AMERICAN UNIVERSITY OF BEIRUT

OXIDATIVE STRESS IN NON-TRANSFUSION DEPENDENT THALASSEMIA: A JOURNEY FROM NADPH OXIDASES TO CYTOCHROME P450

by

RAYAN IMAD BOU-FAKHREDIN

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Anatomy, Cell Biology, and Physiological Sciences
of the Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
May 2020

AMERICAN UNIVERSITY OF BEIRUT

OXIDATIVE STRESS IN NON-TRANSFUSION DEPENDENT THALASSEMIA: A JOURNEY FROM NADPH OXIDASES TO CYTOCHROME P450

by

RAYAN IMAD BOU-FAKHREDIN

Approved by:

Assaad A. Eid, DSs, HDR, Associate Professor — Advisor
Department of Anatomy, Cell Biology, and Physiological Sciences, American University of Beirut, Beirut, Lebanon

Ali T. Taher, MD, PhD, FRCP, Professor — Co-Advisor
Division of Hematology and Oncology, Department of Internal Medicine, American University of Beirut Medical Center, Beirut, Lebanon

Abdo Jurjus, PhD, Professor — Member of Committee
Department of Anatomy, Cell Biology, and Physiological Sciences, American University of Beirut, Beirut, Lebanon

Maria Domenica Cappellini, MD, Professor — Member of Committee
Department of Clinical Sciences and Community, University of Milan, IRCCS Ca' Granda Foundation Maggiore Policlinico Hospital, Milan, Italy

Stefano Rivella, PhD, Professor — Member of Committee
Children's Hospital of Philadelphia (CHoP), Cell and Molecular Biology Graduate Group (CAMB), University of Pennsylvania, Philadelphia, PA, USA

Date of thesis defense: May 11, 2020

AMERICAN UNIVERSITY OF BEIRUT

THESIS, DISSERTATION, PROJECT RELEASE FORM

Student Name: BOU-FAKHREDIN RAYAN IMAD
Last First Middle

● Master's Thesis ○ Master's Project ○ Doctoral Dissertation

☐ I authorize the American University of Beirut to: (a) reproduce hard or electronic copies of my thesis, dissertation, or project; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes.

☐ I authorize the American University of Beirut, to: (a) reproduce hard or electronic copies of it; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes after:

One ---- year from the date of submission of my thesis, dissertation, or project.
Two ---- years from the date of submission of my thesis, dissertation, or project.
Three -X- years from the date of submission of my thesis, dissertation, or project.

Signature Date

ACKNOWLEDGMENTS

I would first like to thank Dr. Assaad A. Eid for his continuous support over the last years and for always being there by my side. As my mentor and advisor, Dr. Eid always provided guidance and opportunities for me to develop my individual abilities. He never imposed his opinions on me, but rather helped me to independently recognize, evaluate, and pursue potential opportunities. I am sure that Dr. Eid will serve as an inspiration to many, just like he inspired me to become a future scientist and educator. It is my hope that I impact future individuals and students in my professional life as profoundly as he affected me.

I would also like to transmit my gratitude to all members of Dr. Eid's lab. It has been a pleasure getting to know them all. I would like to acknowledge one particular individual in the lab, Mrs. Batoul Dia. Batoul was in charge of teaching me everything I needed to know as soon as I joined the lab. She was my reference point at all times. She quickly became one of my closest friends and my support system. Over the last two years, I have witnessed her continuous hard work and dedication. To say the least, she has a wondrous soul and a beautiful heart that is able to give so much love and positive energy to those around her.

I remain indebted to the continuous support and mentorship of Prof. Ali Taher. As an authority and world renowned leader in the field of thalassemia, with a legacy of over 25 years, Prof. Taher has dedicated his life to advance knowledge of this disease, with continuous efforts to improve the health and overall quality of life of thalassemia patients. He is my role model in life and a true source of inspiration. I thank him for giving me the opportunity to create myself and to embody the qualities of a future leader. He not only made me a better scientist, but also a better person. I will be forever grateful for his presence in my life.

As Prof. Taher's student and follower, I hereby pledge to make the same commitment as a researcher to continue investigating and better understanding different aspects of thalassemia and translate these findings into clinical practice, with the hopes of addressing the healthcare needs of all thalassemia patients, enhancing their health and well-being as a result.

Special thanks go to the committee members, Prof. Maria Domenica Cappellini, Prof. Stefano Rivella, and Prof. Abdo Jurjus.

Finally, I would like to thank my family and all relatives and friends for the unconditional love and support throughout my journey.

AN ABSTRACT OF THE THESIS OF

Rayan Imad Bou-Fakhredin for Master of Science
Major: Physiology

Title: Oxidative Stress in Non-Transfusion Dependent Thalassemia: A Journey from NADPH Oxidases to Cytochrome P450

Background: Oxidative damage by reactive oxygen species (ROS) is considered to be one of the main contributors to cell injury and tissue damage in thalassemia patients. Recent studies suggest that ROS generation in non-transfusion-dependent (NTDT) patients occurs as a result of iron overload. This increased ROS production in organs has been associated with multiple pathological outcomes. It has been proposed that sources of ROS production during pathophysiology are disease-specific. Among the different sources of ROS, the NADPH oxidases and CYPs 450 have been proposed to be the driving force in certain diseases. ROS sources in patients with NTDT remain poorly understood.

Aim: We aim to identify the exact source of ROS in NTDT.

Methods: $Hbb^{th3/+}$ mice were used as a model of NTDT. Eight mice were divided into two groups (a control group and a thalassemia group receiving no treatment). A peripheral blood smear was used to confirm the diagnosis of thalassemia. Liver tissue iron content was measured using high-performance liquid chromatography (HPLC). Assessment of superoxide production was done using HPLC for H_2O_2 production. Enzymatic activity of NADPH oxidases was assessed using the NADPH oxidase assay. Detection and quantification of NADPH oxidase and CYP450 mRNA levels and protein levels were performed by Real-time Polymerase Chain Reaction and western blotting, respectively. Epoxyeicosatrienoic Acids (EET) and 20-Hydroxyeicosatetraenoic Acid (20-HETE) activity were assessed by HPLC. Degree of expression of CYP450 was also evaluated by immunohistochemistry. Statistical analyses were done via an unpaired t-test. *p*-values < 0.05 were considered as statistically significant. All experiments were performed on liver tissues of $Hbb^{th3/+}$ mice.

Results: Increased tissue iron levels were detected in the liver of $Hbb^{th3/+}$ mice compared to control. There was an increased state of oxidative stress and elevated NADPH oxidase enzymatic activity in $Hbb^{th3/+}$ mice. At the mRNA level, no significant changes were observed in the expression of NOX1, NOX2 and NOX4 in $Hbb^{th3/+}$ mice compared to control. However, there was a decreased expression of in the protein levels of NOX1, NOX2 and NOX4 in $Hbb^{th3/+}$ mice compared to control. Additionally, no significant changes were observed in the expression of CYP1A, CYP2B, CYP4A and CYP4F mRNA levels in $Hbb^{th3/+}$ mice. A significant over-expression of CYP1A, CYP2B, CYP4A and

CYP4F protein levels were observed in $Hbb^{th3/+}$ mice compared to control. There was an increase in EET activity, which correlated with the increased expression of its respective CYPs (CYP1A and CYP2B). There was also an increase in 20-HETE activity, which correlated with the increased expression of its respective CYPs (CYP4A and CYP4F). Immunohistochemistry staining of liver tissue sections also showed over expression of CYP1A and CYP4A in $Hbb^{th3/+}$ mice.

Conclusions: This is the first report indicating that CYP450 is the NADPH oxidase dependent ROS-producer responsible for superoxide and H_2O_2 production in the liver of thalassemic ($Hbb^{th3/+}$) mice. Targeting CYP450 (by activating EET production and inhibiting 20-HETE production) merits evaluation as a novel therapeutic approach in thalassemia.

CONTENTS

ACKNOWLEDMENTS……………………………………………………………...v

ABSTRACT……………………………………………………………………….vi

LIST OF ILLUSTRATIONS……………………………………………………x

LIST OF TABLES……………………………………………………………….xi

LIST OF ABBREVIATIONS…………………………………………………….xii

Chapter

I. INTRODUCTION……………………………………………………………....1

A. The Thalassemias: Epidemiology, Molecular Forms and Clinical Classification………………………………………………………………...1

B. Pathophysiology and Associated Morbidity in Non-Transfusion Dependent Thalassemia……………………………………………………………………….3

C. Mechanism of Iron Overload in Non-Transfusion Dependent Thalassemia…...5

D. Evolutionary Perspective of a Redox Balance and Sources of Reactive Oxygen Species………………………………………………………………………….6

E. The NADPH Oxidase Family…………………………………………………..9

F. Cytochrome P450 and Arachidonic Acid Metabolism………………………..11

G. Physiological Roles of EETs and 20-HETE…………………………………..13

H. Role of Reactive Oxygen Species in β-Thalassemia………………………….14

I. Aim of the study and Hypothesis……………………………………………...16

II. MATERIALS AND METHODS……………………………………….17

A. Animal Model………………………………………………………………17

B. High Performance Liquid Chromatography (HPLC)……………………….18

C. NADPH Oxidase Activity Assay…………………………………………...19

D. Western Blot Analysis………………………………………………………20

E. Real-time Polymerase Chain Reaction (PCR)……………………………...21

F. Immunohistochemistry……………………………………………………...23

G. Statistical Analysis…………………………………………………...…..24

III. RESULTS…………………………………………………………………….25

A. Increased tissue iron levels in the liver of Hbb$^{th3/+}$ mice……………………….25

B. Reactive Oxygen Species production in Hbb$^{th3/+}$ mice is induced through the NADPH oxidases……………………………………………………..…26

C. Decreased expression levels of the NOX isoforms in Hbb$^{th3/+}$ mice……….....27

D. Over expression of CYP1A, CYP2B, CYP4A, and CYP4F in Hbb$^{th3/+}$ mice....29

E. Increase in EET and 20-HETE activity in Hbb$^{th3/+}$ mice………..…………….32

IV. DISCUSSION………………………………………………………………33

V. REFERENCES……………………………………………………………..38

ILLUSTRATIONS

Figures Page

1. Mechanism of Iron Overload in NTDT …………………………………….. 6

2. Sources of Reactive Oxygen Species in Cells …………………………..... 9

3. Pathways of Arachidonic Acid Metabolism ……………………………... 13

4. Increased tissue iron levels in the liver of Hbb$^{th3/+}$ mice ……………………. 25

5. Enhancement of superoxide production and NADPH oxidase activity in liver tissues isolated from Hbb$^{th3/+}$ mice …………………………………………….. 26

6. Decreased expression levels of the NOX isoforms in Hbb$^{th3/+}$ mice……........ 28

7. Over expression of CYP1A, CYP2B, CYP4A, and CYP4F in Hbb$^{th3/+}$ mice…31

8. Increase in EET and 20-HETE activity in NTDT mice ……………………... 32

TABLES

Table Page

1. Oligonucleotide primer sequences employed for RT-PCR………………….. 22

ABBREVIATIONS

TDT:	Transfusion-Dependent Thalassemia
NTDT	Non-Transfusion Dependent Thalassemia
HCC:	Hepatocellular carcinoma
LIC:	Liver Iron Concentration
GDF-15:	Growth Differentiation Factor-15
TWSG1:	Twisted-gastrulation 1
TGF-β:	Transforming Growth Factor-β
ROS:	Reactive oxygen species
NOS:	Nitric Oxide Synthase
CYP450:	Cytochrome P450
COX:	Cyclooxygenases
LIPOX:	Lipooxygenases
NADPH:	Nicotinamide Adenine Dinucleotide Phosphate
FAD:	Favin Adenine Dinucleotide
EETs:	Epoxyeicosatrienoic acids
DHETs:	Dihydroxyeicosatrienoic acids
20-HETE:	20- hydroxyeicosatetraenoic acid
sEH:	Soluble Epoxide Hydrolase
EGF:	Epidermal Growth Factor
VEGF:	Vascular Endothelial Growth Factor

NFkB:	Nuclear Factor kappa B subunit
NAFLD:	Non-alcoholic Fatty Liver Disease
PPARα:	Peroxisome Proliferator-activated Receptor α
NTBI:	Non-transferrin Bound Iron
LPI:	Labile Plasma Iron
RBCs:	Red Blood Cells
PS:	Phosphatidylserine
HPLC:	High Performance Liquid Chromatography
DHE:	Dihydroethidium
EOH:	2-hydroxyethidium
RIPA:	Radioimmunoprecipitation assay
PMSF:	phenylmethylsulfonyl fluoride
TPP:	Triphenylphosphine
DMF:	N,N-dimethyl-formamide
TMB:	3,3',5,5'-Tetramethylbenzidine
PBS:	Phosphate-Buffered Saline
BSA:	Bovine Serum Albumin

CHAPTER I

INTRODUCTION

A. The Thalassemias: Epidemiology, Molecular Forms and Clinical Classification

The thalassemias are among the most common inherited monogenic diseases worldwide, and are characterized by defects in the production of adult hemoglobin [1]. The epidemiology of various forms of the thalassemias remains poorly recognized. However, the disease is vastly prevalent in regions that extend from sub-Saharan Africa, through the Mediterranean region and Middle East, to the Indian subcontinent and East and Southeast Asia [2-5]. Continued and recent migrations also meant that the thalassemias can now be found in Europe and North America, making this disease a global health concern [6-8].

Hemoglobin synthesis is controlled by two multigene clusters. These are known to be located on chromosome 16 (encoding the α-like globins) and chromosome 11 (encoding the β-like globins) [9]. The genes in such clusters are arranged along the chromosome in the order in which they are expressed during development to produce different hemoglobin tetramers during embryonic, fetal, and adult life. During fetal life, the predominant form of hemoglobin produced is HbF ($\alpha_2\gamma_2$), which is then switched to adult-type hemoglobin HbA ($\alpha_2\beta_2$) after birth [10, 11]. Defects in the α-globin or β-globin gene clusters at the molecular level form the basis of defective hemoglobin synthesis. This in turn leads to different inherited forms of either α-thalassemias or β-thalassemias. The type and severity of these clinical forms can also rely on additional and independent intrinsic and extrinsic factors [10].

For the purpose of this study, we will focus on β-thalassemia. Patients with β-thalassemia have been previously categorized as minor, major or intermedia based on their α-/β-globin chain imbalance, severity of anemia, and clinical features and presentation. β-thalassemia minor (trait or carrier) represents the heterozygous inheritance of a β-thalassemia mutation. These patients are often clinically asymptomatic. Patients with β-thalassemia major often present to the clinic with severe anemia in infancy and become transfusion dependent for the rest of their life, whereas patients with β-thalassemia intermedia can present later in life with mild-to-moderate anemia and may occasionally require blood transfusion [12-14].

A major transition in the classification of the thalassemias has occurred over the last decade. While the standard and old classification was based on molecular forms, clinicians moved towards a categorization that is based on clinical-management criteria. Because transfusion therapy is the conventional modality of treatment in patients with thalassemia, the frequency and extent of transfusion requirements indirectly reflects the underlying severity of the disease. The use of blood transfusions in these patients can control most of the underlying pathophysiological mechanisms and it can also contribute to secondary morbidity [12, 15]. Therefore, thalassemia patients today are commonly categorized as having transfusion-dependent thalassemia (TDT) or non-transfusion dependent thalassemia (NTDT). Patients with TDT commonly present with severe anemia in their early childhood that requires lifelong blood transfusions for survival. NTDT patients, on the other hand, usually present with mild/moderate anemia during their late childhood or even in adulthood that only necessitates occasional transfusions in certain

clinical settings and for the prevention or management of certain disease manifestations [16].

B. Pathophysiology and Associated Morbidity in Non-Transfusion Dependent Thalassemia

The hallmarks of this disease are the α- to β-globin chain imbalance leading to ineffective erythropoiesis, chronic hemolytic anemia, and iron overload. These in turn lead to multiple morbidities in different organs. Despite being transfusion-independent, NTDT patients often experience many clinical complications. In 2006, one of the first studies examining the wide scope of clinical morbidities in NTDT patients was conducted in Lebanon and Italy. This study clearly identified that NTDT patients could experience greater morbidities than previously recognized and that these morbidities are dissimilar from those observed in TDT patients [17]. The OPTIMAL CARE study was able to confirm the high prevalence of morbidities in NTDT patients, which increases with age [17].

Clinical complications in NTDT patients arise from a combination of multiple pathophysiological factors [18, 19]. Ineffective erythropoiesis contributes to anemia and tissue hypoxia and may lead to the development of extramedullary hematopoietic pseudotumors and leg ulcers [14, 20-22]. Ineffective erythropoiesis and hemolysis also lead to a hypercoagulable state, and ultimately to a high incidence of thrombosis and silent cerebral infarcts [23-26]. Patients with NTDT are also at risk of pulmonary hypertension. This increases with high liver iron concentration (LIC), advancing age, and splenectomy [27, 28]. Ineffective erythropoiesis was also found to be associated with low hepcidin

levels, which subsequently lead to iron overload. Iron overload in NTDT patients is a cumulative process that can lead to significant morbidity (liver, endocrine, vasculature) and mortality [29-32]. Unlike in TDT, there exists no indications of cardiac iron overload in NTDT patients [33-36].

Several case reports have suggested that there exists a relationship between iron overload and hepatocellular carcinoma (HCC) in hepatitis C-negative NTDT patients, suggesting that HCC is a newly emerging adverse complication as a consequence of the prolonged survival of thalassemia patients [37-42]. In fact, progress in the management of thalassemia have increased the life expectancy of thalassemia patients and improved their quality of life. This however has led to the emergence of numerous new morbidities, including different types of solid and hematologic malignancies [43]. Studies looking at the association between thalassemia and cancer and hematologic malignancies continue to be in its early stages. While it is possible that these malignancies are becoming more apparent due to the significant increase in lifespan of this population, multiple predisposing risk factors do exist and may play a role as well. While the definite mechanism of how cancer develops in thalassemia patients is unclear, many possible hypotheses have been made. On one hand, iron overload is a risk factor for cancer development in patients with NTDT. On the other hand, transfusion-transmitted oncogenic viruses may lead to the development of hematologic malignancies.

Renal dysfunction in NTDT has also been described and is caused by hypoxia, anemia, and severe iron overload [44-46]. It is important to note that renal dysfunction in NTDT patients can eventually lead to end-stage renal disease [47]. Longitudinal studies

investigating the incidence, mechanisms, and consequences of renal abnormalities are warranted. Moreover, because of the increased life expectancy of NTDT patients, renal function much be closely monitored and followed-up in order to identify those who are at risk of severe renal disease.

All the above-mentioned observations indicate that the diagnosis of NTDT can be associated with greater morbidities than previously recognized, suggesting an important role for early diagnosis and prompt intervention.

C. Mechanism of Iron Overload in Non-Transfusion Dependent Thalassemia

In patients with NTDT, ineffective erythropoiesis leads to increased iron absorption and primary iron overload, which is mediated by the hepatic hormone hepcidin. It has been hypothesised that an erythroid factor communicates to the liver the need of iron for the incoming red cells. This factor would be produced by erythroid cells, especially under condition of ineffective erythropoiesis, and its function would be to suppress hepcidin synthesis in the liver leading to increased intestinal iron absorption and increased release of recycled iron from the reticuloendothelial system. Several erythroid factors have been proposed to play a role in iron overload development in NTDT. These include growth differentiation factor 15 (GDF15), twisted-gastrulation 1 (TWSG1) and erythroferrone. Erythroferrone, a 340-amino acid soluble protein, directly acts on the liver and inhibits hepcidin production [48]. This erythroid factor was the only one that was shown to be

increased in animals with β-thalassemia [49, 50]. **Figure 1** summarizes the mechanism of iron overload development in NTDT.

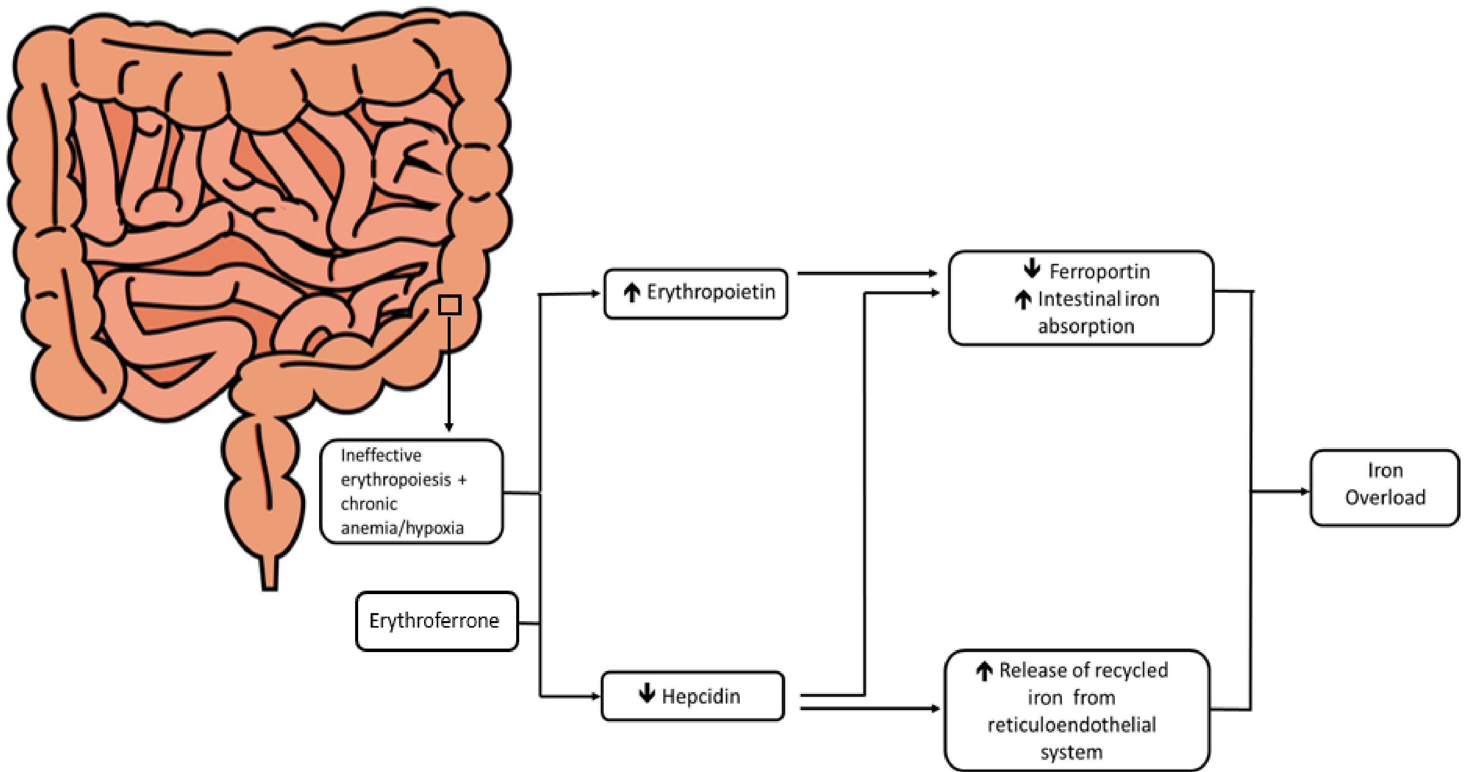


Figure 1. Mechanism of iron overload in NTDT.

D. Evolutionary Perspective of a Redox Balance and Sources of Reactive Oxygen Species

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen that are formed as a byproduct of cellular metabolic reactions. They comprise hydrogen peroxide (H_2O_2), superoxide free radicals ($O_2^{\cdot-}$) as well as nitrogen based free radical species such as peroxynitrite, nitric oxide and singlet oxygen. ROS are significant cellular entities by cause of their contribution to cellular proliferation, signal transduction,

host defense, homeostatic preservation and gene expression [51]. ROS are under homeostatic and regulatory control by antioxidant defense mechanisms [52].

ROS are heritable biological adaptations that have evolved concurrently with natural and environmental modifications. Such modifications are crucial for our understanding since their mechanisms of action provide insights into the evolutionarily preferred mechanistic physiologies of the cells. One strategy of major significance in physiological functions is that cellular events revolve around fixed and coordinated set points. This phenomenon is physiologically referred to as homeostasis, and ROS are tightly counterbalanced by various cellular antioxidants to promote a tight "redox homeostasis".

Aerobic physiology is largely dependent on oxidative species which are oxygen-containing, short lived and highly diverse molecules produced by numerous generators and enzymes in different cell compartments. ROS are recognized as specific mediators and second messengers of cell signaling related to vascular tone, immune responses, cell protection and hormonal actions [53-55]. In sync with this homeostatic balance, these functions are primarily maintained and counter-balanced by anti-oxidative mechanisms which regulate the bioavailability of oxidative species. However, under pathogenic conditions, oxidative species appear in conditions where they are produced in aberrant and cytotoxic concentrations resulting in oxidative stress.

Several reports have indicated escalated ROS levels to be directly correlated with irreversible oxidation of cellular components (genetic material, proteins and lipids) that

eventually contribute to cellular dysfunction and necrosis [56]. This overproduction of ROS has been described to play a role in the pathophysiology and pathogenesis of many different diseases. Several studies have validated the implications of redox alternations in distinct pathologies. However, attempts to reduce bioactive ROS to minimal levels have been shown to be fatal and detrimental. This highlights the significance of the redox system in cellular physiology and the homeostatic balance between oxidant and antioxidant species [57]. Extensive research is currently aiming at identifying the cellular sources of ROS production and those specifically altered in a cell and disease specific manner.

All types of cells can produce ROS. This is generated from non-enzymatic processes such as electron transport chain in the mitochondria and other enzymatic reactions including those catalyzed by NADPH oxidases, and cytochrome P450 (CYP450) **(Figure 2)**. All these sources are shown to vary in their physiological role and importance in organs and related disease [58-60]. Additional sources of ROS include the mitochondria, xanthine oxidase, uncoupled nitric oxide synthase (NOS), the cyclooxygenases (COX) and lipooxygenases (LIPOX) [61] **(Figure 2)**. ROS production overwhelms the cellular defense mechanisms. Injury thus results in the form of altered metabolism, protein and lipid oxidation, activation of extracellular and intracellular transport and signaling pathways, and ultimately apoptosis [62].

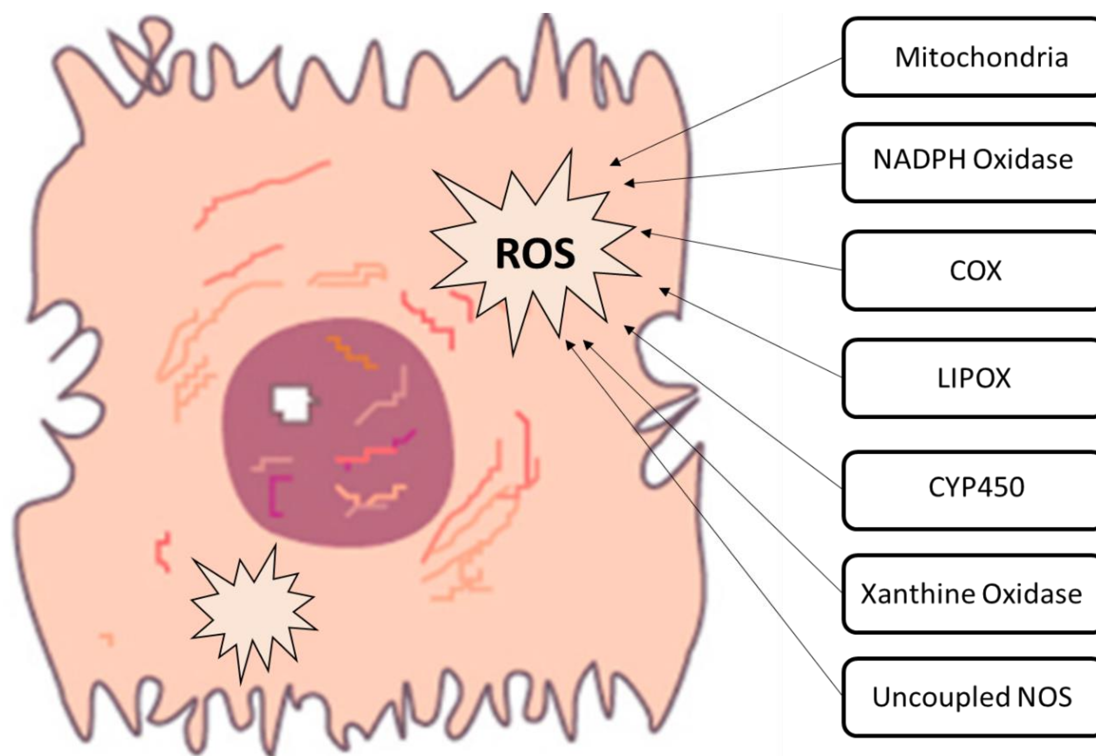


Figure 2: Sources of Reactive Oxygen Species in Cells

E. The NADPH Oxidase Family

The Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidases are a family of proteins responsible for ROS generation in different biological cell membranes. Seven members of the NOX family have been identified in humans and include: NOX1, NOX2, NOX3, NOX4 and NOX5, DUOX1 and DUOX2. Each of these NOX family members is characterized by different activation mechanisms and different expression levels in various tissues [63]**.** In terms of structural properties, these transmembrane proteins have a lot in common: a NADPH-binding site, a flavin adenine dinucleotide (FAD)-binding region, six

conserved transmembrane domains, and four highly conserved heme-binding histidines [63]. For the scope of this study, the focus will be on three isoforms: NOX1, NOX2 and NOX4.

As the focus of our study is on the liver, it is important to mention that hepatocytes generate these different NADPH oxidase isoforms as a response mechanism to many endogenous and exogenous stimuli. Studies measuring total liver mRNA showed large amounts of NOX2 and trace amounts of NOX4 [64, 65]. Other studies conducted on rats showed that their hepatocytes expressed NOX1, NOX2, and NOX4 mRNAs [66]. The main function attributed to NOX-derived ROS in hepatocytes is apoptosis [66, 67]. One study conducted on hepatocytes showed that ROS generation in response to Transforming Growth Factor-β (TGF-β) led to apoptosis through NOX activation [68]. The liver is also made of many non-parenchymal cells types, including endothelial cells, Kupffer cells, and hepatic stellate cells. Hepatic stellate cells express $p22^{phox}$ [69]. Both NOX1 (mRNA) and NOX2 (mRNA and protein) have also been shown to be expressed in hepatic stellate cells primary culture and cell lines [70, 71]. Kupffer cells have also been shown to express NOX2 and its subunits [72, 73]. It is believed that one of the mechanisms of hepatic carcinogenesis involves NADPH-induced (specifically NOX2) DNA damage and/or mitogenic signaling. Increased NADPH oxidase activity has also been implicated in the progression of nonalcoholic fatty liver disease [74]. Other studies have shown that NOX2 could play a role in liver ischemia/reperfusion injury. ROS generation by Kupffer cells have also been shown to generate ROS, presumably NOX2. This has been shown to play an important role in liver reperfusion injury after cold preservation [75]. Importantly, NOX2-deficient mice show reduced hepatic reperfusion injury [76].

F. Cytochrome P450 and Arachidonic Acid Metabolism

The CYP450s belong to a large family of hemoproteins predominantly involved in the metabolism of endogenous and exogenous substances. They are bound to either the membranes of the mitochondria or endoplasmic reticulum and are known to play a role in redox reactions [77]. Additionally, CYP450s have been shown to be major sources of ROS in various tissues, with implications in different disease conditions [78-82]. One of the physiologically relevant reactions catalyzed by CYP450 enzymes is arachidonic acid metabolism. The activation of phospholipase A2 from the phospholipid membrane induces the release of arachidonic acid. Free arachidonic acid is then metabolized by the cyclooxygenase, lipooxygenase and moonoxygenase pathways.

The major products of CYP450-catalyzed arachidonic acid monooxygenase pathway are regiospecific and stereospecific epoxyeicosatrienoic acids (EETs) and their corresponding dihydroxyeicosatrienoic acids (DHETs), and 20- hydroxyeicosatetraenoic acid (20-HETE) [83, 84] **(Figure 3)**. Cytochrome P450-derived eicosanoids are produced in a cell and tissue-specific manner, with numerous biological functions. They play a major role as second messengers, regulating vascular tone and ion transport [85, 86]. Recently, many studies have shown that these eicosanoids also play a role in other critical biological processes, including control of cellular proliferation, inflammation, and hemostasis [84, 87].

Previous studies have shown that numerous members of the cytochrome P450 family can metabolize arachidonic acid to EETs. These include: CYP1A, CYP2B, CYP2C,

CYP2E, and CYP2J subfamilies, also known as the epoxigenases [84, 87, 88]. Once EETs are formed, they can be metabolized even further through a hydration reaction by soluble epoxide hydrolase (sEH), conjugation by glutathione S transferases and esterification to glycerophospholipids [87]. The ω-hydroxylation of arachidonic acids produces 20-HETE. This reaction is catalyzed by the ω-hydroxylase CYP450, which include CYP4A and CYP4F [84, 85, 89]. While the ω-hydroxylation by CYP4A can take place in many organs, it is more prevalent in the kidney where it has been associated with important functions [84, 90-93]. Members of the CYP4F subfamily are also known to catalyze ω/ω-1 hydroxylation of arachidonic acid, with a predominant role in the kidney [93, 94].

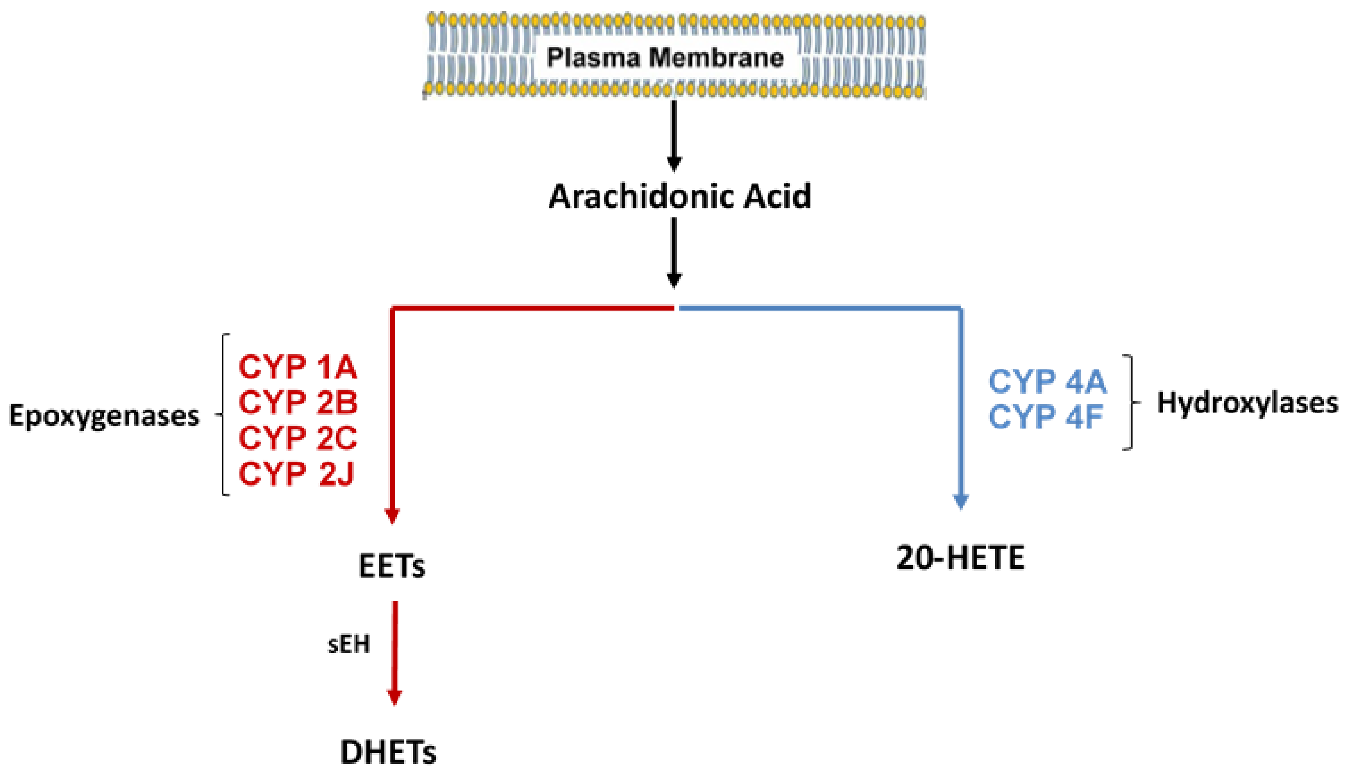


Figure 3. Pathways of Arachidonic Acid Metabolism. Free Arachidonic Acid is metabolized via the CYP450 enzymes, CYP1A, CYP2B, CYP2C and CYP2J (which belong to the epoxigenase family) to produce EETs and by CYP4A or CYP4F enzymes (which belong to the hydroxylase family) to produce 20-HETE.

G. Physiological Roles of EETs and 20-HETE

The EETs were initially labelled as vasoactive lipids [95, 96]. Their vasoactive properties were related to their role in activating calcium sensitive K^+ channels in vascular smooth muscles [97, 98]. EETs are also known to be anti-inflammatory, as they were shown to inhibit cytokine-induced endothelial cell adhesion molecule expression and prevent leukocyte adhesion to the vascular wall [99-101]. Other functional roles for EETs in cells have also been identified Many studies have identified including renal Na^+ excretion, Ca^{2+} mobilization, epidermal growth factor (EGF) signaling, nociception, mitogenic kinase activation, cell replication, vascular endothelial growth factor (VEGF) stimulated angiogenesis, nuclear factor kappa B subunit (NFkB) signaling, tumorgenesis and metastatic growth [90, 102-105]. As for the role of EETs in the liver, one study conducted in mice showed that the induction of non-alcoholic fatty liver disease (NAFLD) markedly suppressed CYP epoxygenase expression and activity, and both hepatic and circulating levels of EETs. Likewise, in another study also conducted on mice, disruption of the *Ephx2* gene (the gene encoding sEH) restored hepatic and circulating EET levels and significantly attenuated induction of hepatic inflammation and injury.

Numerous functional roles for 20-HETE have been reported. These include regulation of renal Na^+/K^+ ATPase activity, Ca^{2+} and Cl^- fluxes, vascular remodeling, and tumor metastasis. It has also been shown to play a role in hormonal signaling through EGF and VEGF, angiotensin, vasopressin, and norepinephrine [90-92, 106, 107]. 20-HETE is also plays a role in the hemodynamics and regulation of metabolic activity in the liver [108]. Being a potent activator of peroxisome proliferator-activated receptor α ($PPAR_{\alpha}$),

20-HETE was also found to play an important role in lipid homeostasis and in controlling fat-dependent energy supply and metabolism [109, 110]. In addition, the presence of 20-HETE in liver disease-abnormalities, such as fibrosis and cirrhosis was also reported in some studies [108, 111]. Data from studies conducted on patients with hepatic cirrhosis showed that 20-HETE production was increased in the pre-glomerular microcirculation zone, and led to the constriction of renal vasculature, reduction of renal blood flow and decrease in renal hemodynamics [108, 111].

H. Role of Reactive Oxygen Species in β-Thalassemia

Excess α-globin chains in β-thalassemia form unstable tetramers. These in turn dissociate into monomers and then are oxidized to hemichromes that eventually precipitate with time. Heme and free iron are then released followed by the precipitation of the globin protein, including on the plasma membrane where it is most injurious. The end result of this chain of events is the enhanced formation of ROS [112]. One study showed that oxidative stress in developing thalassemia erythroid precursors was associated with an increase in apoptosis, characterized by externalization of phosphatidylserine (PS). This in turn suggested that oxidative stress can also lead to ineffective erythropoiesis [113, 114].

An additional contributor to oxidative stress in β-thalassemia is excess iron. Long-term uptake and accumulation of non-transferrin bound iron (NTBI) and labile plasma iron (LPI), its redox active component, lead to increased levels of storage iron and labile cellular iron [115]. The intracellular LPI is redox active, catalyzing the Fenton and Haber–

Weiss reactions and thus generating ROS [116]. Therefore, iron-overload associated cell damage leads excessive levels of LPI which promote the production of ROS [117, 118].

Several clinical manifestations of NTDT can also be credited to a state of chronic hypoxia that is created by low hemoglobin levels. Therefore, another source of ROS in these patients is the underlying insufficient and uneven oxygen which creates a disturbed cellular physiology [119]. The mitochondria is known to be one of the major contributors to hypoxia-induced ROS in cells. Reducing agents such as NADH and FADH2, which play a role in the electron transport chain where oxygen is an integral part, accumulate due to the disruption in the chain. This buildup makes electrons readily available for production of ROS [120].

The role of oxidative stress in red blood cells (RBCs) has also been widely reported. One study found higher baseline level of free radicals in thalassemic RBCs compared to normal RBCs [121]. Analysis of other factors of oxidative stress showed that RBCs from β-thalassemia patients have increased lipid peroxidation and PS externalization and decreased glutathione compared with their normal counterparts. It has been shown that increased ROS in RBCs leads to numerous abnormalities in the cell membrane, including protein thiol oxidation and protein crosslinking, and lipid peroxidation [122]. The presence of high ROS levels also alters and increases the RBC membrane permeability to potassium ions [123-125]. Thalassemic RBCs were also shown to have an increased tendency to undergo intravascular and extravascular hemolysis [114].

I. Aims of the Study and Hypothesis

Oxidative damage by ROS is considered to be a major contributor to cell injury and tissue damage in patients with thalassemia. Recent studies suggest that ROS generation in NTDT patients occurs mainly as a consequence of iron overload. This increased ROS production has been linked to multiple pathological outcomes in various organs. Sources of ROS production in pathophysiology are thought to be disease-specific. Among the different sources of ROS, the NADPH oxidases and CYPs 450 have been proposed to be the driving force in certain diseases. The aim of this study is to therefore identify the exact source of ROS in NTDT.

CHAPTER II

MATERIALS AND METHODS

A. Animal Model

All animals (C57BL/6 background) were bred at the animal care facility of the American University of Beirut. We used $Hbb^{th3/+}$ mice (The Jackson Laboratory-B6; 129P2-*Hbb-b1*^{tm1Unc} *Hbb-b2*^{tm1Unc}/J) as a model of NTDT. These mice carry a double knock-out of the *Hbb-b1 and Hbb-b2* adult β-globin genes. Eight mice were divided into two groups (one control group, one $Hbb^{th3/+}$ group receiving no treatment). A peripheral blood smear was used to confirm diagnosis of thalassemia. All animal-model experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee of the American University of Beirut. All animals were kept in a temperature-controlled room and on a 12/12-dark/light cycle and had standard chow and water access. At the day of the sacrifice, all mice were euthanized and sacrificed, and all organs were collected accordingly. In our study, the liver was the organ of interest. The liver was therefore isolated and cut into different sections; some were frozen in liquid nitrogen and stored at -80°C, while the remaining were fixed with 4% formaldehyde for histological analysis.

B. High Performance Liquid Chromatography (HPLC)

In order to determine and quantify the liver tissue iron content of our samples, an HPLC performed. The basis of this technique was developed by Tesoro et al. and is based on the measurement of the chelate ferrioxamine [126]. The chromatography was performed on a stainless steel XTerra MS C18 column (Waters; 250 mm × 4.6 mm i.d., 5µm) containing a gradient of Tris–HCl buffer (10 mM, pH 5) and acetonitrile. Data from $Hbb^{th3/+}$ mice were represented as fold changes when compared to control counterparts.

HPLC analysis of dihydroethidium (DHE)-derived oxidation products was used to assess cellular superoxide production in liver tissues. This HPLC-based assay separates superoxide-specific 2-hydroxyethidium (EOH) from the nonspecific ethidium. For this technique, homogenates from liver tissues were washed twice with Hanks' balanced salt solution (HBSS)-diethylenetriaminepentaacetic acid (DTPA) and incubated for 30 min with 50 µM DHE (Sigma-Aldrich) in HBSS–100 µM DTPA. Tissues were then collected in acetonitrile and centrifuged (12,000 X g for 10 min at 4°C). Samples were then left under vacuum to dry and were analyzed by HPLC with fluorescence detectors. Quantification of DHE, EOH, and ethidium concentrations was performed by comparison of integrated peak areas between both the obtained and the standard curves of each product under chromatographic conditions identical to those described above. EOH and ethidium concentrations were monitored by fluorescence detection with an excitation wavelength of 510 nm and emission wavelength of 595 nm. DHE concentration was monitored by UV absorption at 370 nm. Results were expressed as the amount of EOH produced (nmol)

normalized for the amount of DHE consumed (i.e., initial minus remaining DHE in the sample; μmol).

Levels of EET and 20-HETE were also measured by HPLC in isolated microsomes. The process begins by drying [1-^{14}C]-labeled arachidonic acid (50–100 μmol/l) and then re-suspending it in a solution containing 30 mmol/l isocitrate, 50μg microsomes, and 0.2 units of isocitrate dehydrogenase in reaction buffer (100 mmol/l potassium phosphate, pH 7.4, 5 mmol/l magnesium chloride, and 1 mmol/l EDTA). After a 5-minute incubation at 37°C, a solution of NADPH was added to initiate a reaction. Different aliquots were taken at 30, 60, and 90 min, and a solution 100% methanol was added to stop the reaction. The samples were then centrifuged to collect the pelleted precipitated proteins stored at −20°C. A separation process via HPLC was then performed on a C-18 column using an acetonitrile/H20 gradient was performed and metabolites were identified by coelution with labeled standards [127].

C. NADPH Oxidase Activity Assay

NADPH oxidase activity was measured in liver tissues. Proteins were first extracted from liver tissues using a douncer and remnants were suspended in a lysis buffer (20 mM KH_2PO_4 (pH 7.0), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EGTA, 10 μg/ml aprotinin, and 0.5 μg/ml leupeptin). In order to initiate the assay, 25 μg of homogenates were added to 50 mM phosphate buffer (pH 7.0) containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin, and 100 μM NADPH (acting as the substrate for the NADPH oxidase). The photon emission, expressed as relative light units (RLU), was

measured every 20 or 30 seconds for 10 minutes in a luminometer. A buffer blank (<5% of the cell signal) was subtracted from each reading. Superoxide production was expressed as relative light units/min/mg of protein. Protein content was measured using the Bio-Rad protein assay reagent.

D. Western Blot Analysis

Liver tissues were lysed using a radioimmunoprecipitation assay (RIPA) buffer containing 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 150 mM sodium chloride, 100mM EDTA, 50 mM Tris-hydrochloride, 1% Tergitol (NP40), 1mM PMSF, 50mM of Tris-HCl, 1% NP-40, protease inhibitor cocktail, and phosphatase inhibitor cocktail. Homogenates were incubated for two hours at 4°C and centrifuged at 13,600 rpm for 30 min at 4°C. The supernatant containing the proteins was collected and stored at -20°C. Protein concentration was measured using the Lowry Protein Assay. For immunoblotting, 20-40 μg of proteins were separated on 12.5% polyacrylamide gel Electrophoresis (Bio-Rad Laboratory, CA, USA) and transferred to nitrocellulose membranes (Bio-Rad Laboratory, CA, USA). After transfer, the membranes were blocked with 5% BSA for 1 hour and then incubated overnight with rabbit polyclonal anti-NOX1 (1:500, Santa Cruz Biotechnology), rabbit polyclonal anti-NOX2/gp91phox (1:500, Abcam), rabbit polyclonal anti-NOX4 (1:500, Santa Cruz Biotechnology), mouse polyclonal anti-CYP1A (1:500, Detroit R&D), mouse polyclonal anti-CYP2B (1:1000, Abcam), rabbit polyclonal anti-CYP4A (1:2000, Abcam), rabbit monoclonal anti-CYP4F (1:1000, Abcam). The primary antibodies were then detected using horseradish

peroxidase–conjugated IgG (1:1000, Bio-Rad). Bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using Image J software.

E. Real-time Polymerase Chain Reaction (PCR)

Messenger RNA (mRNA) expression in liver tissue was analyzed by real-time RT-PCR using the ΔΔCt method and the SYBR green system. Total RNA was extracted from the a small piece of liver tissue using TRIZOL reagent (Sigma Aldrich, Steinheim, Germany) and was then converted into cDNA using the Revert First Strand cDNA Synthesis Kit as per protocol. The cDNA was quantified using RT- PCR Biorad CFX384 with SYBR green dye and mouse RT2qPCR Primers (Humanizing Genomics, Macrogen, Seoul, South Korea), for NOX1, NOX2, NOX4, CYP1A, CYP2B, CYP4A and CYP4F . YWAZ was used as the internal reference gene **(Table 1).**

Primers	Sequence
NOX1	F: 5’-TCGACACACAGGAATCAGGA-3’ R: 5’-TTACACGAGAGAAATTCTTGGG-3’
NOX2	F: 5’-TCATTCTGGTGTGGTTGGGG-3’ R: 5’-CAGTGCTGACCCAAGGAGTT-3’
NOX4	F: 5’-TCAGGACAGATGCAGATGCT-3’ R: 5’-CTGGAAAACCTTCCTGCTGT-3’
CYP1A	F: 5’-AGGCTCTTCTCACGCAACTC-3’ R: 5’-CTGGGGCTACAAAGGGTGAT-3’
CYP2B	F: 5’-GCGCATGGAGAAGGAGAAGTC-3’ R: 5’-CCTGGAGATTTGGAGACATGC-3’
CYP4A	F: 5’-TTGCCCAAAGGTATCATGGTC-3’ R: 5’-GTTTCCCAATGCAGTTCCTTGAT-3’
CYP4F	F: 5’-GGGAAACACAGTGCTCCTGA-3’ R: 5’-ACTTGGCGTGCATGATGTGTG-3’
YWAZ	F: 5’-GGTGATGACAAGAAAGGAATTGTG-3’ R: 5’-GCATCTCCTTTTTGCTGATTTCA-3’

Table 1. Oligonucleotide primer sequences employed for RT-PCR

F. Immunohistochemistry

Formalin-fixed liver tissues were embedded in paraffin blocks and cut into 6μm sections and fixed on glass slides. The sections where then deparaffinized, immersed in xylol/xylene and rehydrated. Necessary washes in distilled water were made. The sections were then immersed in a citrate antigen retrieval buffer (0.1 M citric acid, 0.1M Na Citrate, 2N NaOH droplets and distilled water) for one hour, in a container with boiling water. The slides were then left to sit at room temperature to dry and cool. After the necessary washes with distilled water and phosphate-buffered saline (PBS) 1X (as per protocol), sections were neutralized using Peroxidase Block, and incubated with the protein block. Sections were then incubated overnight with the primary antibody solution (Normal goat serum (NGS), Triton 1X, Bovine Serum Albumin (BSA), PBS 1X, mouse polyclonal anti-CYP1A (1:150, Detroit R&D), rabbit polyclonal anti-CYP4A (1:150, Abcam). The next day, and after the necessary washes with PBS 1X, sections were incubated with either the post-primary (for CYP1A), and with Novolink Polymer (for CYP4A). All sections were then incubated under dim light with 3,3'-diaminobenzidine (DAB) working solution (DAB Chromogen + Novolink DAB Substrate Buffer). After necessary washes with distilled water, the sections were counterstained with 0.1% hematoxylin, rinsed, dehydrated, and mounted. Sections were examined under the light microscope and analysis of the sections was performed using Image J software.

G. Statistical Analysis

Results were expressed as mean ± standard errors (SE) from multiple independent experiments. Statistical significance was assessed by the student's unpaired t-test. A *p-value* <0.05 was considered as statistically significant. When comparing control and thalassemic, one asterisk (*) is used to indicate $p<0.05$. All statistical analyses were performed with Prism 6 Software (GraphPad Software).

CHAPTER III

RESULTS

A- Increased tissue iron levels in the liver of Hbb$^{th3/+}$ mice

The liver tissue iron content was increased in thalassemic mice compared with their control littermates **(Figure 4).**

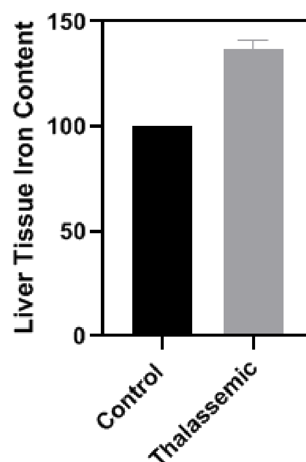


Figure 4. Increased tissue iron levels in the liver of Hbb$^{th3/+}$ mice. Assessment of tissue iron content using HPLC. Values are the means ± SE from 4 different mice in each group (n=4). *, $p < 0.05$ versus control.

B- Reactive Oxygen Species production in Hbb$^{th3/+}$ mice is induced through the NADPH oxidases

Superoxide generation in liver tissues was increased in thalassemic mice compared with their control littermates (**Figure 5A**). Concurrently, this iron-overload induced ROS generation was accompanied by an increase in NADPH oxidase activity **(Figure 5B).**

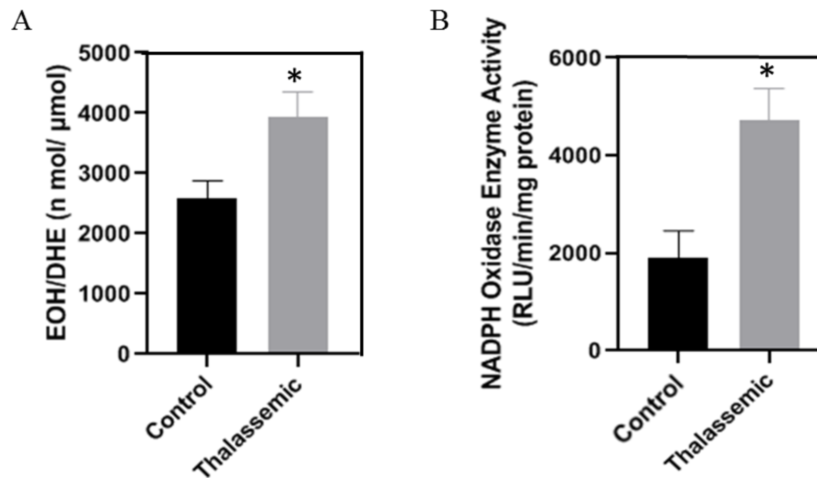


Figure 5. Enhancement of superoxide production and NADPH oxidase activity in liver tissues isolated from Hbb$^{th3/+}$ mice. (A) Superoxide generation evaluated using HPLC. (B) NADPH-dependent superoxide generation assessed by lucigenin-enhanced chemiluminescence. Values are the means ± SE from 4 different mice in each group (n=4). *, $p < 0.05$ versus control.

C- Decreased expression levels of the NOX isoforms in $Hbb^{th3/+}$ mice

After seeing an increase in the NADPH oxidase activity in thalassemic mice as compared to their control littermates, mRNA levels and protein levels of three isoforms of the NADPH oxidases (NOX1, NOX2 and NOX4) were measured by real-time PCR and western blot. At the mRNA level, no significant changes were observed in the expression of NOX1, NOX2 and NOX4 in $Hbb^{th3/+}$ mice compared to control **(Figure 6A-C).** However, at the protein level, there was a decreased expression of all three NOX isoforms in the thalassemic group compared with controls **(Figure 6D-F).**

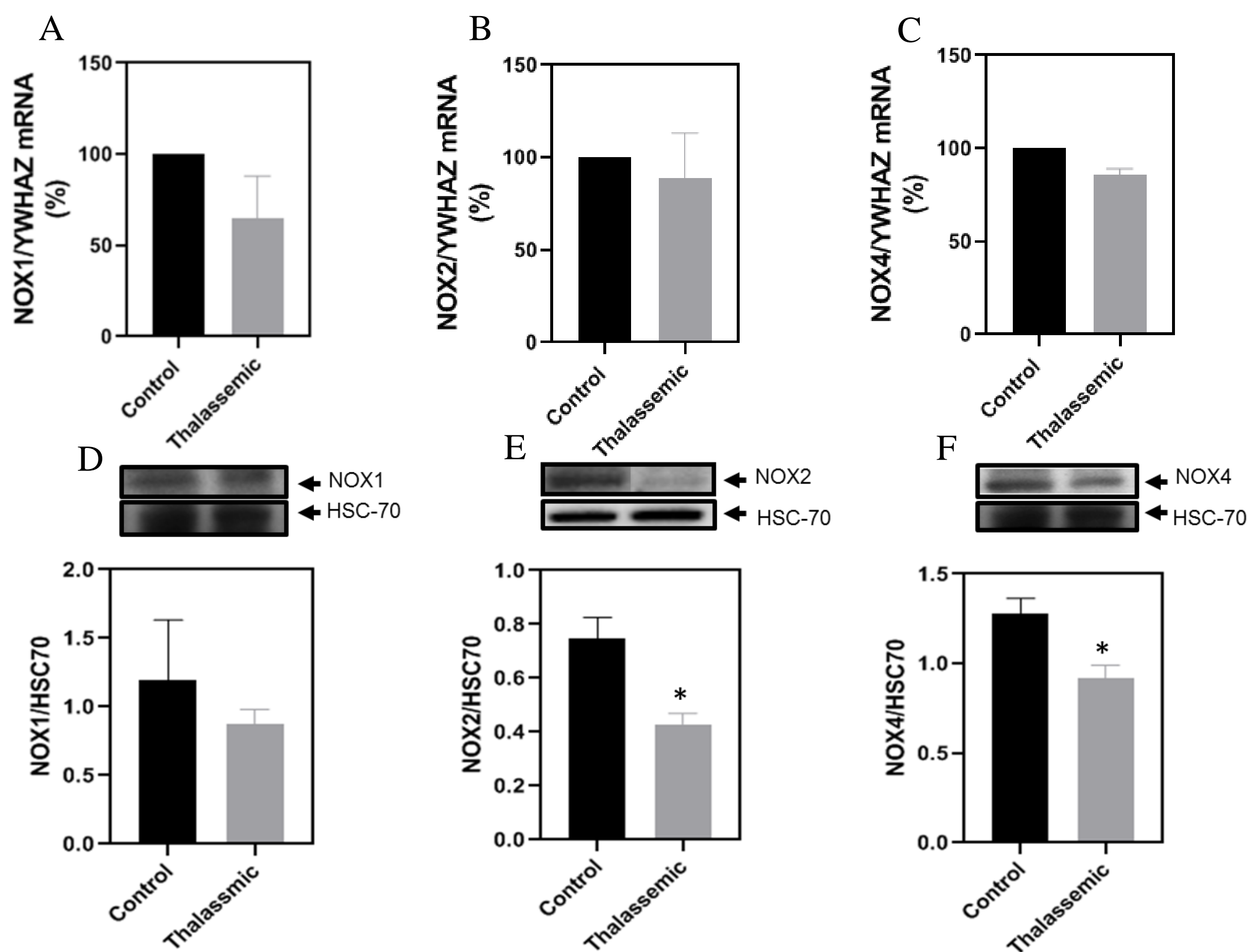
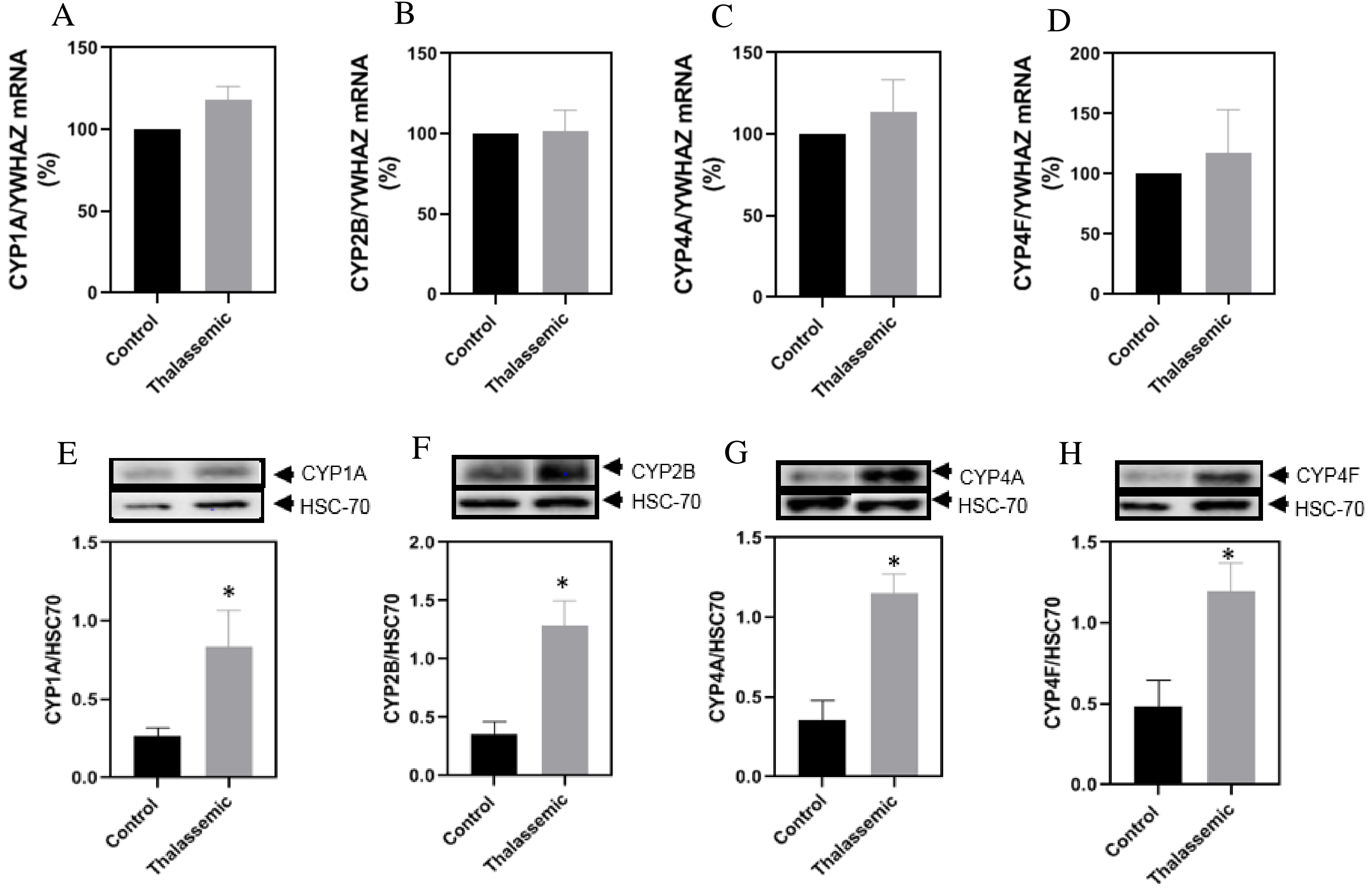


Figure 6. Decreased expression levels of the NOX isoforms in Hbb$^{th3/+}$ mice. Relative mRNA levels of (A) NOX1, (B) NOX2, (C) NOX4. Representative Western blot of (D) NOX1, (E) NOX2, (F) NOX4 and HSC70 levels with the respective densitometric quantification in liver tissues of control and Hbb$^{th3/+}$ mice. Values are the means ± SE from 4 different mice in each group (n=4). *, $p < 0.05$ versus control.

D- Over expression of CYP1A, CYP2B, CYP4A, and CYP4F in Hbb$^{th3/+}$ mice

A second family of ROS that is NADPH dependent is the CYP450. This is because CYPs of the monooxygenase subfamily is made of different components. These include a heme-based catalytic center, an NADPH reductase subunit and an NADH/NADPH oxidase as cofactors. No significant changes were observed in the expression of CYP1A, CYP2B, CYP4A and CYP4F mRNA levels in Hbb$^{th3/+}$ mice compared to control. On the other hand, there was a statistically significant increase in CYP1A, CYP2B, CYP4A and CYP4F protein levels in livers of Hbb$^{th3/+}$ mice compared to control **(Figure 7A-H).** Immunohistochemistry staining of liver tissue sections also showed over expression of CYP1A and CYP4A in Hbb$^{th3/+}$ mice compared with their control littermates **(Figure 7I-L).**


A
B
C
D
CYP1A/YWHAZ mRNA (%)
CYP2B/YWHAZ mRNA (%)
CYP4A/YWHAZ mRNA (%)
CYP4F/YWHAZ mRNA (%)
Control
Thalassemic
E
F
G
H
CYP1A
HSC-70
CYP2B
CYP4A
CYP4F
CYP1A/HSC70
CYP2B/HSC70
CYP4A/HSC70
CYP4F/HSC70

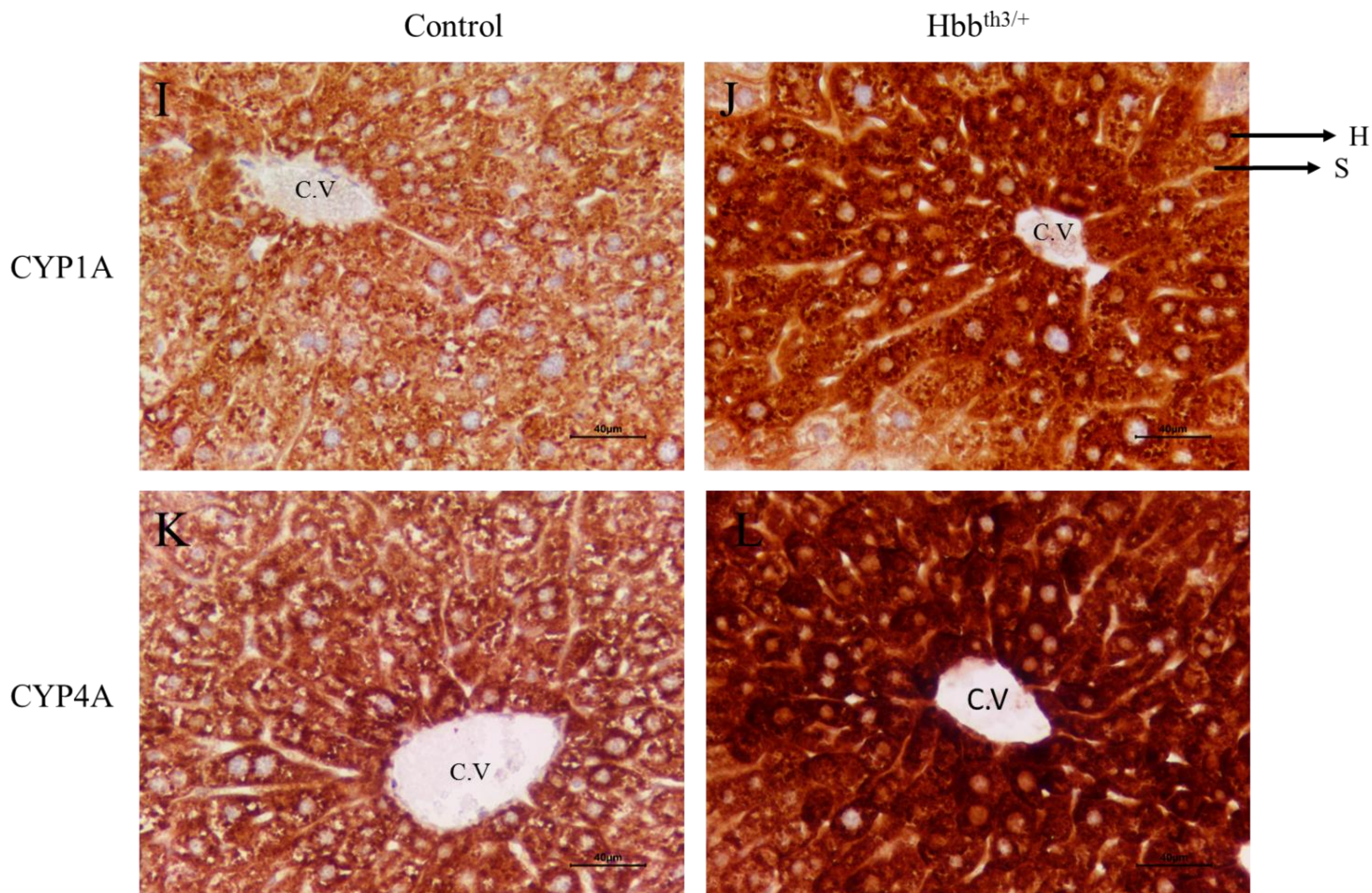


Figure 7. Over expression of CYP1A, CYP2B, CYP4A, and CYP4F in Hbb$^{th3/+}$ mice. Relative mRNA levels of (A) CYP1A, (B) CYP2B, (C) CYP4A, and (D) CYP4F. Representative Western blot of (E) CYP1A, (F) CYP2B, (G) CYP4A, and (H) CYP4F and HSC70 levels with the respective densitometric quantification in liver tissues of control and Hbb$^{th3/+}$ mice. Immunohistochemistry staining of liver tissue sections at 20X for (I) CYP1A expression in control mouse, (J) CYP1A expression Hbb$^{th3/+}$ mouse, (K) CYP4A expression in control mouse, and (L) CYP4A expression in Hbb$^{th3/+}$ mouse. Scale bar represents 40µm.Values are the means ± SE from 4 different mice in each group (n=4). *, $p < 0.05$ versus control. C.V: Central vein; H: Hepatocyte; S: Sinusoid

E- Increase in EET and 20-HETE activity in $Hbb^{th3/+}$ mice

The activities of EET and 20-HETE, which are the metabolites produced by these cytochromes, were tested using HPLC. There was an increase in both EET and 20-HETE activity in thalassemic mice compared with their control littermates, which correlated with the increased expression of their respective CYPs seen earlier **(Figure 8A-B).**

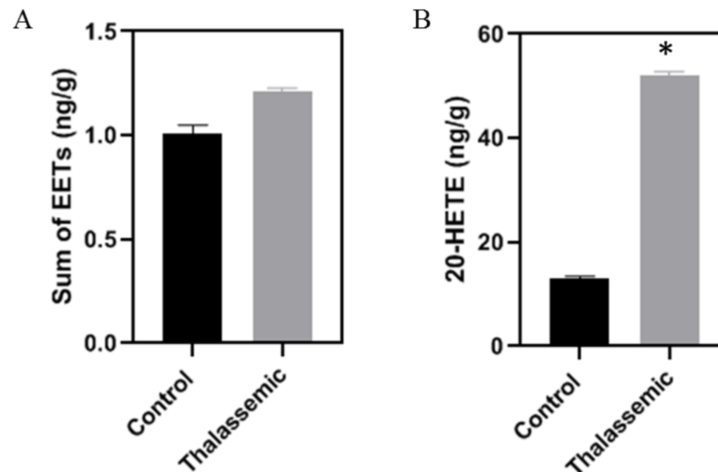


Figure 8. Increase in EET and 20-HETE activity in $Hbb^{th3/+}$ mice. (A) Assessment of EET activity (metabolite produced by CYP1A and CYP2B) by HPLC. (B) Assessment of 20-HETE activity (metabolite produced by CYP4A and CYP4F) by HPLC. Values are the means ± SE from 4 different mice in each group (n=4). *, $p < 0.05$ versus control.

CHAPTER IV

DISCUSSION

Oxidative damage by ROS is a major contributor to cell injury and tissue damage in patients with thalassemia [128]. Recent studies suggest that ROS generation in NTDT patients occurs as a result of iron overload [129]. This increased ROS production in organs has been associated with multiple pathological outcomes. Sources of ROS production in pathophysiology have been proposed to be disease-specific. Despite all the advances in the thalassemia field, no study in the literature was able to provide evidence-based data identifying the exact sources of ROS in NTDT.

Our *in vivo* results showed that superoxide generation in liver tissues is increased in thalassemic mice compared with their control littermates. Concurrently, this iron-overload induced ROS generation was accompanied by an increase in NADPH oxidase activity. The NOX family members are transmembrane proteins responsible for transporting electrons across biological membranes to reduce oxygen to superoxide. Different NOX isoforms have been described, with different structures and functions. After seeing an increase in the NADPH oxidase activity in thalassemic mice as compared with their control littermates, protein levels of three isoforms of the NADPH oxidases (NOX1, NOX2 and NOX4) were measured by western blot. We then were able to show that protein levels of three isoforms of the NADPH oxidases (NOX1, NOX2 and NOX4) were decreased in the thalassemic group compared with controls. This finding was key, as to our knowledge, we are the first

to rule out the role of NADPH oxidases in the liver of Hbb$^{th3/+}$ mice. Whether or not there is a potential role for NADPH oxidases in other organs is yet to be investigated.

While these findings did not provide any evidence on the role of NADPH oxidases, it did pave the way for further investigation into another source of ROS, the CYP450 family. The CYP450s are a large family of hemoproteins that are primarily responsible for metabolism of endogenous, and exogenous molecules. They are bound to the membranes of either the mitochondria or endoplasmic reticulum and are known to play a role in redox reactions. Additionally, CYPs are reported to be major sources of ROS in numerous tissues with implications in different disease conditions. Enzymes of the CYP1A, CYP2B, CYP4A and CYP4F subfamilies have not been investigated nor reported in the NTDT. Subsequently, we first examined whether these CYPs could be expressed in thalassemic mice. The present study is the first to demonstrate the over-expression of CYP1A, CYP2B, CYP4A and CYP4F protein levels in livers of Hbb$^{th3/+}$ mice. We also demonstrated CYPA and CYP4A overexpression by immunohistochemistry.

Major products of CYP450-catalyzed arachidonic acid monooxygenase pathway include EETs and 20- 20-HETE [83, 84]. These metabolites have numerous biological functions and are produced in a cell and tissue-specific manner. The modification EETs and 20-HETE formation and degradation may potentially provide therapeutic benefits, given that these two metabolites have multiple and opposing functions, depending on their degree of expression and location of their production [127, 130-132]. In our study, we tested for the activity of EET and 20-HETE, and we saw that there was an increase in both EET and 20-HETE activity in Hbb$^{th3/+}$ mice, which correlated with the increased

expression of their respective CYPs that we saw earlier. We believe that in $Hbb^{th3/+}$ mice, 20-HETE may play an injurious role, whereas EET may have a protective role. Moreover, there is a cross talk happening between EET and 20-HETE. As a result of the increase in 20-HETE, we see an accompanied increase in EET production, which is trying to compensate for the 20-HETE increase. However, this increase in EET production is not enough to restore 20-HETE levels back to normal.

In summary, our data is the first to report that CYP450 is the NADPH oxidase dependent ROS-producer responsible for superoxide and H_2O_2 production in in the liver of $Hbb^{th3/+}$ mice. One limitation of the study is the number of mice. It is important to replicate our work on a large cohort of mice. Another limitation is the fact that our study was conducted on solely one organ. It would have also been better if we had replicated our work in the context of a different organ, such as a kidney, which is also very much affected by iron overload in patients with NTDT. Whether or not, we would have seen similar results in other target organs yet to be determined. However, the liver was chosen to be studied as it is the major storage organ and it reflects total body iron levels.

In order to strengthen our study, we aim to undertake some further work. Our future work on animal models will involve the addition of different treatment cohorts and inhibit specific types of CYP450. Specifically, we aim to activate EET production (produced by CYP1A and CYP2B) and inhibit 20-HETE production (produced by CYP4A and CYP4F). In order to perform this, we will have different groups of mice. One group will be a control group (C57BL/6), and one group will be made of $Hbb^{th3/+}$ mice who will receive no treatment. Our treatment cohort of $Hbb^{th3/+}$ mice will be divided into 4 groups:

one group will receive AUDA, one group will receive HET0016, one group will receive AUDA + HET0016, and the last group will receive AUDA+HETE0016 + iron chelator (deferiprone or deferasirox). AUDA is an inhibitor of soluble epoxide hydrolase (sEH) enzyme which is responsible for the metabolism of EET. HET0016 is a potent and selective inhibitor of 20-HETE.Treatment duration will be for about 6 weeks and at the end of treatment, all mice will be sacrificed and organs will be collected. We aim to assess improvements in physiological parameters such as iron parameters, ineffective erythropoiesis, RBC lifespan, anemia, splenomegaly, hypoxia, and apoptosis. We will also take a cohort of thalassemia patients in order to confirm the presence of oxidative stress markers in fluids (e.g. venous blood and urine) and blood cells, identify possible genetic polymorphisms and correlate the markers of oxidative stress with genetic polymorphisms to gender variability and aging. Having provided evidence of CYP450 involvement, the future directions of this project also aim to investigate the crosstalk of CYP450 these enzymes and their metabolites with other signaling pathways. Specifically, we aim to look at the PI3K/AKT/mTOR pathway.

Challenges and limitations of the currently available conventional therapies in thalassemia and a thorough understanding of the pathophysiology and overall disease burden of thalassemia has aided clinicians and scientists to optimize disease management approaches. It has also stimulated research towards development of numerous novel therapeutic targets in the field, with ultimate goals of prolonging longevity, reducing symptom burden, improving treatment compliance and adherence for a better quality of life. Throughout the extensive work and investigation that will be done over the next few

years, we hope to show that targeting CYP450 (by activating EET production and inhibiting 20-HETE production), and using it in combination with iron chelation therapy will lead to better outcomes for thalassemia patients. Iron chelators can act as general antioxidants [133]. This is because they can remove both intra- and extracellular-iron species that generate free oxygen radicals. Although ROS are associated with injurious processes, their presence is essential for cellular functions such as gene transcription and cell proliferation, and in maintaining proper blood flow and blood pressure homeostasis [52, 134-138]. These physiological functions of ROS, among other reasons, explain why numerous attempts to treat ROS-associated diseases with general antioxidants have not only failed but have also caused deleterious effects [139, 140]. However, now that we have managed to identify one specific source of ROS, we can directly target the source. We therefore strongly believe that the field of oxidative stress could prove to be the next novel therapeutic approach in the thalassemia realm.

REFERENCES

1. Marengo-Rowe AJ. The thalassemias and related disorders. *Proc (Bayl Univ Med Cent)*. 2007;**20**(1):27-31.

2. Weatherall DJ. The inherited diseases of hemoglobin are an emerging global health burden. *Blood*. 2010;**115**(22):4331-6.

3. Modell B, Darlison M. Global epidemiology of haemoglobin disorders and derived service indicators. *Bull World Health Organ*. 2008;**86**(6):480-7.

4. Olivieri NF, Pakbaz Z, Vichinsky E. Hb E/beta-thalassaemia: a common & clinically diverse disorder. *Indian J Med Res*. 2011;**134**:522-31.

5. Vichinsky E. Complexity of alpha thalassemia: growing health problem with new approaches to screening, diagnosis, and therapy. *Ann N Y Acad Sci*. 2010;**1202**:180-7.

6. Lorey F, Cunningham G, Vichinsky EP, Lubin BH, Witkowska HE, Matsunaga A, et al. Universal newborn screening for Hb H disease in California. *Genet Test*. 2001;**5**(2):93-100.

7. Lorey F. Asian immigration and public health in California: thalassemia in newborns in California. *J Pediatr Hematol Oncol*. 2000;**22**(6):564-6.

8. Michlitsch J, Azimi M, Hoppe C, Walters MC, Lubin B, Lorey F, et al. Newborn screening for hemoglobinopathies in California. *Pediatr Blood Cancer*. 2009;**52**(4):486-90.

9. Higgs DR, Engel JD, Stamatoyannopoulos G. Thalassaemia. *Lancet*. 2012;**379**(9813):373-83.

10. Taher AT. Thalassemia. *Hematol Oncol Clin North Am*. 2018;**32**(2):xv-xvi.

11. Taher AT, Weatherall DJ, Cappellini MD. Thalassaemia. *Lancet*. 2018;**391**(10116):155-67.

12. Taher A, Vichinsky E, Musallam K, Cappellini M-D, Viprakasit V. Guidelines for the management of non transfusion dependent thalassaemia (NTDT): Thalassaemia International Federation, Nicosia, Cyprus; 2013.

13. Musallam KM, Rivella S, Vichinsky E, Rachmilewitz EA. Non-transfusion-dependent thalassemias. *Haematologica*. 2013;**98**(6):833-44.

14. Musallam KM, Taher AT, Rachmilewitz EA. beta-thalassemia intermedia: a clinical perspective. *Cold Spring Harb Perspect Med*. 2012;**2**(7):a013482.

15. In: rd, Cappellini MD, Cohen A, Porter J, Taher A, Viprakasit V, editors. Guidelines for the Management of Transfusion Dependent Thalassaemia (TDT). Nicosia (CY)2014.

16. Taher AT, Cappellini MD. How I manage medical complications of beta-thalassemia in adults. *Blood*. 2018;**132**(17):1781-91.

17. Taher AT, Musallam KM, Karimi M, El-Beshlawy A, Belhoul K, Daar S, et al. Overview on practices in thalassemia intermedia management aiming for lowering complication rates across a region of endemicity: the OPTIMAL CARE study. *Blood*. 2010;**115**(10):1886-92.

18. Sleiman J, Tarhini A, Bou-Fakhredin R, Saliba AN, Cappellini MD, Taher AT. Non-Transfusion-Dependent Thalassemia: An Update on Complications and Management. *Int J Mol Sci*. 2018;**19**(1).

19. Ben Salah N, Bou-Fakhredin R, Mellouli F, Taher AT. Revisiting beta thalassemia intermedia: past, present, and future prospects. *Hematology*. 2017;**22**(10):607-16.

20. Haidar R, Mhaidli H, Taher AT. Paraspinal extramedullary hematopoiesis in patients with thalassemia intermedia. *Eur Spine J*. 2010;**19**(6):871-8.

21. Taher A, Isma'eel H, Cappellini MD. Thalassemia intermedia: revisited. *Blood Cells Mol Dis*. 2006;**37**(1):12-20.

22. Levin C, Koren A. Healing of refractory leg ulcer in a patient with thalassemia intermedia and hypercoagulability after 14 years of unresponsive therapy. *Isr Med Assoc J*. 2011;**13**(5):316-8.

23. Taher A, Isma'eel H, Mehio G, Bignamini D, Kattamis A, Rachmilewitz EA, et al. Prevalence of thromboembolic events among 8,860 patients with thalassaemia major and intermedia in the Mediterranean area and Iran. *Thromb Haemost*. 2006;**96**(4):488-91.

24. Musallam KM, Taher AT, Karimi M, Rachmilewitz EA. Cerebral infarction in beta-thalassemia intermedia: breaking the silence. *Thromb Res*. 2012;**130**(5):695-702.

25. Taher AT, Musallam KM, Inati A. The hypercoagulable state in thalassemia intermedia. *Hemoglobin*. 2009;**33 Suppl 1**:S160-9.

26. Taher AT, Cappellini MD, Bou-Fakhredin R, Coriu D, Musallam KM. Hypercoagulability and Vascular Disease. *Hematol Oncol Clin North Am*. 2018;**32**(2):237-45.

27. Derchi G, Galanello R, Bina P, Cappellini MD, Piga A, Lai ME, et al. Prevalence and risk factors for pulmonary arterial hypertension in a large group of beta-thalassemia

patients using right heart catheterization: a Webthal study. *Circulation*. 2014;**129**(3):338-45.

28. Isma'eel H, El Chafic AH, El Rassi F, Inati A, Koussa S, Daher R, et al. Relation between iron-overload indices, cardiac echo-Doppler, and biochemical markers in thalassemia intermedia. *The American journal of cardiology*. 2008;**102**(3):363-7.

29. Taher A, Musallam KM, El Rassi F, Duca L, Inati A, Koussa S, et al. Levels of non-transferrin-bound iron as an index of iron overload in patients with thalassaemia intermedia. *Brit J Haematol*. 2009;**146**(5):569-72.

30. Taher AT, Musallam KM, El-Beshlawy A, Karimi M, Daar S, Belhoul K, et al. Age-related complications in treatment-naive patients with thalassaemia intermedia. *Brit J Haematol*. 2010;**150**(4):486-9.

31. Taher A, El Rassi F, Isma'eel H, Koussa S, Inati A, Cappellini MD. Correlation of liver iron concentration determined by R2 magnetic resonance imaging with serum ferritin in patients with thalassemia intermedia. *Haematologica*. 2008;**93**(10):1584-6.

32. Musallam KM, Cappellini MD, Wood JC, Taher AT. Iron overload in non-transfusion-dependent thalassemia: a clinical perspective. *Blood Rev*. 2012;**26 Suppl 1**:S16-9.

33. Origa R, Barella S, Argiolas GM, Bina P, Agus A, Galanello R. No evidence of cardiac iron in 20 never-or minimally-transfused patients with thalassemia intermedia. *Haematologica*. 2008;**93**(7):1095-6.

34. Roghi A, Cappellini MD, Wood JC, Musallam KM, Patrizia P, Fasulo MR, et al. Absence of cardiac siderosis despite hepatic iron overload in Italian patients with thalassemia intermedia: an MRI T2* study. *Ann Hematol*. 2010;**89**(6):585-9.

35. Taher AT, Musallam KM, Wood JC, Cappellini MD. Magnetic resonance evaluation of hepatic and myocardial iron deposition in transfusion-independent thalassemia intermedia compared to regularly transfused thalassemia major patients. *Am J Hematol*. 2010;**85**(4):288-90.

36. Mavrogeni S, Gotsis E, Ladis V, Berdousis E, Verganelakis D, Toulas P, et al. Magnetic resonance evaluation of liver and myocardial iron deposition in thalassemia intermedia and b-thalassemia major. *Int J Cardiovasc Imaging*. 2008;**24**(8):849-54.

37. Borgna-Pignatti C, Vergine G, Lombardo T, Cappellini MD, Cianciulli P, Maggio A, et al. Hepatocellular carcinoma in the thalassaemia syndromes. *Br J Haematol*. 2004;**124**(1):114-7.

38. Restivo Pantalone G, Renda D, Valenza F, D'Amato F, Vitrano A, Cassara F, et al. Hepatocellular carcinoma in patients with thalassaemia syndromes: clinical characteristics and outcome in a long term single centre experience. *Br J Haematol*. 2010;**150**(2):245-7.

39. Maakaron JE, Cappellini MD, Graziadei G, Ayache JB, Taher AT. Hepatocellular carcinoma in hepatitis-negative patients with thalassemia intermedia: a closer look at the role of siderosis. *Ann Hepatol*. 2013;**12**(1):142-6.

40. Maakaron JE, Musallam KM, Ayache JB, Jabbour M, Tawil AN, Taher AT. A liver mass in an iron-overloaded thalassaemia intermedia patient. *Br J Haematol*. 2013;**161**(1):1.

41. Mancuso A. Hepatocellular carcinoma in thalassemia: A critical review. *World J Hepatol*. 2010;**2**(5):171-4.

42. Moukhadder HM, Roumi JE, Bou-Fakhredin R, Taher AT. Hepatocellular Carcinoma in a beta-Thalassemia Intermedia Patient: Yet Another Case in the Expanding Epidemic. *Hemoglobin*. 2018;**42**(1):58-60.

43. Hodroj MH, Bou-Fakhredin R, Nour-Eldine W, Noureldine HA, Noureldine MHA, Taher AT. Thalassemia and malignancy: An emerging concern? *Blood Rev*. 2019;**37**:100585.

44. Ziyadeh FN, Musallam KM, Mallat NS, Mallat S, Jaber F, Mohamed AA, et al. Glomerular hyperfiltration and proteinuria in transfusion-independent patients with beta-thalassemia intermedia. *Nephron Clin Pract*. 2012;**121**(3-4):c136-43.

45. Mallat NS, Mallat SG, Musallam KM, Taher AT. Potential mechanisms for renal damage in beta-thalassemia. *J Nephrol*. 2013;**26**(5):821-8.

46. Musallam KM, Taher AT. Mechanisms of renal disease in beta-thalassemia. *J Am Soc Nephrol*. 2012;**23**(8):1299-302.

47. Mallat NS, Musallam KM, Mallat SG, Ziyadeh FN, Koussa S, Taher AT. End stage renal disease in six patients with beta-thalassemia intermedia. *Blood Cells Mol Dis*. 2013;**51**(3):146-8.

48. Coffey R, Ganz T. Erythroferrone: An Erythroid Regulator of Hepcidin and Iron Metabolism. *Hemasphere*. 2018;**2**(2):e35.

49. Camaschella C, Nai A. Ineffective erythropoiesis and regulation of iron status in iron loading anaemias. *Br J Haematol*. 2015.

50. Rivella S. beta-thalassemias: paradigmatic diseases for scientific discoveries and development of innovative therapies. *Haematologica*. 2015;**100**(4):418-30.

51. Turpaev KT. Reactive oxygen species and regulation of gene expression. *Biochemistry (Mosc)*. 2002;**67**(3):281-92.

52. Droge W. Free radicals in the physiological control of cell function. *Physiol Rev*. 2002;**82**(1):47-95.

53. Blackstone NW, Kelly MM, Haridas V, Gutterman JU. Mitochondria as integrators of information in an early-evolving animal: insights from a triterpenoid metabolite. *Proc Biol Sci*. 2005;**272**(1562):527-31.

54. van der Heyde HC, Gu Y, Zhang Q, Sun G, Grisham MB. Nitric oxide is neither necessary nor sufficient for resolution of Plasmodium chabaudi malaria in mice. *J Immunol*. 2000;**165**(6):3317-23.

55. Holm E, Hildebrandt W, Kinscherf R, Droge W. Low postabsorptive net protein degradation in male cancer patients: lack of sensitivity to regulatory amino acids? *Oncol Rep*. 2007;**17**(3):695-700.

56. Scheiber MN, Watson PM, Rumboldt T, Stanley C, Wilson RC, Findlay VJ, et al. FLI1 expression is correlated with breast cancer cellular growth, migration, and invasion and altered gene expression. *Neoplasia*. 2014;**16**(10):801-13.

57. Sies H, Cadenas E. Oxidative stress: damage to intact cells and organs. *Philos Trans R Soc Lond B Biol Sci*. 1985;**311**(1152):617-31.

58. Mullarkey CJ, Edelstein D, Brownlee M. Free radical generation by early glycation products: a mechanism for accelerated atherogenesis in diabetes. *Biochem Biophys Res Commun*. 1990;**173**(3):932-9.

59. Du G, Willet K, Mouithys-Mickalad A, Sluse-Goffart CM, Droy-Lefaix MT, Sluse FE. EGb 761 protects liver mitochondria against injury induced by in vitro anoxia/reoxygenation. *Free Radic Biol Med*. 1999;**27**(5-6):596-604.

60. Nishikawa T, Edelstein D, Brownlee M. The missing link: a single unifying mechanism for diabetic complications. *Kidney Int Suppl*. 2000;**77**:S26-30.

61. Niedowicz DM, Daleke DL. The role of oxidative stress in diabetic complications. *Cell Biochem Biophys*. 2005;**43**(2):289-330.

62. Baynes JW, Thorpe SR. Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. *Diabetes*. 1999;**48**(1):1-9.

63. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev*. 2007;**87**(1):245-313.

64. Cheng G, Cao Z, Xu X, van Meir EG, Lambeth JD. Homologs of gp91phox: cloning and tissue expression of Nox3, Nox4, and Nox5. *Gene*. 2001;**269**(1-2):131-40.

65. Kikuchi H, Hikage M, Miyashita H, Fukumoto M. NADPH oxidase subunit, gp91(phox) homologue, preferentially expressed in human colon epithelial cells. *Gene*. 2000;**254**(1-2):237-43.

66. Reinehr R, Becker S, Eberle A, Grether-Beck S, Haussinger D. Involvement of NADPH oxidase isoforms and Src family kinases in CD95-dependent hepatocyte apoptosis. *J Biol Chem*. 2005;**280**(29):27179-94.

67. Lee YS, Kang YS, Lee JS, Nicolova S, Kim JA. Involvement of NADPH oxidase-mediated generation of reactive oxygen species in the apototic cell death by capsaicin in HepG2 human hepatoma cells. *Free Radic Res*. 2004;**38**(4):405-12.

68. Herrera B, Murillo MM, Alvarez-Barrientos A, Beltran J, Fernandez M, Fabregat I. Source of early reactive oxygen species in the apoptosis induced by transforming growth factor-beta in fetal rat hepatocytes. *Free Radic Biol Med*. 2004;**36**(1):16-26.

69. Bachmann S, Ramasubbu K. Immunohistochemical colocalization of the alpha-subunit of neutrophil NADPH oxidase and ecto-5'-nucleotidase in kidney and liver. *Kidney Int*. 1997;**51**(2):479-82.

70. Bataller R, Schwabe RF, Choi YH, Yang L, Paik YH, Lindquist J, et al. NADPH oxidase signal transduces angiotensin II in hepatic stellate cells and is critical in hepatic fibrosis. *J Clin Invest*. 2003;**112**(9):1383-94.

71. Adachi T, Togashi H, Suzuki A, Kasai S, Ito J, Sugahara K, et al. NAD(P)H oxidase plays a crucial role in PDGF-induced proliferation of hepatic stellate cells. *Hepatology*. 2005;**41**(6):1272-81.

72. Hines IN, Hoffman JM, Scheerens H, Day BJ, Harada H, Pavlick KP, et al. Regulation of postischemic liver injury following different durations of ischemia. *Am J Physiol Gastrointest Liver Physiol*. 2003;**284**(3):G536-45.

73. Rusyn I, Yamashina S, Segal BH, Schoonhoven R, Holland SM, Cattley RC, et al. Oxidants from nicotinamide adenine dinucleotide phosphate oxidase are involved in triggering cell proliferation in the liver due to peroxisome proliferators. *Cancer Res*. 2000;**60**(17):4798-803.

74. Carmiel-Haggai M, Cederbaum AI, Nieto N. A high-fat diet leads to the progression of non-alcoholic fatty liver disease in obese rats. *FASEB J*. 2005;**19**(1):136-8.

75. Shibuya H, Ohkohchi N, Seya K, Satomi S. Kupffer cells generate superoxide anions and modulate reperfusion injury in rat livers after cold preservation. *Hepatology*. 1997;**25**(2):356-60.

76. Harada H, Hines IN, Flores S, Gao B, McCord J, Scheerens H, et al. Role of NADPH oxidase-derived superoxide in reduced size liver ischemia and reperfusion injury. *Arch Biochem Biophys*. 2004;**423**(1):103-8.

77. Rendic S, Di Carlo FJ. Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors. *Drug Metab Rev*. 1997;**29**(1-2):413-580.

78. Bondy SC, Naderi S. Contribution of hepatic cytochrome P450 systems to the generation of reactive oxygen species. *Biochem Pharmacol*. 1994;**48**(1):155-9.

79. Puntarulo S, Cederbaum AI. Production of reactive oxygen species by microsomes enriched in specific human cytochrome P450 enzymes. *Free Radic Biol Med*. 1998;**24**(7-8):1324-30.

80. Fleming I, Michaelis UR, Bredenkotter D, Fisslthaler B, Dehghani F, Brandes RP, et al. Endothelium-derived hyperpolarizing factor synthase (Cytochrome P450 2C9) is a functionally significant source of reactive oxygen species in coronary arteries. *Circ Res*. 2001;**88**(1):44-51.

81. Dunn KM, Renic M, Flasch AK, Harder DR, Falck J, Roman RJ. Elevated production of 20-HETE in the cerebral vasculature contributes to severity of ischemic stroke and oxidative stress in spontaneously hypertensive rats. *Am J Physiol Heart Circ Physiol*. 2008;**295**(6):H2455-65.

82. Medhora M, Chen Y, Gruenloh S, Harland D, Bodiga S, Zielonka J, et al. 20-HETE increases superoxide production and activates NAPDH oxidase in pulmonary artery endothelial cells. *Am J Physiol Lung Cell Mol Physiol*. 2008;**294**(5):L902-11.

83. Capdevila JH, Falck JR. Biochemical and molecular characteristics of the cytochrome P450 arachidonic acid monooxygenase. *Prostaglandins Other Lipid Mediat*. 2000;**62**(3):271-92.

84. Capdevila JH, Falck JR, Harris RC. Cytochrome P450 and arachidonic acid bioactivation. Molecular and functional properties of the arachidonate monooxygenase. *J Lipid Res*. 2000;**41**(2):163-81.

85. Roman RJ, Maier KG, Sun CW, Harder DR, Alonso-Galicia M. Renal and cardiovascular actions of 20-hydroxyeicosatetraenoic acid and epoxyeicosatrienoic acids. *Clin Exp Pharmacol Physiol*. 2000;**27**(11):855-65.

86. Maier KG, Roman RJ. Cytochrome P450 metabolites of arachidonic acid in the control of renal function. *Curr Opin Nephrol Hypertens*. 2001;**10**(1):81-7.

87. Zeldin DC. Epoxygenase pathways of arachidonic acid metabolism. *J Biol Chem*. 2001;**276**(39):36059-62.

88. Imig JD. Epoxygenase metabolites. Epithelial and vascular actions. *Mol Biotechnol*. 2000;**16**(3):233-51.

89. Powell PK, Wolf I, Jin R, Lasker JM. Metabolism of arachidonic acid to 20-hydroxy-5,8,11, 14-eicosatetraenoic acid by P450 enzymes in human liver: involvement of CYP4F2 and CYP4A11. *J Pharmacol Exp Ther*. 1998;**285**(3):1327-36.

90. Roman RJ. P-450 metabolites of arachidonic acid in the control of cardiovascular function. *Physiol Rev*. 2002;**82**(1):131-85.

91. Wu CC, Gupta T, Garcia V, Ding Y, Schwartzman ML. 20-HETE and blood pressure regulation: clinical implications. *Cardiol Rev*. 2014;**22**(1):1-12.

92. Fan F, Roman RJ. Effect of Cytochrome P450 Metabolites of Arachidonic Acid in Nephrology. *J Am Soc Nephrol*. 2017;**28**(10):2845-55.

93. Lasker JM, Chen WB, Wolf I, Bloswick BP, Wilson PD, Powell PK. Formation of 20-hydroxyeicosatetraenoic acid, a vasoactive and natriuretic eicosanoid, in human kidney. Role of Cyp4F2 and Cyp4A11. *J Biol Chem*. 2000;**275**(6):4118-26.

94. Kalsotra A, Strobel HW. Cytochrome P450 4F subfamily: at the crossroads of eicosanoid and drug metabolism. *Pharmacol Ther*. 2006;**112**(3):589-611.

95. Proctor KG, Falck JR, Capdevila J. Intestinal vasodilation by epoxyeicosatrienoic acids: arachidonic acid metabolites produced by a cytochrome P450 monooxygenase. *Circ Res*. 1987;**60**(1):50-9.

96. Carroll MA, Schwartzman M, Capdevila J, Falck JR, McGiff JC. Vasoactivity of arachidonic acid epoxides. *Eur J Pharmacol*. 1987;**138**(2):281-3.

97. Gebremedhin D, Ma YH, Falck JR, Roman RJ, VanRollins M, Harder DR. Mechanism of action of cerebral epoxyeicosatrienoic acids on cerebral arterial smooth muscle. *Am J Physiol*. 1992;**263**(2 Pt 2):H519-25.

98. Hu S, Kim HS. Activation of K+ channel in vascular smooth muscles by cytochrome P450 metabolites of arachidonic acid. *Eur J Pharmacol*. 1993;**230**(2):215-21.

99. Node K, Huo Y, Ruan X, Yang B, Spiecker M, Ley K, et al. Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. *Science*. 1999;**285**(5431):1276-9.

100. Campbell WB. New role for epoxyeicosatrienoic acids as anti-inflammatory mediators. *Trends Pharmacol Sci*. 2000;**21**(4):125-7.

101. Zeldin DC, Liao JK. Reply: cytochrome P450-derived eicosanoids and the vascular wall. *Trends Pharmacol Sci*. 2000;**21**(4):127-8.

102. Jiang JG, Ning YG, Chen C, Ma D, Liu ZJ, Yang S, et al. Cytochrome p450 epoxygenase promotes human cancer metastasis. *Cancer Res*. 2007;**67**(14):6665-74.

103. Pozzi A, Popescu V, Yang S, Mei S, Shi M, Puolitaival SM, et al. The anti-tumorigenic properties of peroxisomal proliferator-activated receptor alpha are arachidonic acid epoxygenase-mediated. *J Biol Chem*. 2010;**285**(17):12840-50.

104. Davis CM, Liu X, Alkayed NJ. Cytochrome P450 eicosanoids in cerebrovascular function and disease. *Pharmacol Ther*. 2017;**179**:31-46.

105. Capdevila JH. Regulation of ion transport and blood pressure by cytochrome p450 monooxygenases. *Curr Opin Nephrol Hypertens*. 2007;**16**(5):465-70.

106. Imig JD. Epoxyeicosatrienoic Acids and 20-Hydroxyeicosatetraenoic Acid on Endothelial and Vascular Function. *Adv Pharmacol*. 2016;**77**:105-41.

107. Chen XW, Yu TJ, Zhang J, Li Y, Chen HL, Yang GF, et al. CYP4A in tumor-associated macrophages promotes pre-metastatic niche formation and metastasis. *Oncogene*. 2017;**36**(35):5045-57.

108. Sacerdoti D, Gatta A, McGiff JC. Role of cytochrome P450-dependent arachidonic acid metabolites in liver physiology and pathophysiology. *Prostaglandins Other Lipid Mediat*. 2003;**72**(1-2):51-71.

109. Antoun J, Goulitquer S, Amet Y, Dreano Y, Salaun JP, Corcos L, et al. CYP4F3B is induced by PGA1 in human liver cells: a regulation of the 20-HETE synthesis. *J Lipid Res*. 2008;**49**(10):2135-41.

110. Plee-Gautier E, Antoun J, Goulitquer S, Le Jossic-Corcos C, Simon B, Amet Y, et al. Statins increase cytochrome P450 4F3-mediated eicosanoids production in human liver cells: a PXR dependent mechanism. *Biochem Pharmacol*. 2012;**84**(4):571-9.

111. Sacerdoti D, Balazy M, Angeli P, Gatta A, McGiff JC. Eicosanoid excretion in hepatic cirrhosis. Predominance of 20-HETE. *J Clin Invest*. 1997;**100**(5):1264-70.

112. Rachmilewitz EA, Peisach J, Bradley TB, Blumberg WE. Role of haemichromes in the formation of inclusion bodies in haemoglobin H disease. *Nature*. 1969;**222**(5190):248-50.

113. Schrier SL, Centis F, Verneris M, Ma L, Angelucci E. The role of oxidant injury in the pathophysiology of human thalassemias. *Redox Rep*. 2003;**8**(5):241-5.

114. Fibach E, Dana M. Oxidative Stress in beta-Thalassemia. *Mol Diagn Ther*. 2019;**23**(2):245-61.

115. Cabantchik ZI. Labile iron in cells and body fluids: physiology, pathology, and pharmacology. *Front Pharmacol*. 2014;**5**:45.

116. Valko M, Jomova K, Rhodes CJ, Kuca K, Musilek K. Redox- and non-redox-metal-induced formation of free radicals and their role in human disease. *Arch Toxicol*. 2016;**90**(1):1-37.

117. Hershko CM, Link GM, Konijn AM, Cabantchik ZI. Iron chelation therapy. *Curr Hematol Rep*. 2005;**4**(2):110-6.

118. Fibach E, Rachmilewitz EA. The role of antioxidants and iron chelators in the treatment of oxidative stress in thalassemia. *Ann N Y Acad Sci*. 2010;**1202**:10-6.

119. Clanton TL. Hypoxia-induced reactive oxygen species formation in skeletal muscle. *J Appl Physiol (1985)*. 2007;**102**(6):2379-88.

120. Turrens JF. Mitochondrial formation of reactive oxygen species. *J Physiol*. 2003;**552**(Pt 2):335-44.

121. Amer J, Goldfarb A, Fibach E. Flow cytometric measurement of reactive oxygen species production by normal and thalassaemic red blood cells. *Eur J Haematol*. 2003;**70**(2):84-90.

122. Rice-Evans C, Omorphos SC, Baysal E. Sickle cell membranes and oxidative damage. *Biochem J*. 1986;**237**(1):265-9.

123. Asakura T, Onishi T, Friedman S, Schwartz E. Abnormal precipitation of oxyhemoglobin S by mechanical shaking. *Proc Natl Acad Sci U S A*. 1974;**71**(5):1594-8.

124. Chiu D, Kuypers F, Lubin B. Lipid peroxidation in human red cells. *Semin Hematol*. 1989;**26**(4):257-76.

125. Ideguchi H. [Effects of abnormal Hb on red cell membranes]. *Rinsho Byori*. 1999;**47**(3):232-7.

126. Tesoro A, Novakovic J, Thiessen JJ, Spino M. Validated HPLC assay for iron determination in biological matrices based on ferrioxamine formation. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2005;**823**(2):177-83.

127. Eid AA, Gorin Y, Fagg BM, Maalouf R, Barnes JL, Block K, et al. Mechanisms of podocyte injury in diabetes: role of cytochrome P450 and NADPH oxidases. *Diabetes*. 2009;**58**(5):1201-11.

128. Fibach E, Rachmilewitz E. The role of oxidative stress in hemolytic anemia. *Curr Mol Med*. 2008;**8**(7):609-19.

129. Fibach E, Rachmilewitz EA. Iron overload in hematological disorders. *Presse Med*. 2017;**46**(12 Pt 2):e296-e305.

130. Zhang B, Lai G, Liu X, Zhao Y. Alteration of epoxyeicosatrienoic acids in the liver and kidney of cytochrome P450 4F2 transgenic mice. *Mol Med Rep*. 2016;**14**(6):5739-45.

131. Imig JD, Hammock BD. Soluble epoxide hydrolase as a therapeutic target for cardiovascular diseases. *Nat Rev Drug Discov*. 2009;**8**(10):794-805.

132. McCarthy ET, Sharma R, Sharma M. Protective effect of 20-hydroxyeicosatetraenoic acid (20-HETE) on glomerular protein permeability barrier. *Kidney Int*. 2005;**67**(1):152-6.

133. Prus E, Fibach E. Effect of iron chelators on labile iron and oxidative status of thalassaemic erythroid cells. *Acta Haematol*. 2010;**123**(1):14-20.

134. Engelhardt JF. Redox-mediated gene therapies for environmental injury: approaches and concepts. *Antioxid Redox Signal*. 1999;**1**(1):5-27.

135. Kaltschmidt B, Sparna T, Kaltschmidt C. Activation of NF-kappa B by reactive oxygen intermediates in the nervous system. *Antioxid Redox Signal*. 1999;**1**(2):129-44.

136. Sen CK, Packer L. Antioxidant and redox regulation of gene transcription. *FASEB J*. 1996;**10**(7):709-20.

137. Shackelford RE, Kaufmann WK, Paules RS. Oxidative stress and cell cycle checkpoint function. *Free Radic Biol Med*. 2000;**28**(9):1387-404.

138. Kim-Shapiro DB, Schechter AN, Gladwin MT. Unraveling the reactions of nitric oxide, nitrite, and hemoglobin in physiology and therapeutics. *Arterioscler Thromb Vasc Biol*. 2006;**26**(4):697-705.

139. Mitchinson MJ, Stephens NG, Parsons A, Bligh E, Schofield PM, Brown MJ. Mortality in the CHAOS trial. *Lancet*. 1999;**353**(9150):381-2.

140. Rapola JM, Virtamo J, Ripatti S, Huttunen JK, Albanes D, Taylor PR, et al. Randomised trial of alpha-tocopherol and beta-carotene supplements on incidence of major coronary events in men with previous myocardial infarction. *Lancet*. 1997;**349**(9067):1715-20.