

compared to controls. Patients with SCD were found to have significantly higher levels for TG and FFA levels than controls (Table 3).

Patients had significantly increased hemolysis reflected by increased LDH ($p < 0.001$), total bilirubin ($p < 0.001$) and direct bilirubin ($p < 0.001$) levels. Median Hpg concentration in the control group was 83.4 mg/dL, while Hpg concentration was undetectable in almost all patients, except three patients with levels of 6.2 mg/dL, 32.4 mg/dL and 88.2 mg/dL.

Compared to controls, hemin levels (as a measure of oxidized heme) were significantly increased among patients with SCD ($p < 0.001$). Ferritin levels were also significantly higher in the patient group ($p < 0.001$).

The inflammation markers, such as SAA and MPO, were significantly increased in patients with SCD. The serum levels of ALT and GGT, as markers of liver function, were significantly increased in patients compared to controls.

The correlation analyses were performed to evaluate the relationships among anemia, lipid profile, inflammation and oxidative stress. The significant correlations among these characteristics of the disease are depicted in Table 4. HDL-C is positively correlated to Hb and negatively correlated to LDL. On the other hand Apo A1 is negatively correlated to SAA. Apo B is negatively correlated to serum iron and positively correlated to ferritin.

Discussion

This study was conducted to understand the metabolic changes that were associated with hypocholesterolemia in the pathological course of SCD. The major findings of the study were the association between hemolysis and decreased HDL-C, as well as the link between inflammation and decreased apolipoprotein A1 levels. We suggest that, during the pathological course of SCD, the production of the HDL particle is altered, or its function is influenced.

Table 3 Laboratory characteristics of patients with sickle cell disease and controls (median, min-max)

	SCD Patients (N = 35)	Controls (*N* = 19)	*P* value
Age (years)	13.5 ± 4.1	13.5 ± 3.5	0.557
Gender (Boys/Girls)	20/15	10/9	0.597
TG (mg/dL)	127 (58–246)	89 (54–223)	0.042
TC (mg/dL)	116 (76–170)	167 (117–240)	0.000
HDL-C (mg/dL)	28 (20–43)	50 (32–82)	0.000
LDL-C (mg/dL)	70 (44–106)	106 (62–143)	0.000
VLDL-C (mg/dL)	25 (12–49)	18 (11–45)	0.035
Apo A1 (mg/dL)	134 (94–378)	198 (136–268)	0.000
Apo B (mg/dL)	73 (54–202)	82 (55–107)	0.016
LCAT (U/L)	133 (8–457)	285 (113–1190)	0.441
LDH (U/L)	487 (251–1122)	205 (122–293)	0.000
T. Bilirubin (mg/dL)	1.7 (0.3–4.4)	0.2 (0.1–0.6)	0.000
D. Bilirubin (mg/dL)	0.4 (0.1–0.9)	0.1 (0.0–0.1)	0.000
Hemin (mg/dL)	2.80 (0.01–22.16)	0.01 (0–0.27)	0.000
Serum Iron (μg/dL)	81 (39–237)	65 (14–143)	0.385
Ferritin (ng/mL)	112 (35–1293)	13 (6–46)	0.000
Uric acid (mg/dL)	4.7 (3.4–8.3)	4.0 (2.3–6.3)	0.025
SAA (mg/dL)	1.14 (0.27–2.63)	0.80 (0.27–1.69)	0.041
MPO (μg/L)	76 (34–189)	43 (29–56)	0.000
ALT (U/L)	21 (10–86)	12 (3–36)	0.000
GGT (U/L)	14 (8–180)	12 (3–24)	0.023

SCD: sickle cell disease, TG: triglycerides, TC: total cholesterol, HDL-C: high-density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol, VLDL-C: very low density lipoprotein cholesterol, Apo A1: apolipoprotein A1, Apo B: apolipoprotein B, FFA: free fatty acids, LCAT: lecithin acyl cholesterol transferase, LDH: lactate dehydrogenase, SAA: serum amyloid A, MPO: myeloperoxidase, ALT: alanine transaminase, GGT: gamma-glutamyl transferase

In this study, pediatric SCD patients had hypocholesterolemia, reflected by lower total cholesterol, HDL-C and LDL-C levels. Median Apo A1 and Apo B levels in the patients were also lower than controls, suggesting either decreased production or increased catabolism of the HDL and LDL particles. The depletion of Hpg and increases in LDH and bilirubin levels demonstrate the

Table 4 Significant correlations among lipid, hemolysis and inflammation parameters in SCD

		r	p
HDL-C	Hb	0.454	0.012
	LDH	−0.590	< 0.001
Apo A1	SAA	−0.482	0.008
Apo B	Serum Iron	− 0.360	0.050
	Ferritin	0.438	0.025
Hemin	MPO	0.383	0.040

HDL-C: high density lipoprotein cholesterol, Hb: hemoglobin, LDH: lactate dehydrogenase, Apo B: apolipoprotein B, SAA: serum amyloid A, Apo A1: apolipoprotein A1
MPO: myeloperoxidase

presence of the characteristic chronic hemolysis in SCD. Increased hemin levels depicted the increased oxidative stress; whereas increased SAA and MPO demonstrated chronic inflammation. Correlation analysis showed that HDL-C levels were associated with hemolysis and anemia as well as chronic inflammation in SCD patients.

Numerous studies have shown that the levels of various lipids are altered in steady-state SCD and vaso-occlusive episodes of the disease [6, 12, 17]. Hypocholesterolemia is a hallmark of SCD and relative increases in TG levels have also been shown. TG concentration has been shown to be associated with hemolysis and inflammation [5, 12, 18, 19]. We also observed decreased TC and increased TG levels in the serum of our pediatric SCD patients, compared to healthy children (Table 1). Serum HDL-C and LDL-C levels of the patients were also lower than controls in this study.

The enzyme LCAT, which functions in reverse cholesterol transport, is carried by the HDL particle and LCAT activity is an important parameter of HDL function [20]. In a recent study, Ozturk et al. reported a reduction in LCAT protein levels in SCD [21]; however, they measured protein levels and not LCAT activity. Whereas, Soupene et al. measured LCAT specific activity and concluded that LCAT specific activity was reduced in patients with SCD, compared to controls [22]. These studies all included adult patients. In the current study, in which pediatric patients were enrolled, LCAT activities were similar in patients and controls, while Apo A1 (its cofactor) was found to be significantly lower in patients. Similar to our findings, a few studies have reported reduced Apo A1 levels at steady state and during vaso-occlusive events in patients with SCD [16, 23].

Severe chronic hemolysis was shown in our pediatric SCD patients by increased LDH levels and almost undetectable Hpg (due to scavenging toxic heme) levels. Hpg has aslo been shown to bind Apo A1 during hemolysis [24], thereby protecting Apo A1 from the detrimental effects of free oxygen radicals produced in the plasma [25]. However, severe hemolysis in SCD exhausts almost all Hpg in the plasma, causing Apo A1 to become open to the constant oxidative stress in the circulation of patients with SCD. This mechanism, the loss of defense against oxidative damage, may be a mechanism that caused the relationships between oxidative/inflammatory parameters and the levels of Apo A1 and HDL-C.

Plasma hemin levels were increased in SCD patients compared to the almost-undetectable levels in controls. Hemin is produced by the oxidation of heme [26], a molecule which was suggested to be a biomarker of disease severity in SCD [27]. It has also been shown that hemin causes oxidative damage to plasma lipoproteins, such as LDL and HDL [28]. In our study, hemin levels were found to be correlated positively with MPO levels, suggesting that hemin may play an important role in the activation of inflammation in patients with SCD, which may in turn aggravate the oxidative damage sustained by erythrocytes, endothelial cells, HDL and LDL. Taken together, these results suggest that hemin levels may contribute to inflammatory activation and possibly the oxidation of the Apo A1 of the HDL particle in SCD patients; thus causing HDL dysfunction. A recent study is linked low levels of lipoproteins to hemolysis markers, particularly to hemopexin concentrations in adults with SCD [29].

In the current study, SAA levels, as a measure of inflammation, were significantly higher among patients at steady state, which is consistent with previous studies [16, 30, 31]. There was also a negative correlation between SAA and Apo A1 levels of the SCD patients, indicating a negative relationship between chronic inflammation and HDL function. SAA is an acute phase protein synthesized by the liver during inflammation. Although vascular injury and infarction lead to increased SAA levels in SCD [32], chronic hemolysis itself is an important stimulus to increase acute phase proteins including SAA. Interestingly, SAA has been shown to have a role in splenic cholesterol transport in a mouse model mimicking hemolytic disorders [15]. Furthermore, SAA is also suggested to promote rapid cholesterol efflux in macrophages that carry phagocyted cellular debris to the spleen, a reticulo-endothelial organ [33]. Therefore, we suggest that chronic hemolysis in SCD may induce SAA production which may then contribute to hypocholesterolemia. This may also have a role in reducing reverse cholesterol transport of HDL particle. Therefore, in addition to the effects of oxidative damage, the replacement of Apo A1 by SAA seems to be associated with the dysfunction in HDL and Apo A1.

In the current study, we found MPO levels to be significantly higher among SCD patients, compared to controls. Besides, MPO levels were positively correlated with hemin levels, which can be explained by the role of MPO in both the oxidative and inflammatory processes. MPO is an enzyme that is crucial to the oxidative burst of neutrophils during the inflammatory response and it has been shown to cause oxidative damage to Apo A1 [34]. Additionally, MPO inhibition was shown to decrease vascular oxidative stress and induce vasodilation in murine models of SCD [35]. Thus, we believe that having a role in both oxidative stress and inflammation, MPO contributes to alteration of HDL particle in SCD.

According to Table 4, both HDL composition and structure seem to be affected by hemolysis and inflammation in our study. A previous study supported our view, by suggesting that Apo A1 lipoprotein composition and function are altered in SCD. The authors suggested that this was associated with the structural and functional changes seen in the HDL_2 and HDL_3 particles in

SCD. As the level of HDL_3 is lowered in SCD, the Apo A1 exchange rate also decreases; thus affecting cholesterol import and export by the HDL particle, which is worsened by the fact that there is an overall reduction in HDL-C levels in patients with SCD [36]. Sexias et al. previously suggested that SCD patients with low HDL and relatively increased TG might have a specific dyslipidemic phenotype of the disease [5]. However, they did not suggest any structural alteration in HDL particle as the cause of this phenotype, but their study also did not determine this with structural evidence, as the study was focused on patient characteristics (similar to ours). However, from a molecular point of view, it is very likely that the pro-inflammatory transformation of the HDL particle in SCD due to oxidative insult [37] causes lipid peroxidation (among other affects) and significantly influences all facets of lipoprotein and apolipoprotein function [14]; therefore, these alterations may be the molecular basis of the changes seen in HDL-C and Apo A1 levels and their relationships with oxidative and inflammatory parameters.

The most important limitation of this study is that, although we measured many parameters that may modify or alter the structure and function of HDL particle, we were unable to investigate the structure and composition of the HDL particle separated from SCD patients' blood. However, we plan to investigate oxidative alterations in the HDL particle's lipidome and proteome, particularly the oxidative post-translational modifications of Apo A1, in future studies. Besides, SCD is a rare disease and we were able to admit 35 children from a total of 70 patients under follow-up in Mersin University Hospital. The patient number is limited due to inclusion criteria limiting the age, sex and clinical condition of patients; as only steady state patients with no crisis and transfusion for the last 3 months were enrolled. Finally, although no relationships were found with regard to the presence/absence of complications within the prior year, we believe including a higher number of patients may provide a chance to assess the results of these complications in patients with SCD.

Conclusion

Pediatric SCD patients in this study had hypocholesterolemia reflected by low TC, LDL-C and HDL-C levels. We suggest that the cumulative effect of chronic hemolysis, oxidative stress and inflammation in SCD may result with a pathological structural alteration in HDL. Several explanations about these relationships were reported in this study under the light of prior research on this topic. Briefly put, the slowing down of reverse cholesterol transport (induced by increased SAA) in response to chronic inflammation and/or oxidative stress may be an important cause of hypocholesterolemia in SCD.

Abbreviations

Apo B: Apolipoprotein B; ApoA1: Apolipoprotein A1; FFA : Free fatty acids; Hb: Hemoglobin; HbS: Hemoglobin sickle; HbSS: Homozygous SCD; HbSß$^+$: Hemoglobin sickle beta thalassemia; HDL: High density lipoprotein; HDL-C: High density lipoprotein cholesterol; Hpg: Haptoglobin; LCAT: Lecithin cholesterol acyl transferase; LDH: Lactate dehydrogenase; LDL: Low density lipoprotein; LDL-C: LDL cholesterol; MPO: Myeloperoxidase; SAA: Serum amyloid A; SCD: Sickle cell disease; TC: Total cholesterol; TG: Triglycerides; VLDL: Very low density lipoprotein; VLDL-C: Very low density lipoprotein cholesterol

Acknowledgements

We thank all the patients and their families for their involvement in this study.

Authors' contributions

A.Y. obtained samples from patients, conducted experiments, performed statistical evaluations, and wrote the draft of the manuscript. S.U. contributed to the design of the study, recorded patient history, assessed the clinical characteristics of patients and performed interviews during the enrolment process, supervised sample collection, and provided critical feedback on the manuscript. Y.O. conceived the idea, designed the study, obtained necessary permissions, provided scientific and statistical support, conducted and supervised all study steps and experiments, revised and wrote the final manuscript.

Funding

This study was supported by a grant from Scientific Research Projects Coordination Unit, Hacettepe University (TSA-2016-7276).

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Ethical approval was obtained from Mersin University Clinical Research Ethical Committee (2014/115). All patients and controls (and their caretakers) provided written informed consent. All steps of the study conformed to the Helsinki Declaration and Good Clinical Practice guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

Author details

[1]Department of Medical Biochemistry, Faculty of Medicine, Hacettepe University, Ankara, Turkey. [2]Department of Pediatric Hematology, Faculty of Medicine, Mersin University, Mersin, Turkey.

Received: 22 July 2019 Accepted: 16 December 2019
Published online: 20 December 2019

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Publisher's Note

Article 4: Oxidative alterations in sickle cell disease: possible involvement in disease pathogenesis.

World Journal of Hematology

Submit a Manuscript: http://www.f6publishing.com

World J Hematol 2017 August 6; 6(3): 55-61

DOI: 10.5315/wjh.v6.i3.55

ISSN 2218-6204 (online)

MINIREVIEWS

Oxidative alterations in sickle cell disease: Possible involvement in disease pathogenesis

Yesim Oztas, Ahmet Yalcinkaya

Yesim Oztas, Ahmet Yalcinkaya, Department of Medical Biochemistry, Faculty of Medicine, Hacettepe University, 06100 Ankara, Turkey

Author contributions: Oztas Y designed the concept of the manuscript and wrote it; Yalcinkaya A contributed in writing some sections.

Conflict-of-interest statement: There is no conflict of interest.

Manuscript source: Invited manuscript

Correspondence to: Yesim Oztas, MD, PhD, Department of Medical Biochemistry, Faculty of Medicine, Hacettepe University, Sihhiye, 06100 Ankara, Turkey. yoztas@hacettepe.edu.tr
Telephone: +90-312-3051652
Fax: +90-312-3245885

Received: January 8, 2017
Peer-review started: January 12, 2017
First decision: February 16, 2017
Revised: April 26, 2017
Accepted: May 21, 2017
Article in press: May 22, 2017
Published online: August 6, 2017

Abstract

Sickle cell disease (SCD) is the first molecular disease in the literature. Although the structural alteration and dysfunction of the sickle hemoglobin (HbS) are well understood, the many factors modifying the clinical signs and symptoms of the disease are under investigation. Besides having an abnormal electrophoretic mobility and solubility, HbS is unstable. The autooxidation rate of the abnormal HbS has been reported to be almost two times of the normal. There are two more components of the oxidative damage in SCD: Free radical induced oxidative damage during vaso-occlusion induced ischemia-reperfusion injury and decreased antioxidant capacity in the erythrocyte and in the circulation. We will discuss the effects of oxidative alterations in the erythrocyte and in the plasma of SCD patients in this review.

Key words: Oxidative stress; Sickle cell disease; Iron; Protein oxidation; Carbonyl group; Sulfhydryl group; Low-density lipoprotein; High-density lipoprotein

Core tip: Oxidative alterations in the plasma and erythrocyte of sickle cell disease may indicate disease progression and phenotype. Detected oxidative modifications may be used as disease markers. Novel drugs targetting oxidative damage of plasma and cellular components may be important as promising therapeutic options.

Oztas Y, Yalcinkaya A. Oxidative alterations in sickle cell disease: Possible involvement in disease pathogenesis. *World J Hematol* 2017; 6(3): 55-61 Available from: URL: http://www.wjgnet.com/2218-6204/full/v6/i3/55.htm DOI: http://dx.doi.org/10.5315/wjh.v6.i3.55

INTRODUCTION

Sickle cell disease (SCD) is an autosomal recessive disease which was first reported by an American

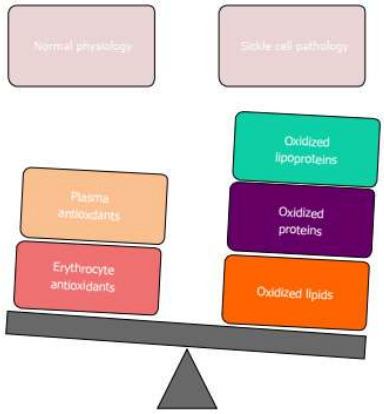

Figure 1 The balance between antioxidants and oxidants in sickle cell disease is altered towards the increase of oxidative stress and production of oxidized lipids, proteins and lipoproteins.

physician, James Herrick in 1904[1]. It was noted for being the first molecular disease after demonstration of the point mutation in beta globin gene in 1949[2]. Acidic glutamate residue at the sixth position was exchanged with a hydrophobic valine in the beta subunit of sickle hemoglobin (HbS). Solubility of abnormal HbS decreases in deoxygenation, dehydration and acidosis resulting with formation of long and solid polymers in the erythrocyte where it interacts with the cytoskeleton forcing the cell to get an almost sickled shape. Although the erythrocyte has a high capacity to move through the narrowest capillaries, the sickle erythrocyte loses its elasticity and tends to slow down and accumulate resulting with vaso-occlusion.

SCD is characterized by anemia, vaso-occlusion and chronic inflammation. Ischemia, and necrosis develop after vaso-occlusion concomitantly resulting with organ dysfunction[3]. Acute vaso-occlusive crisis is the most common clinical presentation that results with hospitalization. Pulmonary hypertension, leg ulcers, priapism and stroke may develop as a complication of vaso-occlusive crisis. On the other hand frequent transfusions result with iron deposition in the tissues and organs of the patients with resultant organ dysfunction[4]. Iron induces generation of free radicals that produce oxidative stress and damages cell membrane, organelles and DNA[5].

Although the structural alteration and dysfunction of the HbS are well understood, the many factors modifying the phenotype of the patients and clinical presentation are under investigation. Understanding the spectrum of biochemical alterations produced by this genetic disease, novel therapeutic approaches can be developed to increase the life quality of the patients.

OXIDATIVE PROCESSES IN THE NORMAL ERYTHROCYTE

The erythrocytes have always been subjected to oxidative stress because they already transport oxygen in the circulation[6]. While there is a continuous flow of oxygen in the erythrocyte, it additionally contains iron (Fe^{2+}) bound to heme in the cytoplasm surrounded by a membrane containing unsaturated fatty acids[7]. However, under normal conditions Fe^{2+} is isolated in the pocket of heme group and the antioxidant enzymes work to prevent or limit the damage of the oxidant stress[8].

When deoxyhemoglobin binds oxygen, an electron from Fe^{2+} of hemoglobin is transferred to oxygen forming oxyhemoglobin also called superoxyhemoglobin[9]. Normally this is reversible, however occasionally O_2 leaves hemoglobin in the form of superoxide and l ferric hemoglobin named methemoglobin (MetHb) is formed. Normal erythrocytes have some amount of MetHb and superoxide formation. As a result hydroxyl radical is formed by dysmutation catalyzed by H_2O_2 and Fe^{2+}. Therefore there is always some amount of oxidative stress in the erythrocyte[10].

However, there is an excessive increase of oxidative stress in the sickle erythrocyte and plasma medium that the balance between antioxidants and oxidants is altered towards an increased production of oxidized lipids, proteins and lipoproteins (Figure 1).

OXIDATIVE PROCESSES IN THE SICKLE ERYTHROCYTE

A point mutation in beta globin gene results with an unstable HbS protein that has an abnormal electrophoretic mobility and solubility[11,12]. Therefore, MetHb formation and decomposition and heme release have tremendously increased[13]. It was first shown by Hebbel *et al*[9] that autooxidation of HbS was increased compared to normal hemoglobin, HbA. The autooxidation rate of HbS has been reported to be almost two times (1.7-1.9) of the normal with an increased formation of superoxide[9-14].

Excessive amount of lipid peroxidation has been observed in sickle erythrocytes[15] where the membrane damage due to peroxidation was demonstrated by increased permeability to potassium ion[16], altered membrane asymmetry[17], decreased erythrocyte deformability[18], dehydration[19,20] and hemolysis[21].

Iron and copper are particular elements that trigger Hb oxidation[22-24]. There are contradictory findings about the concentration of Fe^{2+} and Cu^{2+} in the sickle erythrocyte. Increased[24-26], similar or decreased amounts were reported in the sickle erythrocyte compared to normal[27,28]. Furthermore, there is an iron

deposit on the membrane of the sickle erythrocyte that is different from normal. Heme bound iron[11] and unbound Fe^{2+} ion[29] were shown on the membrane. This is a factor that further increases the oxidative stress on the membrane. In addition, Hb auto-oxidation and radical formation thereby increased as mentioned above.

There are two more components of the oxidative damage in SCD: Free radical induced oxidative damage during vaso-occlusion induced ischemia-reperfusion injury and decreased antioxidant capacity in the erythrocyte and in the circulation[30]. Increased oxidative stress in the sickle erythrocyte disrupts the reducing power and defense mechanisms of the cell, thus facilitates further damage by other oxidative agents. Free heme in the sickle erythrocyte inhibits some enzymes that protect the cell from oxidation; there is a decreased activity of hexose mono phosphate pathway as well as decreased glutathione[30]. Although this metabolic deterioration has not been understood in the sickle erythrocytes, it should have a strong implication on the disease pathogenesis.

Membrane proteins of sickle erythrocytes were reported to have oxidative alterations[20-31]. Irregularities in the membranous distribution of band 3 and glycophorin, show that the membrane structure of the sickle erythrocyte is disrupted[20]. It has been observed that, accumulation of aggregates of hemichrome at the cytoplasmic region of Band 3 results in the merging of Band 3 molecules which in turn increases sickle cell fusion to endothelium and recognition by macrophages through increased immunoglobin G and complement activation at Band 3 merging sites[32]. Spectrin, which is a membrane skeleton protein, cannot properly bind to the sickle membrane as a result of the anomalies in the membrane proteins of the sickle cell. There is direct evidence that membrane proteins such as ankyrin, spectrin, Band 3 and Band 4 may have oxidative damage[31].

It has been shown that, membrane lipids of sickle cells also suffer oxidative damage[15]. Excessive lipid peroxidation accompanied by loss of membrane fluidity in biological membranes result in decreased membrane potential and increased permeability of H^+ and other ions, followed with cell rupture and loss of cell contents and organelles.

ENDOTHELIAL DYSFUNCTION IN SCD AND OXIDATIVE ALTERATIONS IN THE PLASMA PROTEINS

Chronic intravascular hemolysis of SCD results with excessive production of heme that depletes endothelial nitric oxide[33]. Additionally vaso-occlusive crisis end up with ischemia-reperfusion injury where enzymes like xanthine oxidase, NADPH oxidase, nitric oxide synthase were activated inducing excessive production of free radicals[34,35]. Asymmetric dimethyl arginine, a nitric oxide inhibitor was found to be increased in SCD[36]. All these factors contribute to endothelial dysfunction and further aggravate oxidative stress resulting with a depletion of plasma antioxidants in SCD[37].

Plasma protein oxidation is monitored by measurement of protein carbonyl levels[38]. Increased protein carbonyl levels were reported in various diseases and regarded as a factor that might contribute to the disease pathology[39-41]. Carbonyl-modified plasma proteins were demonstrated to trigger endothelial dysfunction[42] which is regarded to be important in the pathogenesis of SCD. We reported increased protein oxidation by carbonyl modification in SCD patients' plasma where carbonyl levels were correlated to plasma iron and hemolysate zinc levels[43]. Sulfhydrl groups measured in the plasma are mostly from proteins[44]. In fact protein sulfhydryl groups are important antioxidants that can break the oxidation chain. Albumin is the major plasma protein and was been shown to be a strong antioxidant[12]. We found decreased sulfhydryl content in the plasma of SCD patients[43]. All these posttranslational modifications occurred as a result of oxidative stress and needs further investigation to understand their effect on protein function and turnover.

Albumin is the major plasma protein that has antioxidant capacity due to its sulfhydryl groups[45]. Therefore it is a major target for oxidative injury. It was previously reported that free ^{34}cysteine residue of albumin was the target for oxidizing agents[46,47]. A study using proteomics approach reported oxidative posttranslational modification of plasma albumin in the form of malondialdehyde adducts in SCD patients with pulmonary hypertension[48]. Our group showed that electrophoretic mobility of albumin from SCD patients was different than that of albumin from healthy controls[49]. The inflammatory and oxidative medium in SCD possibly targets albumin and induces structural modification. Methemalbumin formation was also reported in SCD patients[50]. This may be an antioxidant defense mechanism where plasma albumin binds oxidized heme and may by this way alleviate toxic effects of free heme on other low abundance proteins.

LIPID PEROXIDATION IN SICKLE ERYTHROCYTES

Malonyldialdehyde is a non-enzymatic oxidative by product of lipid peroxidation. Its main sources are oxidation of polyunsaturated fatty acids and cyclic endoperoxides released during eicosanoid synthesis[51]. Peroxidation of membrane lipids results in loss of membrane architecture that is essential for the deformability of the erythrocyte in passing through capillaries[52]. An erythrocyte with such membrane defects has a shorter life span and becomes a target for the reticuloendothelial system.

We previously reported MDA levels in the plasma and in the erythrocyte of SCD patients were higher than healthy controls[53]. Interestingly these patients had significantly lower blood cholesterol levels and there was a negative correlation between MDA and cholesterol in these patients.

Oxysterols are cholesterol oxidation products having metabolic roles as well[54]. 7-ketocholesterol is an oxysterol that is mostly formed due to increased oxidative stress[55]. There are two studies investigating cholesterol oxidation in the sickle erythrocytes. One study found sickle erythrocyte membranes contained higher 7-ketocholesterol levels than normal erythrocyte membranes[56]. In the other study, increased 7-ketocholesterol in sickle erythrocyte membrane was suggested to alter membrane dynamics and packaging capacity, therefore contributing to membrane pathology in SCD[57]. We found increased 7-ketocholesterol levels in SCD patients who also had hypocholesterolemia[58]. We suggested this cholesterol oxidation product, 7-ketocholesterol may modulate cholesterol biosynthesis at cytoplasmic or nuclear level.

LIPOPROTEIN OXIDATION

Low-density lipoprotein (LDL) oxidation is a complex procedure in which both the proteins and lipids of the LDL are oxidized, resulting in extensive damage to its structure[59,60]. Macrophages, through increased proteoglycan binding, recognize and scavenge this cytotoxic remnant of native LDL forming foam cells[61,62]. The oxidation of LDL particles draws attention primarily because of their effect on atherosclerosis and coronary syndromes[63]. However, LDL leakage across endothelium and its subsequent oxidation by radicals can result in macrophage activation in all vascular structures. Furthermore, it is known that without oxidation, LDL particles do not result in the accumulation of cholesterol esters in blood vessels[64,65]; we can infer that if LDL is being oxidized, the result will be damage in vascular structure.

For example, oxidation of apolipoprotein B-100 component of LDL resulted in conformational change and increased endothelial uptake of LDL[66]. Being reported previously in patients with thalassemia[67], increased oxidation of LDL in patients with SCD patients might result with its increased clearance from plasma. This may be an explanation for decreased LDL as well as cholesterol levels in patients with SCA[68]. Possibly chronic hemolysis and increased erythropoietic activity are more important in the consumption of plasma pool of cholesterol and the development of hypocholesterolemia in patients with SCD[69]. However, the possible link between LDL oxidation and hypocholesterolemia should be investigated in further studies.

High-density lipoprotein (HDL) is known as the apolipoprotein that carries cholesterol back into the liver[70]; although HDL function is not as simple as this sentence suggests, its primary ability to accept cholesterol from LDL and macrophage foam cells is why HDL is considered protective against atherosclerosis[71,72]. Oxidized HDL on the other hand, loses its ability to remove cholesterol[73]. Contrary studies exist, it has been shown that specific forms of oxidized HDL (tyrosylated HDL) may in fact increase cholesterol uptake and decrease atherosclerotic plaque formation[74]. However, the specific nature of these oxidations and the lack of data about the *in vivo* formation of oxidized HDL raise questions on the reliability of this data for in vivo consideration.

Another important role of HDL is its anti-inflammatory function[75]. Oxidized HDL loses this function almost entirely and may even act pro-inflammatory during the acute phase response[75,76]. Furthermore, HDL levels are also decreased by ongoing inflammation[77,78]. This data suggests that the ongoing inflammatory state, increased acute phase reactants, and the constant oxidative stress that SCD patients undergo can result in a vicious cycle that is a major contributor to HDL dysfunction in SCD[79].

HDLs have additional functions; lipopolysaccharide binding, endothelial cell movement and function modulation, platelet-activating factor inhibition, anticoagulant activity inhibition, anti-oxidant enzyme effects, prostacyclin binding, stimulation of NO release; these are either direct effects through their plasma lipid transport role or effects through enzymes that travel alongside the apolipoprotein[78,80,81]. Paroxonase is one of these enzymes and was shown to have a decreased activity in SCD and researchers suggested that pediatric patients with SCD who had chronic oxidative stress might have a higher incidence of vaso-occlusive crisis[82]. However, SCD patients who had hydroxyurea had normal paroxonase activity. HDL has important antioxidant capacity and HDL mimetic peptides keep a potential to be a therapeutic agent in vascular inflammation[83]. 4F, an HDL mimetic, was shown to be beneficial against endothelial dysfunction in a mouse model of SCD[84].

CONCLUSION

SCD is regarded as a high oxidative stress situation, because of HbS. It is not unexpected that iron of heme can trigger many oxidative events that may damage erythrocyte and plasma macromolecules. Besides iron, vaso-occlusion induced ischemia-reperfusion injury and chronic inflammation also trigger oxidative damage at the cellular and at the circulation. There are many oxidative markers being studied in SCD. The clinical correlations of molecular alteration of proteins and lipids are important and they may modify disease presentation. New options of therapy in SCD will possibly involve antioxidants-either being synthetic or being biomimetic as adjuvant.

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P- Reviewer: Acuna-Castroviejo D, Al-Haggar M, Li Z
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Li D

Article 5: Hypocholesterolemia and increased plasma 7-ketocholesterol levels in pediatric sickle cell patients.

Acta Medica 2017; 48(1) : 1-4

acta medica

ORIGINAL ARTICLE

Hypocholesterolemia and Increased Plasma 7-ketocholesterol Levels in Pediatric Sickle Cell Patients

Ahmet Yalçınkaya[1], [MD]
Incilay Lay[1], [MD]
Afshin Samadi[1], [MD]
Selma Ünal[2], [MD]
Filiz Akbıyık[1], [MD]
Yesim Öztaş[1]*

1 Hacettepe University Faculty of Medicine, Department of Medical Biochemistry, Ankara, Turkey
2 Mersin Universtiy, Department of Pediatric Hematology, Mersin, Turkey
* Corresponding Author: Yesim Oztas, Hacettepe University Faculty of Medicine, Department of Medical Biochemistry, Ankara, Turkey
e-mail: yoztas@hacettepe.edu.tr

* This work was accepted as a speed talk to be presented in Young Scientist Forum, FEBS 2016 Congress and published in the abstract book as a supplement issue of FEBS Journal.

Received: 30 December 2016, Accepted: 10 March 2016, Published online: 29 March 2017

ABSTRACT

Introduction: Hypocholesterolemia is the most documented lipid abnormality in sickle cell disease which is also characterized by increased oxidative stress. We investigated plasma levels of oxysterols, oxidized cholesterol derivatives, in the plasma of sickle cell disease patients and compared with controls.

Material and Methods: Twenty steady state sickle cell disease patients and 8 healthy controls were enrolled in the study. 7-ketocholesterol and cholestane-3β,5α,6β-triol levels, were measured by an LC-MS/MS method. Total cholesterol levels were determined by enzymatic colorimetric method.

Results: Mean plasma total cholesterol levels were significantly lower (109.0±17.4 mg/dl versus 149.6±28.8 mg/dl) (p=0.002) and 7-ketocholesterol levels were higher (10.6±1.9 mg/dl versus 9.0 ±1.2 mg/dl) (p=0.033) in the sickle cell disease group versus control group. When patients were grouped according to their genotype, Sβ patients (N=10) had higher cholestane-3β,5α,6β-triol levels (p=0.033).

Discussion: To our knowledge this is the first study investigating plasma oxysterols, particularly 7-ketocholesterol and cholestane-3β,5α,6β-triol in sickle cell disease . We found a significant increase in mean 7-ketocholesterol levels in sickle cell disease patients compared to controls. Patients also had hypocholesterolemia that is typical in many sickle cell disease patients. Apart from being an oxidative stress marker, 7-ketocholesterol may be a modifier of plasma cholesterol concentration. The metabolic roles of cholesterol oxidation products in diseases warrant further research to explain the tremendous nuclear effects of these oxysterols.

Key words: Oxysterol, 7-ketocholesterol, cholesterol, hypocholesterolemia, oxidative stress

INTRODUCTION

Sickle cell disease (SCD) is a hemoglobinopathy caused by a point mutation in the beta globin gene. It is a chronic condition characterized by anemia, vaso-occlusion, inflammation and increased oxidative stress [1-3]. Patients with sickle cell disease have increased morbidity and mortality due to involvement of organs such as liver, spleen, kidney, lungs and brain [4]. Mutated hemoglobin (Hb) is unstable and decomposes easily to heme and globulin (9). This free heme further releases its iron that both heme and iron attacks membrane lipids producing oxidized lipids in various forms (10). Ischemia reperfusion injury developed as a result of vasoocclusive crisis also aggravates free radical production. There is an increasing number of reports on the lipid abnormalities in serum and erythrocytes of patients with SCD such as hypocholesterolemia [5-6], relative hypertriglyceridemia decreased HDL [6], decreased apolipoprotein AI and apolipoprotein B [7] compared to controls. Anemia, inflammation and oxidative stress are three pathological processes that may result with lipid abnormalities in sickle cell disease [8]. Various abnormalities concerning lipid metabolism were reported in patients with SCD in which hypocholesterolemia is the most documented lipid abnormality [5-6]. The chronic anemia results with overstimulation of erythropoiesis that consumes plasma cholesterol pool for new membrane synthesis [9-10]. There is a previous report which suggested a possible link between oxidative stress and

hypocholesterolemia in SCD patients whose plasma cholesterol levels were negatively correlated to hemolysate MDA levels [11]. Cholesterol is converted to more polar compounds by the addition of oxygen containing groups such as epoxy, hydroxy or ketone and these oxidative decomposition products of cholesterol are named oxysterols [12]. Various oxysterols are produced in body at lower quantities during cholesterol metabolism to perform recently understood metabolic roles. However, oxysterol concentrations increase in pathologic conditions. 7-ketocholesterol is the major, oxysterol assumed to be produced in vivo mainly by free-radical attack on biomembranes and on low density lipoprotein particle (LDL) [13]. Cholestane-3β,5α,6β-triol is another oxysterol produced during increased oxidative stress. Cholesterol oxidation products were previously investigated in the erythrocytes from SCD patients by two consecutive studies. In the first study, various oxysterols were inserted into normal and sickle RBC membranes to observe the effect on the membrane fluidity and it is concluded that oxidized cholesterol derivatives perturbate membrane dynamics and that this might contribute to membrane pathology in SCD [14]. In the next study 7-ketocholesterol levels were measured in the membrane of sickle erythrocytes and it was found that membranes from sickle erythrocytes contained higher 7-ketocholesterol levels than normal erythrocytes [15]. 7-position derivatives of cholesterol were also shown to induce cytotoxic effects on macrophages in vitro [16]. To our knowledge there is no report in the literature investigating oxysterol levels in the plasma of SCD patients.

MATERIALS and METHODS

Patients

We enrolled SCD patients who were under routine follow-up, free of vaso-occlusive crisis and transfusion for the last three months in the study. Two patients recovering from crisis were also analyzed for the below parameters. The diagnosis of SCD was established according to hemoglobin (Hb) electrophoresis and β globin gene mutation analysis. The control group was consisted of healthy children were of similar age without any acute or chronic disease with normal Hb electrophoresis. The study was approved by the Ethics Committee of Mersin University (2014-115).

Sampling

Blood was drawn into EDTA containing tubes kept on ice at least for 5 min, centrifuged at 2500 rpm for 10 minutes (Hettich, Germany). Plasma samples were separated, aliquoted and frozen immediately at –80°C.

Cholesterol and oxysterol measurements

We measured plasma total cholesterol levels with the enzymatic colorimetric method (Beckman-Coulter, Pasadena, CA, USA) and 7-ketocholesterol and cholestane-3β,5α,6β-triol levels by the LC-MS/MS (Schimadzu, Japan) with method from Jiang et al. [17].Statistics: The results were expressed in terms of arithmetic means ±standard deviation (SD). Nonparametric statistics were used. Difference between the two groups was determined by Mann–Whitney U-test. Correlation between the parameters was calculated by Spearman correlation coefficient, $p < 0.05$ was considered statistically significant. SPSS (SPSS 19.0 for Windows, Chicago, IL, USA) was used for statistical analysis.

RESULTS

Demographic characteristics and laboratory values of the patients are given on Table 1. Mean plasma total cholesterol levels were significantly lower and 7-ketocholesterol levels were significantly higher in the steady state SCD group when compared to the healthy control group as shown in Table 1. There was no significant difference between groups

Table 1. Age, sex, white blood cell (WBC) count, cholesterol and oxysterol levels in SCD patients and healthy children.

	Sickle cell disease N=20	Healthy N=8
Age	14.4 ±3.7	14.2 ±3.6
Sex (M/F)	13/7	6/2
WBC (x103cells/µl)	10.970 ±5.61 *	8.00 ±0.89
Total cholesterol (mg/dl)	109.0 ±17.4**	149.6±28.8
7-ketocholesterol (ng/ml)	10.6±1.9***	9.0 ±1.2
cholestane-3β,5α,6β-triol (ng/ml)	6.5 ±2.4	5.3±2.6

*p=0.001
**p=0.002
***p=0.033

Table 2. Total cholesterol and oxysterol levels in SCD patients grouped according to their genotype.

	SS N=10	Sβ N=10
Total cholesterol (mg/dl)	103 ±18.4	115.1 ±14.7
7-ketocholesterol (ng/ml)	10.2 ±1.5	10.9 ±2.2
cholestane-3β,5α,6β-triol (ng/ml)	5.0 ±2.4	8.0±1.2*

*p=0.02

for cholestane-3β, 5α, 6β-triol. However, when the patients were grouped according to genotype, either being SS or Sβ, cholestane-3β, 5α, 6β-triol levels were found to be significantly higher in Sβ group (Table 2).

DISCUSSION

To our knowledge this is the first study investigating plasma oxysterols, particularly 7-ketocholesterol and cholestane-3β, 5α, 6β-triol in the plasma of patients with SCD. We found a significant increase in mean 7-ketocholesterol levels in SCD patients compared to controls. Patients also had hypocholesterolemia that is typical in many SCD patients. Cholestane-3β, 5α, 6β-triol levels were higher in the patients with Sβ genotype than SS genotype..Oxysterols mediate various roles in cholesterol metabolism in addition to being cholesterol metabolites themselves. 7-Ketocholesterol is suggested to have a role in the inhibition of cellular cholesterol synthesis by binding to oxysterol-related-binding-protein related protein-2, depletion of which reduces cholesterol biosynthesis [18]. Such an inhibition may contribute to other factors causing low blood cholesterol such as anemia in SCD patients.7-ketocholesterol was shown to reduce nitric oxide synthesis and release by vascular endothelial cells [19]. Increased 7-ketocholesterol levels in the plasma of SCD patients should be investigated further for any role in the vascular pathology such as chronic inflammation and vaso-occlusions observed in these patients. Oxysterols also effect sterol synthesis by regulating sterol element-binding protein (SREBP) function [20] and this may be an important contribution to hypocholesterolemia in SCD patients. SREBPs are transcription factors which are bound to the nuclear membrane and endoplasmic reticulum membrane when inactive. When activated they are cleaved in the Golgi apparatus and subsequently move into the nucleus to bind specific DNA sequences (Sterol Response Elements, SREs), initiating sterol synthesis by upregulating the synthesis of required enzymes [21]. SREBP activation relies on the level of cholesterol in the cell, which is when the SREBP cleavage-activating protein (SCAP) comes to effect. SCAP is a sensor for cholesterol, when cholesterol is low, SCAP escorts SREBP to the Golgi apparatus. In the Golgi, SREBP is cleaved and the amino terminal domain (referred to as nSREBP) moves to the nucleus and performs its aforementioned function [20]. Cholesterol is a natural inhibitor of this path as it is the end product. However, 7α-Hydroxycholesterol, 7β-Hydroxycholesterol, and 7-Ketocholesterol have been shown to inhibit SCAP escort of SREBP with high potency [22], by inducing SCAP binding with insig-1 and insig-2 which are ER membrane anchor proteins; thus blocking the SCAP-SREBP complex's movement to the Golgi [23-24]. In light of these studies, the elevated 7-ketocholesterol levels that we found in the SCD group may play an important role in the hypocholesterolemia of SCD. It is worth noting that while the mechanism of SCAP inhibition by cholesterol is known; it is as of yet unclear how oxysterols induce the SCAP/insig binding [25]. Besides relevance of all the above literature in explaining a possible association between 7-ketocholesterol and SCD pathology such as hypocholesterolemia, the source of plasma 7-ketocholesterol is also an important concern. Increased plasma 7-ketocholesterol in SCD may presumably be a result of increased LDL oxidation in SCD as reported previously [26]. Another study reported a positive correlation between plasma and erythrocyte 7-ketocholesterol levels in diabetic patients [27]. Therefore, 7-ketocholesterols from erythrocyte membrane may also contribute to measured plasma concentration of 7-ketocholesterol. We also investigated cholestane-3β, 5α, 6β-triol levels which were suggested to cause endothelial damage in animal studies [28]. However, we did not find a significant difference for cholestane-3β, 5α, 6β-triol levels between patients and controls. SB patients who had a better disease course than SS patients had a higher concentration of cholestane-3β, 5α, 6β-triol. There is a positive correlation between cholestane-3β, 5α, 6β-triol and total cholesterol levels in the patient group (r=0.0434, p=0.039). This may explain the increased cholestane-3β, 5α, 6β-triol levels

in Sβ patients. This is a preliminary report with a limited number of patients and controls as part of a larger project investigating lipid metabolism in SCD. Patients also had hypocholesterolemia that is typical in many SCD patients. Apart from being an oxidative stress marker, 7-ketocholesterol may be a modifier of plasma cholesterol concentration. The metabolic roles of cholesterol oxidation products in diseases warrant further research to explain the tremendous nuclear effects of these oxysterols.

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