UNIVERSITY OF TURIN

Phd School in life and Health Sciences
Molecular Medicine

XXXII Cycle
Academic Years: 2016-2020

Assessment of 4-hydroxynonenal conjugates as blood oxidative stress markers in malaria anemia

Tutor: Evelin Schwarzer

Candidate: Rosalba La Grotta

Coordinator: Prof. Francesco Novelli

Table of Contents

List of abbreviations 4
CHAPTER 1 8
Introduction 8
1.1 Pathogenic factors in malaria 8
1.1.1 The malaria parasite P. falciparum 9
1.1.2 Malaria pigment hemozoin 12
1.2 Parasite elicited oxidative stress and lipoperoxidation in the host 18
1.2.1 Lipoperoxidation product 4-hydroxynonenal: A powerful reactive aldehyde 19
1.2.2 Malaria and 4-hydroxynonenal 25
1.2.3 Hemoglobin oxidation and formation of hemichromes in the host cells 28
1.3 Anemia in malaria-endemic regions and malaria anemia 31
1.3.1 Pathomechanism of malaria anemia 32
1.3.2 Non-malarial anemia forms in malaria regions 40
1.3.3 Oxidative modifications in RBC membranes from anemic patients. State of the art 43
1.4 Objectives 45
CHAPTER 2 47
Materials and methods 47
2.1 Analysis of RBC membrane modifications of anemic children 47
2.1.1 Patients 47
2.1.2 Membrane protein analysis of RBC 48
2.1.2.1 Preparation of membranes from fresh and frozen RBC 48
2.1.2.2 Protein quantification 48
2.1.2.3 SDS-PAGE 49
2.1.2.4 4-hydroxynonenal conjugated protein analysis by western blot 49
2.1.3 Measurement of hemoglobin (Hb) and heme bound to RBC membranes 50
2.1.3.1 Solubilization of ghosts 50
2.1.3.2 Luminescence assay 50
2.1.4 Statistical methods 51
2.2 Lipoperoxidation as function of RBC senescence 52
2.2.1 Density separation of human RBC 52
2.2.1.1 White blood cells and thrombocytes deprivation from whole blood by cellulose 52
2.2.1.2 Determination of hematocrit 52
2.2.1.3 Senescence- dependent fractionation of RBC on percoll gradient 52
2.2.2 Physical parameters of RBC fractions assessed by FACS 53

2.2.3 Senescence parameters of RBC fractions assessed by FACS 53
2.2.4 Reticulocyte count in RBC fractions by Brilliant Cresyl Blue staining 55
2.2.5 4-hydroxynonenal conjugates in intact RBC of different senescence stages by FACS analysis 55
2.2.6 4-hydroxynonenal conjugates in isolated RBC membranes assessed by immunochemistry 55
2.2.6.1 Electrophoretic separation of ghost proteins 56
2.2.6.2 Analysis of 4-HNE- conjugated membrane proteins by western blot 56
2.2.7 Statistical methods 56
CHAPTER 3 57
Results 57
3.1 Correlation of blood hemoglobin with membrane-bound heme and 4-hydroxynonenal-protein conjugates in RBC of infants 57
3.1.1 Impact of RBC storage on membrane-bound heme and 4-hydroxynonenal-protein conjugates in RBC 57
3.1.2 Correlation between RBC membrane protein-bound 4-hydroxynonenal and blood hemoglobin in study participants 60
3.1.3 Membrane-bound Hb and hemichromes in analyzed RBC from study participants 63
3.2 Increased 4-hydroxynonenal-conjugate levels in moderate and severe anemia 66
3.3 Increased 4-hydroxynonenal-conjugates in malaria anemia and non-malaria anemia 67
3.4 Correlation between 4-hydroxynonenal conjugation levels in RBC and blood parameters 69
3.4.1 Correlation of 4-hydroxynonenal-protein conjugates with parasite count 70
3.4.2 Correlation of 4-hydroxynonenal-protein conjugates with C-reactive protein 72
3.4.3 Correlation of 4-hydroxynonenal-protein conjugates with plasma iron and soluble transferrin receptor 76
3.4.4 Correlation of 4-hydroxynonenal-protein conjugates with Vitamin A 79
3.4.5 Correlation of 4-hydroxynonenal-protein conjugates with erythropoietin 82
3.4.6 Correlation of 4-hydroxynonenal-protein conjugates with lactate dehydrogenase activity 85
3.5 Mechanistic role of 4-hydroxynonenal-conjugation in anemia 88
3.5.1 4-hydroxynonenal-membrane protein conjugates in RBC of different cell age analyzed by FACS without prior cell separation 88
3.5.2 4-hydroxynonenal-membrane protein conjugates in RBC subpopulations of different cell age 90
3.5.2.1 Physical parameters, reticulocyte count and surface antigen expression characterize cell age of density fractionated RBC 90
3.5.2.2 Impact of cell age on 4-hydroxynonenal-binding to intact RBC surface proteins 93
3.5.2.3 Impact of cell age on 4-hydroxynonenal-membrane protein conjugates of RBC membrane extracts 96

3.5.3 4-hydroxynonenal- conjugation of membrane skeleton protein spectrin increases in senescent RBC 98
3.5.4 4-hydroxynonenal-conjugation of membrane skeleton protein spectrin in RBC of anemic children 100
CHAPTER 4 101
Discussion 101
References 114
Acknowledgements 125

List of abbreviations

3-AT	3-amino-1,2,4-triazole
4-HNE	4-hydroxynonenal
ACTs	Artemisinin-based combination therapies
AU	Arbitrary units
BSA	Bovine serum albumin
CD235a	Glycophorin A
CD47	Integrin associated protein
CD71	Transferrin receptor
CRP	C-reactive protein
DC	Dendritic cell
Epo	Erythropoietin
FACS	Fluorescence Activated Cell Sorting
FITC	Fluorescein isothiocyanate
G6PD	Glucose-6-phosphate-dehydrogenase
Hb	Hemoglobin
Hct	Hematocrit
HRP	Horseradish peroxidase
HZ	Hemozoin
ID	Iron deficiency
IgG	Immunoglobulin G
LDH	Lactate dehydrogenase
MDA	Malondialdehyde
NAC	N-acetyl-L-cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
nHZ	Natural hemozoin
npRBC	Not parasitized RBC
OD	Optical density

P.	*Plasmodium*
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBS	Phosphate buffered saline
PBS-G	Phosphate buffered saline-supplemented with glucose
PBS-G-FCS	Phosphate buffered saline-glucose-fetal calf serum
Pf	*P. falciparum*
PfEMP1	*P. falciparum* erythrocyte membrane protein 1
PKC	Protein kinase C
pRBC	Parasitized RBC
PS	Phosphatidylserine
PUFAs	Polyunsaturated fatty acids
RB	Residual body
RBC	Red blood cell/s
ROS	Reactive oxygen species
RpRBC	Ring-parasitized RBC
RT	Room temperature
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
sHZ	Synthetic HZ
sTfR	Soluble transferrin receptor
TBARS	Thiobarbituric acid reactive substances
TEMED	Tetramethyl ethylenediamine
TNF-α	Tumor necrosis factor-α
TpRBC	Trophozoite-parasitized RBC
WHO	World Health Organization

“Coloro che amiamo e che abbiamo perduto

non sono più dove erano

ma sono ovunque noi siamo”.

Alla mia adorata nonna Margherita,

che manca tanto ma è nel mio cuore,

sempre.

CHAPTER 1
Introduction

1.1 Pathogenic factors in malaria

Malaria is a global health problem and caused 219 million of new cases in 2017 and nearly 450000 deaths. The ten most burdened African countries saw an estimated 3.5 million more cases than in 2016 (WHO, 2018).

Malaria is a parasitic infection caused by *Plasmodium (P.)* species and transmitted by the Anopheles mosquito that leads to acute potentially life-threatening disease and poses a significant global health threat (Buck and Finnigan, 2019).

There are five *Plasmodium* species that can infect humans: *P. falciparum, P. vivax, P. malariae, P. ovale curtisii* and *P. ovale wallikeri,* and *P. knowlesi*.

The global burden of human malaria is caused almost exclusively by two species of the parasite, *P. falciparum* and *P. vivax*. For long, research efforts have largely been focused on *P. falciparum* malaria because of its higher mortality rates, especially in Africa. The high mortality, is mainly caused by the severe forms of malaria, malaria anemia and cerebral malaria (Hay et al., 2005).

Recently, vivax malaria has re-emerged in regions formerly considered malaria free (Severini et al., 2004) (Kim et al., 2009) (Bitoh et al., 2011).

Worldwide, about 2.85 billion people have been estimated to be at risk of infection by *P. vivax* (Price et al., 2007) (Guerra et al., 2010) (Battle et al., 2012) (Gething et al., 2012), is more spread than P. falciparum and represents a major public health threat affecting populous regions in Asia, the horn of Africa, and Central and South America (Gething et al., 2012).

It can cause a wide spectrum of clinical manifestation, ranging from benign to severe and even fatal anemia, mainly in children (Genton et al., 2008) (Tjitra et al., 2008) and pregnant women. (Rodriguez-Morales et al., 2006).

Malaria due to *P. ovale curtisii, P. ovale wallikeri*, and *P. malariae* are much less common while *P. knowlesi* has become an important cause of malaria in Southeast Asia. *P. knowlesi* is predominantly a zoonotic parasite of South-east Asian macaques, with yet no definitive evidence for primary human-to-human transmission (Ahmed and Cox-Singh, 2015) (Leclerc et al., 2004). Nevertheless, *Plasmodium falciparum* is responsible for 99.7% of all malaria cases in the World Health Organization (WHO)(WHO, 2017) African region, and accounted for 93% of all malarial deaths in 2017. The occurrence of *falciparum* resistance against the common antimalarial drugs and the vector resistance against insecticides motivate further studies of the pathomechanisms behind severe malaria, such as malaria anemia caused by falciparum.

One of the greatest obstacles in the control of malaria has been the spread of drug resistance almost worldwide. Currently, the typical treatment of uncomplicated malaria in regions where malaria infection remains widespread, consists of artemisinin-based combination therapies (ACTs), while chloroquine combined with primaquine is the treatment of choice for chloroquine-sensitive infections. For the treatment of severe malaria there are two classes of drugs available: the cinchona alkaloids (quinine and quinidine) and artemisinin derivatives (artesunate and artemether) (Organization, 2015) The incredible dissemination in the world of Plasmodium falciparum is also due to its resistance to this principle pharmacological approach.

Thus, considering the high incidence and mortality of *P. falciparum* malaria and the difficulty to treat severe falciparum malaria with specific drugs, I decided to focus on this particular plasmodium species.

1.1.1 The malaria parasite P. falciparum

For human malaria, pathogenesis is very complex and it involves many players, settings, and potential outcomes.

The Plasmodium life cycle takes place in two different hosts: the definitive host or vector, which is the mosquito of the genus Anopheles, and a vertebrate intermediate host, such as the human (Cowman et al., 2017).

The real success of the malaria parasite is its transition without interruption from mosquito to human to mosquito, not causing particular damage to either the carrier or the guest.

After infection of a host, the parasite undergoes a period of replication in the liver. Here, sporozoites inoculated by mosquitos invade hepatocytes, and become liver merozoites. Liver merozoites are released into the bloodstream where they invade RBC. They develop through ring, trophozoite, and schizont stages replicating to produce 16–32 merozoites that are released from the host RBC after schizogony. The free merozoites invade new erythrocytes to continue the asexual blood-stage life cycle. Some intraerythrocytic parasites develop into sexual forms, the male microgametocyte and the female macrogametocytes. These are taken up by the mosquito during feeding and develop into gametes in the insect gut and fuse to form zygotes. The zygote develops to an invasive ookinete, which traverses the midgut and transforms into an oocyst, from which sporozoites are released that migrate to the salivary glands for injection into a new human host during the next blood meal.

Merozoites in the human host have a single purpose: to invade erythrocytes. This is also evident from their morphology: they are polarized cells that in the apical part contain particular structures some of which are responsible for binding to the membrane of the erythrocyte, others instead to form the parasitophorous vacuole, the membrane-surrounded compartment within the host cell cytosol wherein merozoites develop to mature parasite stages that divide to have new daughter cells.

In the parasitophorous vacuole, proteolytic processes take place to facilitate merozoite egress.

Despite differences in host cell preference, length of the intraerythrocytic lifecycle and the number of progeny produced, the basics of the intraerythrocytic lifecycle of the species are very similar: after binding to an erythrocyte, the parasite invades the host cell by pushing itself into the host cell, causing an indentation in the membrane of the host (Sherling and van Ooij, 2016).

To survive, the malaria parasite has to induce fundamental changes inside the host cell. The principle of these changes is the formation of membranous compartments (exomembrane system). Once the parasite enters the erythrocyte, it hooks onto its membrane and does not disengage until it has formed a system of membranes around itself. Only when the parasite is enveloped by the membrane which is called parasitophorous vacuole membrane, it detaches from the RBC membrane and can move into the erythrocyte cytosol.

Phenotype changes of the parasitized RBC take place as parasite proteins are transported into the host cell, reshaping it according the parasite's needs (Miller, 1969). For example, the increased adherence of infected RBC to the endothelium, which removes mature parasites from the bloodstream, is due to the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (Leech et al., 1984) (Baruch et al., 1995) (Smith et al., 1995) (Su et al., 1995). This is a parasite protein that migrates to erythrocyte surface and is part of the knob system.

PfEMP1 are variant surface antigens which owing to their hypervariability, enable the parasite to evade host immune system. PfEMP1 also bind to various host-receptors such as CD36, ICAM, thrombospondin, chondroitin sulphate A, endothelial protein C receptor, complement receptor CR1, etc. thereby causing infected RBC to cytoadhere. Cytoadhesion to endothelial cells enables infected RBC to get sequestered in deep microvasculature, thereby, avoiding splenic clearance.; Cytoadhesion to not parasitized RBC (npRBC), also called rosetting, makes it easy for merozoites to quickly invade new host cells. Therefore, knobs are central to the virulence of *P. falciparum* and may contribute to increased phagocytosis of npRBC and anemia.

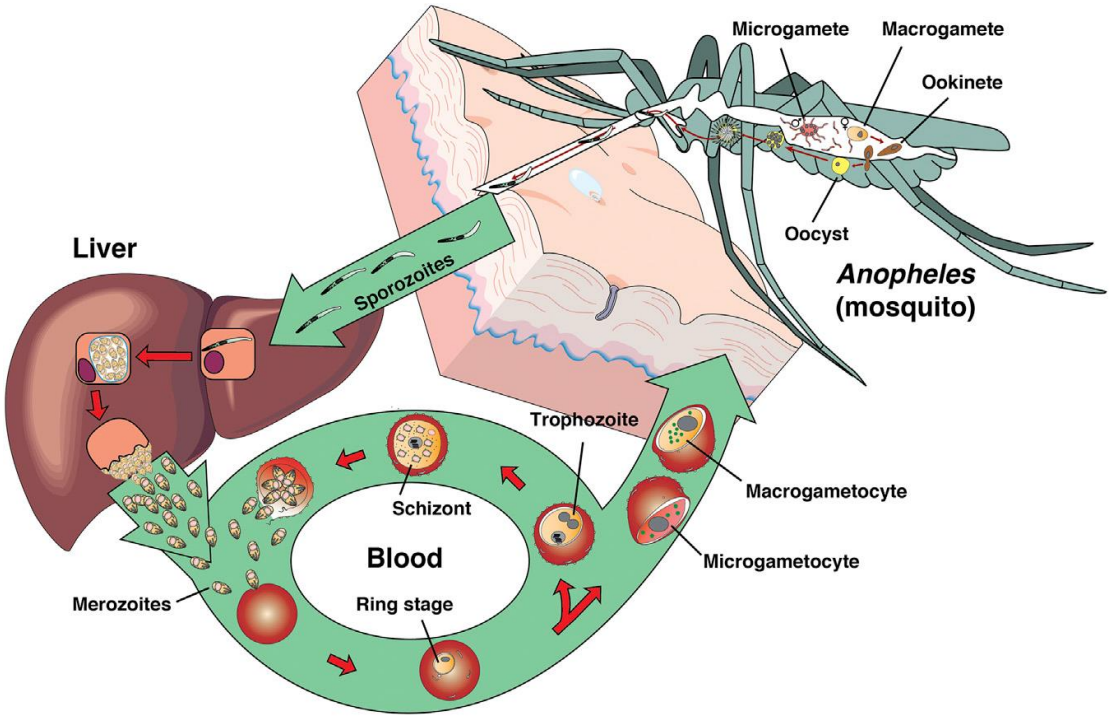


Figure 1. Life cycle of P. falciparum in the human host and mosquito vector. Asexual replication takes place in RBC of the human host while sexual replication occurs in the mosquito vector (Cowman et al., 2017).

The main clinical symptoms of malaria are caused by the 48h lasting intra-erythrocytic asexual development of the parasite, during which one merozoite-stage parasite gives rise to a progeny of about 16–32 merozoites. Most of that time is spent at the trophozoite stage, during which the parasite will grow to fill up to 50% of the volume of RBC, digesting the host cell cytoplasm (Krugliak et al., 2002). In the last 12–15 h parasite proceeds to multinucleated schizont stage and differentiation and release of new merozoites.

The success of the parasite infection is due to its ability to remodel its host erythrocyte (RBC) by exporting PfEMP1 to the RBC surface. PfEMP1 is an adhesin that mediates the adhesion of infected RBC to host cells by interacting with a variety of host cell surface receptors, and is anchored at the RBC surface in parasite-induced protuberances called knobs. However, export of parasite proteins to the host RBC is not restricted to the knob components. Indeed, an increasing number of exported parasite proteins, as well as parasite-hijacked host cell proteins, have been identified that might be essential for the parasite growth, survival and dissemination.

1.1.2 Malaria pigment hemozoin

Beside exporting own proteins into the host cell and its membrane, the parasite engulfs and digests the major part of host RBC hemoglobin (Hb) and polymerizes Hb-heme to hemozoin (HZ), the malaria pigment. Hb is composed of a protein portion (globin chains) and the heme group (a complex porphyrin ring with a central iron atom which binds oxygen and serves for gas exchange during breathing. Malaria parasites take up Hb from RBC by the cytostome and transport Hb-containing vesicles across their cytosol into a specialized membrane surrounded acidic organelle, the digestive or food vacuole. The pH of the vacuole is maintained between 5.0 - 5.4 by an ATPase pump that creates a proton gradient. In the acidic pH of the digestive vacuole, specific proteases (plasmepsins I and II and falcipain) degrade the globin chains to peptides and amino acids that are used by the parasite for protein synthesis, while the released heme remains indigestible. The latter is toxic to the parasite and for this reason the detoxification of heme is crucial for the survival of the parasite (Meshnick, 2002). The presence of iron (II) within heme and the acidic environment in the parasitic vacuole, allows the formation of reactive oxygen species (ROS) thanks to the Fenton reaction, especially hydroxyl radicals that could lead to membrane lipid peroxidation (Kirschner-Zilber et al., 1982) (Vincent, 1989). In absence of heme-oxygenase activity in plasmodium heme is detoxified by hemozoin formation in the food vacuole of developing intra-erythrocytic parasites. Heme released during digestion of Hb-globin forms dimers, the basic unit for beta-hematin formation. Heme molecules are linked into dimers through reciprocal iron–carboxylate bonds to one of the propionic side chains of each porphyrin, and the dimers form chains linked by hydrogen bonds in the crystalline beta-hematin. The growth of the beta-hematin crystal is most likely aided by lipids. Thus, beta-hematin is the core of hemozoin and is complexed with lipids and proteins (Pagola et al., 2000). (Pisciotta et al., 2007). The detoxification of heme starts with the self-oxidation of Fe (II) in the heme group into Fe (III) to form potentially toxic hydroxy ferriprotoporphyrin IX (alpha-hematin, HO-Fe (III) PPIX) and continues with the formation of highly insoluble brown crystals, the beta-hematin, which is the core compound in malaria pigment or hemozoin (Figure 2).

These three terms, beta-hematin, malaria pigment and hemozoin are often used as synonyms in the older but also recent scientific literature despite the presence of organic compounds such as lipids and proteins additionally to beta-hematin in naturally occurring hemozoin (nHZ). A laxity in usage of these terms often causes confusion and misinterpretations of experimental results obtained with HZ of different composition.

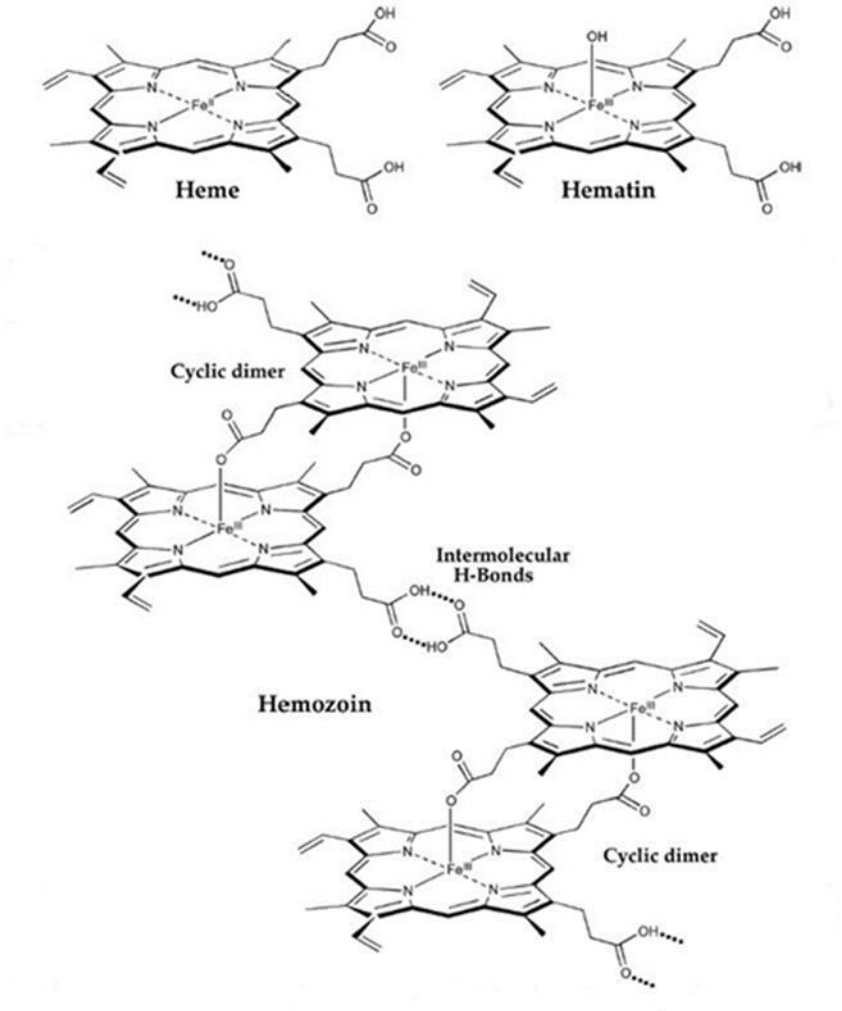


Figure 2. Chemical structure of heme, hematin and beta-hematin.

The exact mechanism underlying the formation of hemozoin are still unclear both *in vitro* and *in vivo* despite the numerous studies that led to various assumptions on its formation: spontaneous, auto-catalyzed, enzyme-catalyzed or lipid-catalyzed (Vanderesse et al., 2016).

The hypothesis of spontaneous formation derives from *in vitro* studies conducted in acetate solution, which shows that β-hematin can be formed at a moderate low pH comparable to the acidic digestive food vacuole (Orjih et al., 2012).

The most recent and widely accepted view on the synthesis of beta-hematin is the biocrystallization of heme to beta-hematin in presence of lipids as promotor of the process in the food vacuole (Ambele and Egan, 2012), which supports also the observed peroxidation processes during this process (Tilley et al., 2010).

Though beneficial as heme depot for the parasite, hemozoin and its constituents are not at all inert but biologically rather active in the host. HZ affects the function of host cells, especially immune cells. Schwarzer, Arese and colleagues could show a the profound modulation of immune cell

function by HZ resulting in anti-inflammatory immune impairing as well as proinflammatory responses of monocyte-derived phagocytes (Schwarzer et al., 1992a).

Ani-inflammatory effect

When a monocyte recognizes and avidly phagocytoses a mature infected RBC or nHZ, that is released during schizogony, HZ persists in the monocyte lysosome, provoking cellular dysfunction. The HZ-laden monocytes and monocyte-derived macrophages are unable to digest HZ or to repeat phagocytosis (Schwarzer et al., 1992a), to generate the oxidative burst upon appropriate stimulation (Schwarzer and Arese, 1996) and to kill ingested bacteria, fungi, or tumor cells (Fiori et al., 1993).

Additionally to NADPH-oxidase malfunction another crucial player in immune cell regulation is impaired: the membrane translocation and activity of protein kinase C (PKC) were precociously and severely impaired (Schwarzer et al., 1993).

The compromised PKC activity may also contribute to inhibited phagocyte movement (directed and undirected migration) and phagocytosis, though HZ-elicited lipoperoxidation modifies the crucial plyer of cell motility actin by conjugation with 4-hydroxynonenal (4-HNE) (Skorokhod et al., 2014). Similarly, HZ- dependent lipoperoxidation impairs the differentiation to macrophages and DCs, and maturation to immune competent DC (Skorokhod et al., 2004) and antigen presentation (Scorza et al., 1999).

Pro-inflammatory effects

The initial event after contact of nHZ to the phagocyte is a strong and long-lasting oxidative burst accompanied by an increase of MCP-1 and TNF alpha (Barrera et al., 2011), that further stimulates neighbor cells to move and to become activated. The overwhelming ROS burst is considered crucial for peroxidations and subsequent functional impairments driven by lipoperoxidation breakdown products, such as 4-HNE (as detailed in paragraph 1.2.1).

Moreover, some experiments demonstrate that HZ-fed human and murine monocytes/macrophages were found to release large amounts of 4-HNE, nitric oxide, considered as a protection for malaria because it causes parasite death and inhibits endothelial adhesion by parasites.

But in 2007, Skorokhod et al. suggested to be careful with this generalized affirmation as he demonstrated that phagocytosis of HZ or synthetic HZ (sHZ) by monocytes obtained from non-immune Caucasian donors did not increase NO-production, iNOS protein and mRNA expression either after stimulation by IFN-gamma or other strong proinflammatory cytokines and stimulus like LPS. By contrast, in HZ/sHZ-laden murine macrophages, identical treatment with IFN-gamma and the cytokine-LPS mix, elicited significant increases in protein and mRNA expression of iNOS and NOS metabolites production, in agreement with literature data (Malarial pigment haemozoin, IFN-gamma, TNF-alpha, IL-1beta and LPS do not stimulate expression of inducible nitric oxide

synthase and production of nitric oxide in immuno-purified human monocytes (Skorokhod et al., 2007b).

These data do not support the hypothesis that monocytes are mediators of anti-parasitic defense in clinical malaria via activation of iNOS and production of NO, and suggest caution in extrapolating data obtained with murine or hybrid systems to human malaria and I will not focus on NO-dependent processes in my own studies.

Further anti-malarial, long-term proinflammatory responses of monocyte-derived phagocytes to HZ- phagocytosis include a long-lasting TNF-alpha, IL-1-beta, and metalloprotease production and secretion (Prato et al., 2005).

In animal mouse models HZ elicited proinflammatory responses in tissue macrophages of lung and liver (Scaccabarozzi et al., 2018), while strong proofs for immune-suppressive responses to HZ are coming from co-infection studies and exposure of animals to HZ with subsequent bacteria challenge (Harding et al., 2020).

Therefore, the malarial pigment HZ, represents an ’intelligent tool’ of the parasite to tune the immune response of the host not only to survive the immediate cellular immune attacks, but also to avoid excessive immune responses that could kill the host.

Inhibition of erythropoiesis

Beside of the immune-modulatory effects HZ is hypothesized to have a role in malaria anemia that is due to abnormalities of erythroid cell differentiation and proliferation.

In vitro and *ex vivo* data have supposed HZ to play role in inhibition of erythropoiesis, either directly or indirectly via interaction with phagocytes.

Studying abnormal erythropoiesis during malarial anemia, Giribaldi et al. (Giribaldi et al., 2004) show that HZ impairs the *ex vivo* growth of erythroid colonies in semisolid culture medium. They hypothesize the contribution of HZ in malaria anemia and dyserytrhopoiesis, demonstrating an inhibition of erythroid-progenitor growth by supernatants of HZ and HZ-fed monocytes. Researchers propose that this mechanism could be the principal way to impair erythropoiesis in malaria. In fact they suggest that lipid peroxidation could play a very important role through shed molecules that mediate the inhibition of erythroid cells (Giribaldi et al., 2004).

Based on this important finding, many researchers have investigated this potential role of HZ.

Casals et al. demonstrated that there is a suppression of erythropoiesis in malarial anemia associated with HZ *in vitro* and confirmed the data *in vivo*. They added HZ (derived from trophozoite-lysate) to erythroid cells (CD34+) on different days, noticing that there is a significant inhibition of cells expansion. HZ was associated with decreased reticulocyte count in patients. Furthermore, they compare the role of TNF-α and HZ during malaria abnormal erythropoiesis: in vitro absence of TNF does not prevent HZ from interfering with erythroid cell development (Casals-Pascual et al., 2006).

Lamikanra et al. (Lamikanra et al., 2009) demonstrated that regardless the immune response to HZ, HZ still has an inhibitory effect on erythropoiesis by its own.

They noticed that co-cultured macrophages protect erythroid cells from HZ and that reduced erythroid expansion was accompanied by increased apoptosis in a liquid culture system (Fibach et al., 1989). The absence of inflammation mediators and macrophages allows to suggest that accumulation of HZ in the bone marrow could contribute to the severity of anemia in children with chronic malarial infection. (Lamikanra et al., 2009).

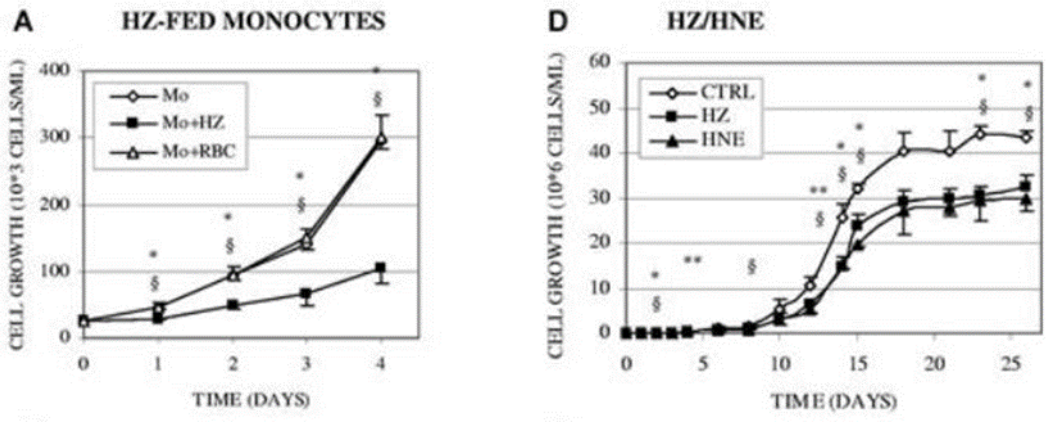


Figure 3. Two histograms to indicate cell growth in the case of HZ-fed monocytes (A) and in case of HZ/HNE from the study of Skorokhod (Skorokhod et al., 2007b).

Skorokhod et al. published important results on the mechanism of HZ to inhibit erythropoiesis and the HZ-fed monocytes effect on erythropoiesis. For the first time, the whole process of CD34+ cell differentiation and maturation to erythroid progenitors and precursors and finally mature erythrocytes was followed in presence of exogenously added nHZ in liquid culture *ex vivo*, making use of the Giarratana protocol (Giarratana et al., 2005). HZ was shown to slow the erythroid cell cycle by the rise of 2 critical proteins in cell-cycle regulation, p53 and p21, and the retinoblastoma protein, central regulator of G_1-to-S-phase transition, was consequently hypophosphorylated. By contrast, GATA-1, the most important transcription factor in erythropoiesis, was reduced. Cyclin A and D2 decreased, retarding cell-cycle progression in erythroid cells.

Additionally, he studied the effect of HZ that was taken up by monocytes and co-cultured with erythroid cells as a model of the erythroid island, in which central bone marrow macrophages are surrounded by erythroid cells, functioning as wet nurses for the latter.

For this he has shown the inhibitory role of HZ, identify HNE as one HZ-generated inhibitory molecule during erythroid progenitors growth possibly involved in erythropoiesis inhibition in malaria anemia.

Ruth Aguilar finally, could confirm the presence of HZ in bone marrow macrophages of children with malaria anemia, a fact that underlines the pathomechanistic importance of the findings from the Oxford and Torino laboratories referred above.

1.2 Parasite elicited oxidative stress and lipoperoxidation in the host

The relationship between the redox status of malarial parasites and that of their host is complex and the mechanistic role of oxidative stress during malaria infection needs further, and mainly *in vivo* investigations.

The malaria parasite generates large quantities of reactive oxygen species firstly, in its own metabolism and secondly by interaction with phagocytes.

The breakdown of hemoglobin by the parasite in the acidic food vacuole results in formation of toxic free heme and generates large amounts of reactive oxygen species (ROS), causing an imbalance between the activity of antioxidants and formation of oxidizing species. (Becker et al., 2004).

The biochemical mechanism of oxidative stress in malaria-infected cells has not been explained yet but it has been suggested that it could involve Hb, iron or the electron transport of the parasite mitochondrion (Atamna and Ginsburg, 1993). To mimic the environment of the parasite food vacuole, authors adjusted the pH of RBC lysate to acidic values. They observed that: oxyhemoglobin, but not deoxyhemoglobin, is quickly converted to methemoglobin. The conversion rate is further increased in the presence of the catalase inhibitor 3-amino-1,2,4-triazole (3-AT) and catalase inhibition increased with acidification, suggesting that H_2O_2 is thus produced by spontaneous dismutation of superoxide radicals generated during the formation of methemoglobin. Human red blood cells (RBC) parasitized with *P. falciparum* trophozoites (TpRBC) have been shown to produce about twice as much H_2O_2 and $^{\cdot}OH$ radicals as normal RBC, as demonstrated by inhibition of endogenous catalase activity and degradation of deoxyribose, respectively. Increased H_2O_2 levels and catalase activity were found in both host cell and parasite compartments. No increase in H_2O_2 production compared to that observed in uninfected RBC could be detected in the annular phase when digestion of host Hb is absent (Atamna and Ginsburg, 1993).

In response to malaria parasite infection, natural defense mechanisms are activated by macrophages and neutrophils and oxidative burst produces superoxide radicals that are subsequently converted into other ROS forms (Malaguarnera and Musumeci, 2002).

The increase of ROS is not restricted to the parasite and its host cell but leads to the development of systemic complications caused by malaria. Oxidative stress resulting from increased production of free radicals, causes a lipid peroxidation and consequently formation of reactive aldehydes, important lipid peroxidation markers.

1.2.1 Lipoperoxidation product 4-hydroxynonenal: A powerful reactive aldehyde

Among a series of aldehydic products derived from lipid peroxidation, hydroxy aldehydes, like 4-HNE, are of particular interest in biological systems because they reach relatively high local concentrations, are more stable as radicals and able to diffuse inside or even outside the cell to reach distant target molecules that they attack to form covalent conjugates. Target molecules are proteins, DNA, and phospholipids (Schwarzer et al., 2014).

4-HNE was discovered for the first time in 1964 by Schauenstein (Schauenstein et al., 1964). In 1980 Esterbauer and colleagues correctly characterized it (Benedetti et al., 1980).

When discovered, it was regarded as a mere by-product of autoxidation of unsaturated fatty acids, when triglycerides become rancid. Things changed when it turned out that 4-HNE is a normal constituent of mammalian tissue membranes (Comporti, 1998) and recent interest is not only based on the fact that 4-HNE is a specific marker of oxidative stress, but on its likely role as modulator of cellular processes.

Generation of 4-HNE

The generation of 4-HNE starts when omega-6-polyunsaturated fatty acids (PUFAs), such as the essential fatty acids linoleic and arachidonic acids “offers” their first conjugated double bound to a free radical specie, e.g., OH. After the abstraction of a hydrogen, PUFA will transform in PUFA radical that, binding molecular oxygen, becomes a reactive peroxide (Figure 4, Figure 5). This peroxide continues with the abstraction of another H of a new PUFA. This generates two molecules, firstly a new PUFA radical that continues the chain reaction and secondly, a PUFA hydroperoxide, not stable yet. In fact, thanks to Hock cleavage, the bond between carbons 9 and 10 of the fatty acid chain of the hydroperoxyl fatty acid is destabilized and breaks. The product of this cleavage is an aldehyde that contains the last nine carbons of the omega-6 fatty acid, a nonenal. By rearrangement of the double bound, the binding of oxygen takes place at the fourth position and generates 4-hydroperoxynonenal which is reduced to final product, 4-HNE. These reactions can occur without the intervention of any enzyme (Schwarzer et al., 2014).

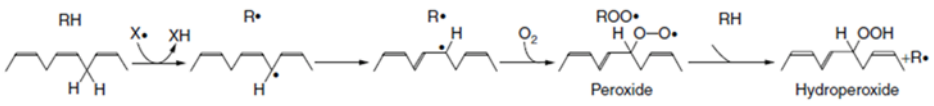


Figure 4. First stages of peroxidation of PUFAs: hydrogen abstraction, shift of double bond, binding of oxygen.

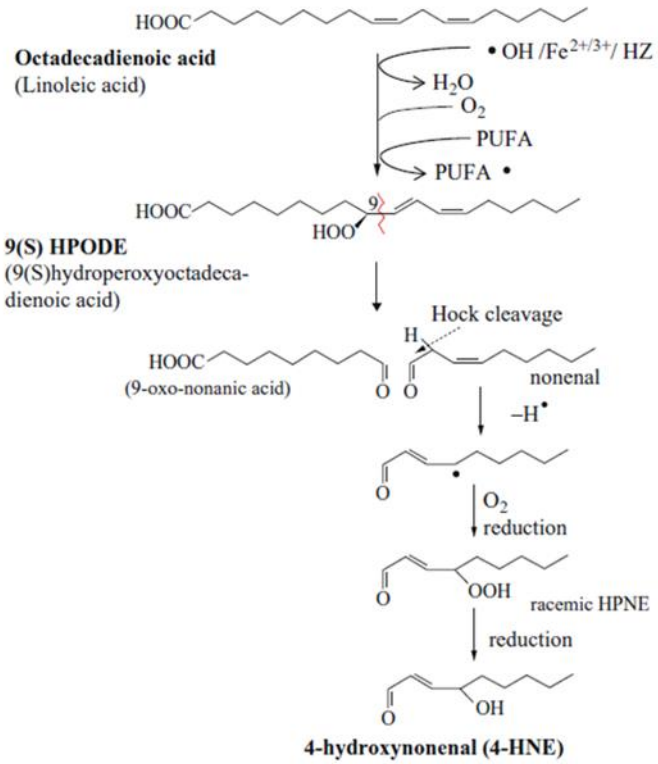


Figure 5. The formation of 4-HNE from linoleic acid by lipid peroxidation chain break and hydroxylation (Schwarzer et al., 2014).

In malaria, iron and HZ is supposed to catalyze the PUFA peroxidation. This leads to the formation of a chemically unstable hydroperoxide of PUFA, which breaks into 9-oxo-nonanic acid and nonenal in case of linoleic acid as substrate of peroxidation. Nonenal is further transformed in 4-hydroperoxynonenal (HPNE) and finally reduced to 4-HNE.

Generation of 4-HNE *in vivo*

Biological membranes and lipoproteins are rich in PUFAs, esterified in phospholipids. Membrane phospholipids represent excellent targets for lipoperoxidation and become sources for 4-HNE.

The normal human serum concentration of HNE does not exceed 0.05-0.15 µmole per liter (Parola et al., 1999) and is controlled by degrading metabolic pathways.

Obviously, the accumulation of this biologically active compound, has to be controlled, even under high oxidative stress conditions.

Main 4-HNE-metabolizing enzymes are: i) the alcohol dehydrogenases (NADH-dependent reduction of the carbonyl group), ii) the aldehyde dehydrogenases (NADP-dependent oxidation of the carbonyl group), and iii) the glutathione S-transferases (Michael addition of thiols). Glutathione S-transferases (Berhane et al., 1994) (Singhal et al., 1994) and aldehyde dehydrogenases are house-keeping enzymes of all mammalian including human cells. Moreover, other enzymes or non-enzymatic reactions should be taken into account for 4-HNE metabolism, such as the super

family of aldo-keto reductases (AKR) with an isoform considered very important for the detoxification of lipid peroxidation products, the AKR1B (Srivastava et al., 1984) (Spycher et al., 1997).

The glutathione S-transferase catalyzed reaction is more than 600 times faster in comparison with the non-enzymatic Michael addition. Under pathological conditions, when the formation of 4-HNE is accelerated, or when 4-HNE is added to cells, a fast decrease of intracellular GSH concentration is observed (Figure 6) and the important protective molecule against membrane oxidations may become limiting. For this reason, for 4-HNE it's easier to accumulate, it can proceed undisturbed and result dangerous for cells.

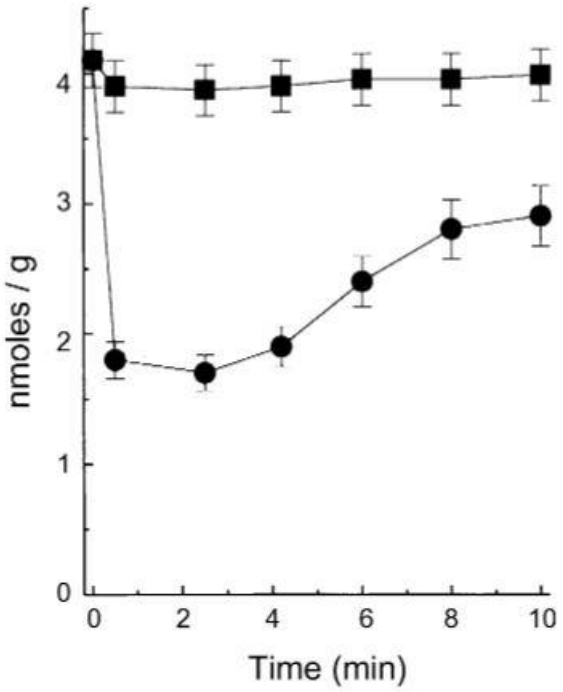


Figure 6. GSH decrease after the formation or addition of 4-HNE. The change of GSH was monitored in rat hepatocytes without (square) or with addition of 100 µM HNE (circle) at time 0 (Siems et al., 1997).

Despite the rapid 4-HNE catabolism, the 4-HNE level can increase and protein modification by 4-HNE does occur.

Under pathological conditions 4-HNE levels can exceed 100 µmole per liter in blood. Thus, high levels of 4-HNE have been detected in several diseases accompanied by oxidative stress. The values vary between 1 and 100 µmole per liter according to the cell type in kidney tubular cells, hepatocytes and monocytes. A concentration of 100 µmole per liter of 4-HNE in the whole organ may seem almost impossible but it is more plausible that the concentration of 4-HNE increases locally, e.g., near peroxidized membranes.

During the immediate immune response oxidative burst reaction produces oxygen radicals directed against non-self-targets. Thus, monocytes produce high concentrations of 4-HNE, in case of HZ -

phagocytosis approximated 40 µmoles per liter or higher (Schwarzer et al., 2014) (Schwarzer et al., 1996).

Reactions of 4-HNE with proteins and its regulatory role in health and disease

The binding behavior of 4-HNE to exposed sites in macromolecules in and outside the cell can affect and modulate cell functions at very low concentrations without being toxic to cells. Both facts have opened the door for studying the role of the molecule in diseases accompanied by oxidative stress focusing on the involvement of 4-HNE in inflammation-related events, in cell signaling and in gene expression. The growing knowledge of 4-HNE reactivity, metabolism, signaling and modulatory effect in the various human organs should provide a solid background for investigating the aldehyde's contribution to the pathogenesis of human major chronic diseases and would likely promote advanced and oriented applications not only in diagnosis and prevention but also in molecular treatment of human diseases. The implication of increasing levels of 4-HNE has been found in 39 different human diseases: either the affected organ or generalized in the blood plasma or both (Figure 7). This lead to consider the reactive aldehyde directly involved in the disease pattern and progression.

Acute myocardial infarction	Dilated cardiomyopathy
Adult respiratory distress syndrome	Deep venous thrombosis
AIDS	Genetic hemochromatosis
Alzheimer's disease	Global cerebral ischemia
Amyotrophic lateral sclerosis	Huntington's disease
Atherosclerosis	Lupus erythematosus
Brain ischemia/reperfusion injury	Macular degeneration of the retina
Chronic alcoholic disease	Multiple sclerosis
Chronic hepatitis B and C	Non-alcoholic fatty liver disease
Chronic exposure to ozone	Non-alcoholic steatohepatitis
Chronic iron overload	Parkinson's disease
Chronic kidney failure	Perinatal asphyxia
Chronic obstructive pulmonary disease	Post-surgery lymphedema
Congenital nephrotic syndrome	Primary biliary cirrhosis
Crohn's disease	Rheumatoid arthritis
Circulatory shock	Spinal cord traumatic injury
Cirrhosis	Stroke
Cognitive impairment	Systemic inflammatory response syndrome
Creutzfeldt–Jakob disease	Wilson's disease
Diabetes mellitus	

Figure 7. Example of human disease associated with 4-HNE.

Molecular modifications by 4-HNE

4-HNE is a stable lipoperoxidation product but biologically rather reactive. What gives it reactive are the following chemical properties: a double bond between C2 and C3 conjugated with an aldehyde group and the OH group at C4.

4-HNE is divided in two portions: the first part with aldehyde and hydroxyl groups is hydrophilic while the second part from C5 to C9 forms a hydrophobic tail. The amphiphilic properties allow 4-HNE to leave the cell of origin, crossing the membrane and to interfere with cell membrane structures of other cells.

The position C3 is an excellent target for nucleophilic molecules and makes 4-HNE specifically reactive to form Michael adducts, and the aldehyde group of 4-HNE react with molecules containing amino groups to form Schiff bases (Figure 8).

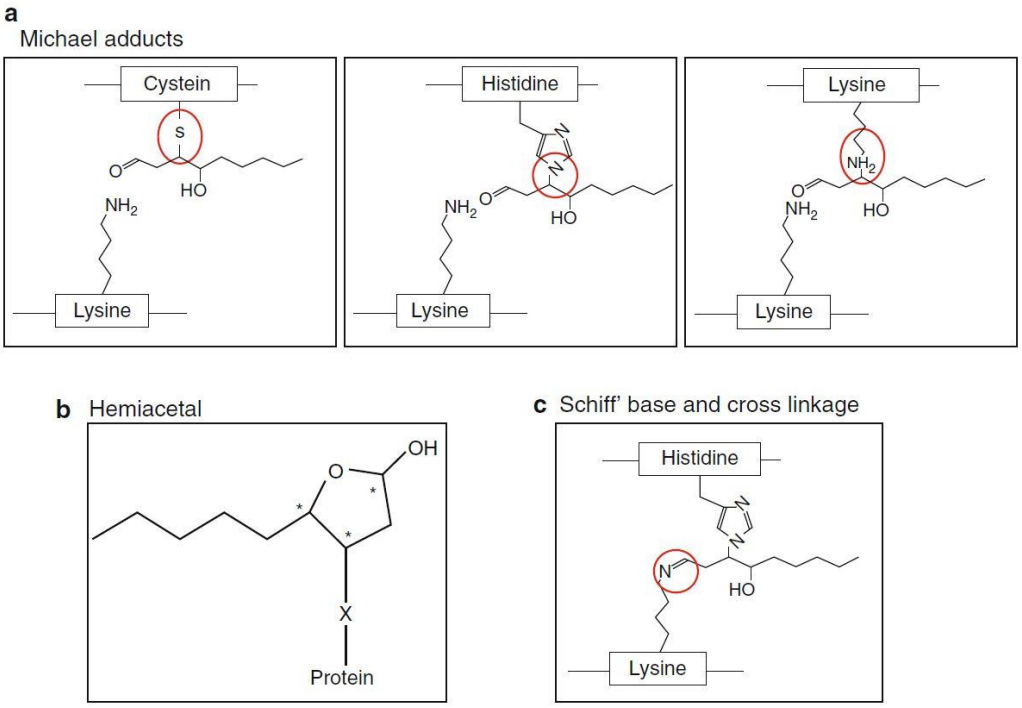


Figure 8. Binding of 4-HNE to proteins by adduction (a) or Schiff base formation with the amino acids cysteine, histidine, and lysine (c) (Schwarzer et al., 2014).

It appears clear that 4-HNE will tend to conjugate using proteins, peptides, nucleotides, and phospholipids.

Most covalent binding occurs with specific amino acid residues cysteine, histidine, lysine and sometimes arginine. Once the modification has taken place, the properties of the proteins affected will no longer be the original ones and functions are likely modified.

Protein modification represents the best known mechanism by which 4-HNE can influence cellular processes.

The capability of 4-HNE to leave the cell of origin, crossing the membrane and to reach other cells provokes the covalent posttranslational modification of proteins also distant from the site of origin of 4-HNE and to interfere with changes of their function.

Because of cumulative effect in the membrane, 4-HNE can attack crucial target proteins in or near the lipid bilayer. A high and increasing number of 4-HNE target proteins have been identified. The binding sites of 4-HNE to the protein chain were determined by mass spectrometry for several proteins, e.g. actin binding sites for 4-HNE were identified and supposed to have consequences for cell motility (Schwarzer et al., 2014).

The study of Poli et al. on modified proteins by 4-HNE reveals a wide spectrum of functional proteins which includes enzymes in energy metabolism, protein kinases and phospholipases involved in cellular signaling, enzymes involved in intracellular proteolysis by the proteasome, extracellular matrix metalloproteinases, membrane receptors for neurotransmitter and growth factors, membrane ion transporter and carriers in the plasma membrane, some plasma proteins, cytoskeletal proteins, heat-shock proteins, and proteins involved in translation (Poli et al., 2008).

1.2.2 Malaria and 4-hydroxynonenal

In 1988, Buffinton et al. (Buffinton et al., 1988) described unconjugated 4-HNE in RBC of *P. vinckei*-infected mice, the first malaria-related report. In the early 90th high levels of HNE were found in phagocytes that had ingested malarial pigment HZ (Schwarzer et al., 1996). Later, *P. falciparum* -pRBC and free HZ were shown to carry substantial amounts of 4-HNE (Skorokhod et al., 2007a).

During malaria infection, there are several sources for 4-HNE as summarized in Figure 9. These are: i) the mature intraerythrocytic stages of plasmodium , the late trophozoite and schizont stage (upper left panel), ii) HZ that is released in the blood plasma as residual body during the rupture of schizonts and merozoite egress, iii) monocyte derived phagocytes when they get in contact with pRBC or residual bodies and respond with an extraordinary strong and long lasting oxidative burst elicited by Toll-like receptor 4 (TLR4) signaling and iv) the HZ-laden phagocyte after ingestion of HZ or HZ containing parasites. HZ persists in the vital but functionally impaired monocytes.

HZ is able to catalyze in *ex vivo* cultured primary cells PUFA peroxidation with formation of 4-HNE and hydroxy-fatty acids such as hydroxylated arachidonic acid (hydroxy eicosatetraenoic acid, HETE) or hydroxylated linoleic acid (hydroxy octadecadienoic acid, HODE).Parasite growth is accompanied by rising levels of HZ, matching with increasing 4-HNE levels in that in mature intraerythrocytic parasite stages, late trophozoites and schizonts exceed the physiological stationary level of 4-HNE. Thus 15-fold higher values were observed in hemozoin-rich schizonts compared to young hemozoin-poor ring forms (Skorokhod et al., 2007a). As experimentally shown, HZ-containing parasite release 4-HNE which reaches neighbor cells and modifies them (Uyoga et al., 2012). At the end of the 48-h parasite growth cycle in the RBC, the daughter parasites leave the RBC to infect new RBCs (schizogony). In parallel the residual body (RB) or nHZ, that is the membrane-enveloped, HZ-containing digestive vacuole, is expelled into the bloodstream. RBs in the supernatant of *P. falciparum* cultures contained 50 micromoles per liter 4-HNE (Skorokhod et al., 2005).

RBs released into plasma bind preferentially host fibrinogen. Barrera and colleagues found 10–100,000 fibrinogen molecules per residual body, stably bound to nHZ collected from plasma-cultured parasites. Bound fibrinogen is recognized by TLR4 on monocytes, thus RBs become excellent ligands for TLR4 and elicit an immediate, extensive and long-lasting oxidative burst in human monocytes. ROS production by RBs exceeds that by opsonized RBCs 100-fold (Barrera et al., 2011). As consequence of the violent oxidative burst, HZ phagocytosis provoked significantly higher levels of lipid peroxides in monocytes as compared to RBC phagocytosis, (Schwarzer et al., 1992a). Close contact of HZ with the cell membrane, together with extensive oxidative burst during HZ phagocytosis, resulted in fast and persistent 6-fold increase of lipid peroxides in RB-fed monocytes compared to the transient 2-fold increase after RBC phagocytosis (Schwarzer et al., 1992a).

Importantly, the increase of free 4-HNE can already be seen after 2 hours of feeding monocytes with HZ.

Schwarzer et al. demonstrated that primary human monocytes which were fed in vitro with HZ show a 50-fold higher 4-HNE level compared to unfed controls at 5 h after phagocytosis and still 15-fold higher levels after 12 hours.

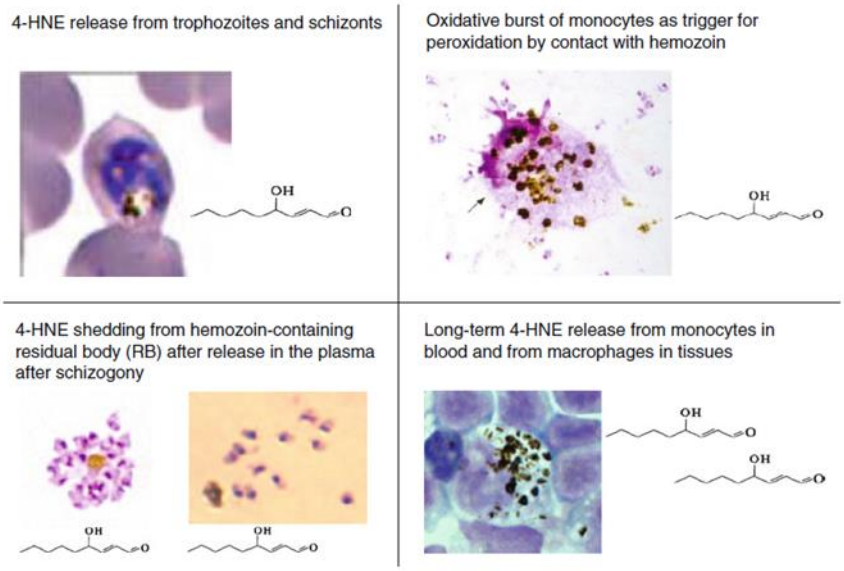


Figure 9. 4-HNE sources related to malaria modified from (Schwarzer et al., 2015).

Structural and functional modifications in host cells by 4-HNE

4-HNE is a reactive molecule and as outlined in detail above paragraph “Generation of 4-HNE in malaria elevated levels of 4-HNE occur. 4-HNE conjugates with proteins were present in falciparum- parasitized RBC, HZ-laden monocytes, macrophages, dendritic cells and erythroid cells most likely as consequence of HZ generation, contact or presence (Schwarzer et al., 2015).

Skorokhod et al. (Skorokhod et al., 2010) focuses on the fact that 4-HNE is the product of HZ or monocytes that have phagocytosed HZ, and could alterate the correct functioning of erythropoiesis and supposes this lipoperoxidation end product as mechanistic link between HZ and functional impairments and modulations. Cocultivation of erythroid cells with HZ, 4-HNE and HZ-fed monocytes, cause a significative inhibition of erythroid cellular growth. In brief, the indigestible heme portion of Hb is organized by the parasite to form HZ in close contact to the membrane of the digestive vacuole (Kapishnikov et al., 2012). *In vitro*, just like free heme, HZ also causes lipid peroxidation of PUFAs and, consequently, degradation to 4-HNE and other products (Miller, 2005). Skorokhod et al. demonstrated in *P. falciparum* cultures that 4-HNE and its membrane protein conjugates increase significantly in pRBC compared to control npRBC. 4-HNE protein conjugates

increase up to 15-fold higher levels in HZ-rich schizonts compared to young HZ-free parasite forms (Skorokhod et al., 2007a).

The work of Uyoga et al. (Uyoga et al., 2012) on severe anemia, adds to the HZ-elicited lipid peroxidation product 4-HNE, as it can be transferred in vitro from pRBC to non-parasite RBC. They noticed also that treating normal RBC with plausible concentration of 4-HNE results in increased phagocytosis. From the results obtained by Uyoga's study, the formation of rosettes is proposed as mechanism for destruction of the large numbers of npRBC as observed *in vivo* (Uyoga et al., 2012).

In patients with malaria, 4-HNE-protein conjugates were significantly increased as compared to age -matched non-malaria control children and were visible on pRBC and npRBC (Aguilar et al., 2014).

1.2.3 Hemoglobin oxidation and formation of hemichromes in the host cells

As outlined in previous chapters the damage caused by oxidative stress is based on many changes that can modify the cell and intervene in many diseases. Sometimes, however, the oxidative changes can act as a signal.

In RBC, Hb is continuously exposed to redox reactions. Redox reactions occur mainly due to the presence of oxygen that produce superoxide and hydrogen peroxide. In turn hydrogen peroxide can react with Hb producing ferryl Hb and eventually degrading heme (Rifkind et al., 2003). An additional source of oxidative stress are nitrite-derived reactive species that are generated after contact with Hb. The erythrocytic cycle in malaria begins when a single merozoite invades a host RBC. This results in most of the red cell cytoplasm, including its hemoglobin, being consumed providing the parasite a source for amino acids and generating toxic reactive oxygen species and free heme.

The binding of Hb to the RBC cell membrane is a hallmark of cell senescence and targeting of RBC for recognition and removal from circulation by macrophages via phagocytosis (Kay, 1975) (Kannan et al., 1988) (Low, 1986).

Physiologically Hb is regularly oxidized to methemoglobin and about 1% of Hb being in this form at any time (Welbourn et al., 2017).

Hb, which is damaged by severe oxidative stress with heme groups being in a redox- and oxygen-binding inactive hemichrome form, has been implicated as the membrane binding species.

When the level of Hb oxidation increases (as for example when RBC become senescent or in the case of a pathological condition), more Hb molecules are membrane bound and increase lipid peroxidation processes at the membrane which may explain the cell damage caused by Hb.

In vivo oxidative stress studies with oxidants that go beyond the level of cell tolerability, showed an increase in hemoglobin membrane binding, especially in older cells with lower levels of antioxidant enzymes. These are then targeted for destruction.

During malaria infection, parasitized-RBC are gradually transformed in non-self-cells to be recognize by human monocytes. The accumulation of erythrocyte profound changes is due to the presence of parasite neo-antigens on the host cell surface and host cell protein modifications as consequence of high levels of oxidation elicited by the growing parasite.

This leads to an increasing number of autologous IgG (Facer, 1980) (Mendis et al., 1983) and complement binding (Stanley et al., 1984, Turrini et al., 1992) with a consequent rise of phagocytosis levels (Celada et al., 1982) (Turrini et al., 1992).

The actual molecular model for parasite elicited phagocytosis is described by the following sequence of events: 1) hemoglobin oxidation to hemichromes by the parasite, 2) hemichrome binding to and oxidation of the cytoplasmic domain of membrane protein band 3, 3) which allows SYK to bind and be activated, 4) with subsequent phosphorylation of band 3, 5) leading to

detachment of the bilayer from the cytoskeleton and 6) subsequent clustering of band 3. Band 3 cluster are recognized by auto-anti-band 3 antibodies.

Note, that the term “hemichrome” in the scientific literature has been attributed to any unreactive low spin form of ferric (met) Hb. Generally, this is due to ligation of the distal histidine residue in the heme pocket, creating a bis-His iron complex, unable to react with external ligands. However, hemichrome formation can be irreversible or reversible.

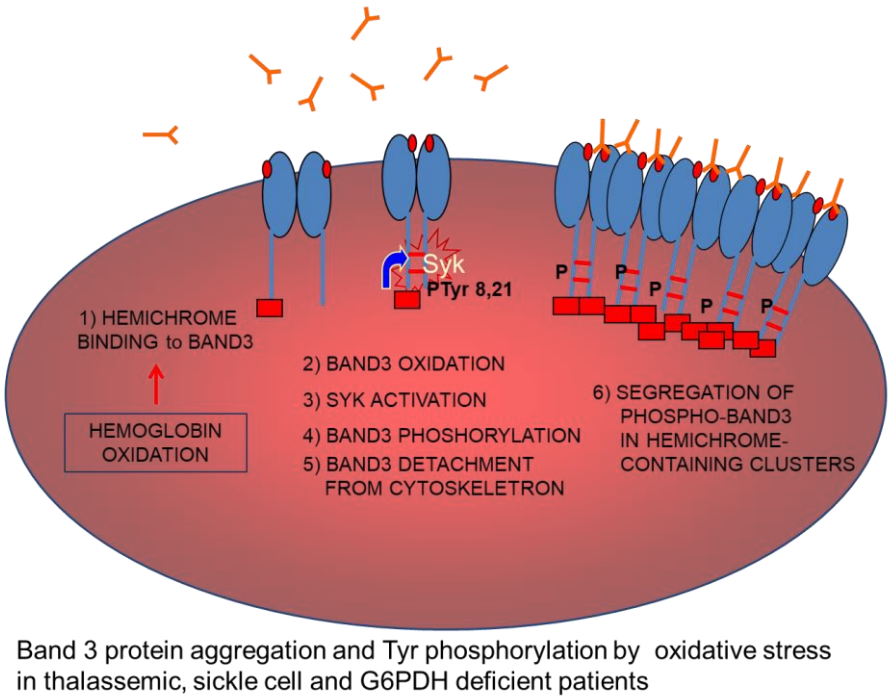


Figure 10. Illustration of a RBC from thalassemic, with sickle cells or G6PDH deficient patients in which is explained the sequence of events after hemoglobin oxidation that leads to clustering of phospho-band 3 and hemicromes (Pantaleo et al., 2008).

SYK activation and band 3 clustering in the RBC membrane may have two consequences , the phagocytic removal of the flagged cell or the expulsion of destabilized membrane microparticles that contain clustered proteins avoiding whole cell eradication.

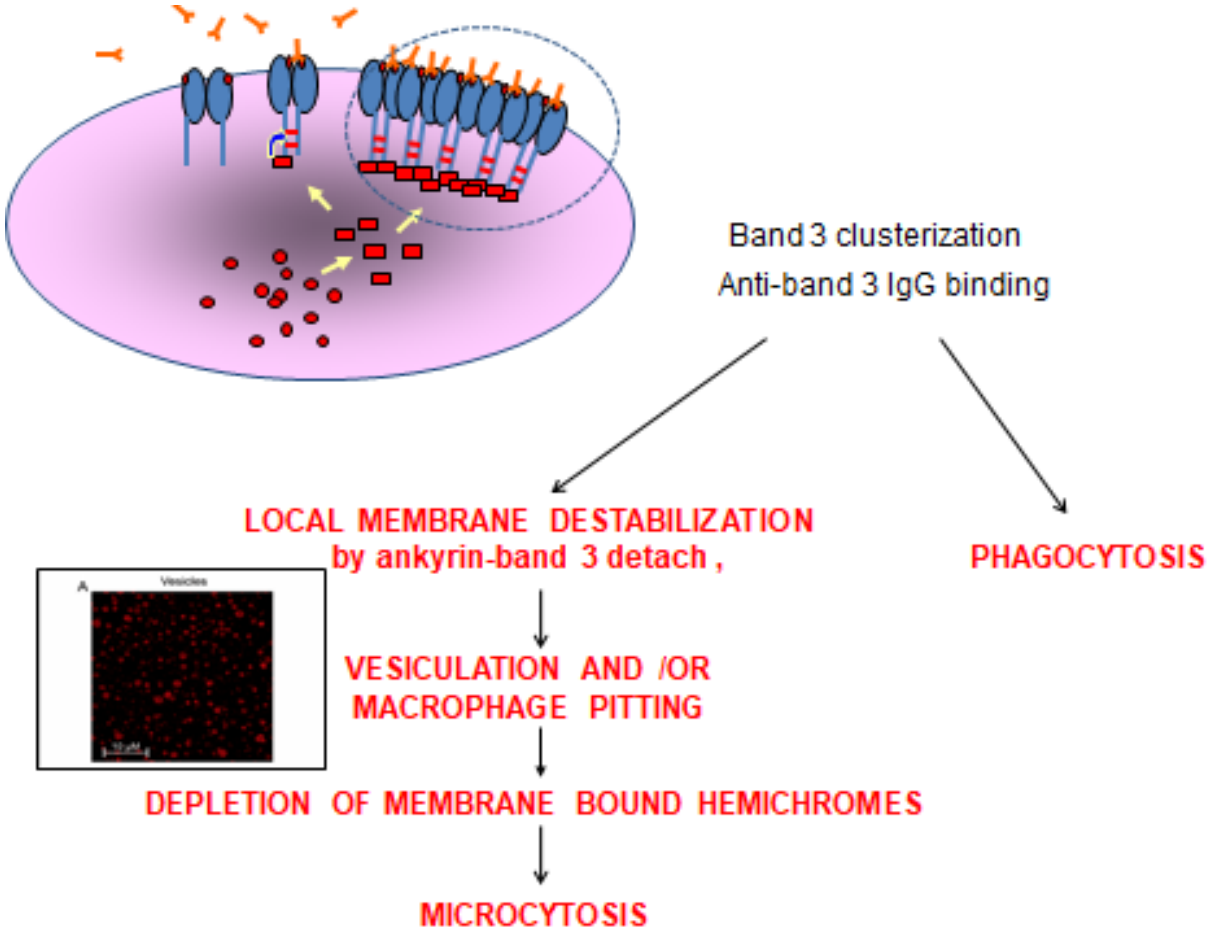


Figure 11. Possible outcomes of protein band 3 clustering in RBC due to oxidative stress: Microparticle shedding and phagocytosis.

1.3 Anemia in malaria-endemic regions and malaria anemia

The WHO has defined anemia as a “condition in which the number of RBC or the Hb concentration within them is lower than normal”. Hemoglobin is needed to carry oxygen and too few or abnormal RBC, or not enough Hb result in a decreased capacity of the blood to carry oxygen to the body’s tissues. This results in symptoms such as fatigue, weakness, dizziness, and shortness of breath, among others. The optimal Hb concentration to meet physiologic needs varies by age, sex, geographic altitude of residence, smoking habits, and pregnancy status. The most common causes of anemia include nutritional deficiencies, particularly iron deficiency and, though deficiencies in folate, vitamins B12 and A are also important causes; haemoglobinopathies; and infectious diseases, such as malaria, tuberculosis, HIV and parasitic infections and neoplastic diseases. The WHO evaluates that 42% of children less than 5 years of age and 40% of pregnant women worldwide are anemic according anemia criteria shown in Table 1 (https://www.who.int/health-topics/anaemia#tab=tab_1).

According to the “Global Burden of Disease Study”, 2.36 billion individuals were affected by anemia worldwide in 2015 (Collaborators, 2015).

Anemia continues to burden large segments of the global population. An earlier study published similar numbers with an estimated 29% of non-pregnant women and 43% of preschool-age children worldwide being anemic (Stevens et al., 2013), and iron-deficiency anemia is the single prominent cause of years lived with disability in children and adolescents worldwide.

In developing countries including Africa, anemia represents an important cause of morbidity and mortality. Long-term anemia is thought to delay a child's development and make children more likely to get infections. The real problem is that the data reflect the situation in hospitalized children, only. Unfortunately, the large share of children who have anemia, do not go to the hospital and do not receive any professional diagnosis or in any case interviews that are not entirely reliable. (Brabin et al., 2001). For this reason, reliable numbers on anemia are unknown.

It appears evident that the pathogenesis of anemia is not easy to understand because of the innumerable factors that can cause anemia. This complicates anemia studies, especially in areas with a high burden of infectious diseases.

Nutritional deficiencies, acute and chronic inflammation, parasitic infections, and inherited or acquired disorders that affect Hb synthesis, compromised erythropoiesis or decreased RBC survival, are possible causes of anemia.

Nowadays anemia remains a major cause of global morbidity and mortality, particularly among children. The successful treatment of anemia depends on causes and severity of anemia. Anemia of any etiology increases the risk of child mortality and morbidity, and specifically iron deficiency (ID) anemia has been associated with harmful effects on cognitive and physical development and on immune function of children (WHO, 2008. Worldwide Prevalence of Anemia 1993–2005: WHO

Global Database on Anaemia. Geneva, Switzerland: World Health Organization). Treatment of ID in childhood is based on the administration of iron supplements and/or food fortification.

Table 1. WHO cut-offs for anemia. Hb (g/L) concentrations to diagnose anemia at sea level (Chaparro and Suchdev, 2019).

		Anemia		
Population	**Nonanemic**	**Mild**	**Moderate**	**Severe**
Children 6–59 months of age	≥110	100–109	70–99	<70
Children 5–11 years of age	≥115	110–114	80–109	<80
Children 12–14 years of age	≥120	110–119	80–109	<80
Nonpregnant women (15 years of age and above)	≥120	110–119	80–109	<80
Pregnant women	≥110	100–109	70–99	<70
Men (15 years of age and above)	≥130	110–129	80–109	<80

1.3.1 Pathomechanism of malaria anemia

One of the most important complication of malaria is anemia. Severe malaria anemia can be life-threatening mainly for children and pregnant women living in malaria-endemic regions of Africa. Moraleda et al. of 2017 searching for etiological aspects of anemia in hospitalized preschool children from a rural area in Mozambique, defined severe anemia by Hb <5 g/dl and moderate anemia by Hb ≥5 g/dl to <11 g/dl (Moraleda et al., 2017).

It should not be a surprise that anemia is one of the most common disorders associated with malaria, because *Plasmodium* parasites undergo an intraerythrocytic schizogony process culminating in lysis of host RBCs, not only circulating RBCs but also those sequestered in deep vasculature and spleen.

In fact, during acute falciparum malaria, when schizonts rupture, anemia develops 48 h after the onset of fever. There is an initial decline of hematocrit (Hct) following treatment owing to rehydration. This is followed by a further fall over the next 4–5 days as long as the parasitemia declines; then the hematocrit continues to fall, often up to two weeks after clearing parasitemia.

In non-immune children and adults infected with *P. falciparum*, the degree of anemia may correlate with parasitemia (Phillips and Pasvol, 1992, Biemba et al., 2000) (Menendez et al., 2000b) and in most experimental rodent malaria models, acute anemia is a consequence of rupture of pRBCs at high parasitemias that reach 50% and more in the animals (Lamikanra et al., 2007). In humans,

malaria anemia occurs usually at parasitemia values below 2% and the destruction of pRBCs alone cannot account for anemia severity in malaria patients.

The frequency of malaria-related anemia and death was inversely correlated to age in a study in Mozambiquan children. The age group most affected was 0 to 1 year old compared to older children as shown in Figure 12. Thus, babies (1-2 years of age) and newborns with malaria infection develop in 10-20% of cases severe anemia with hematocrit values below 15% and need transfusion.

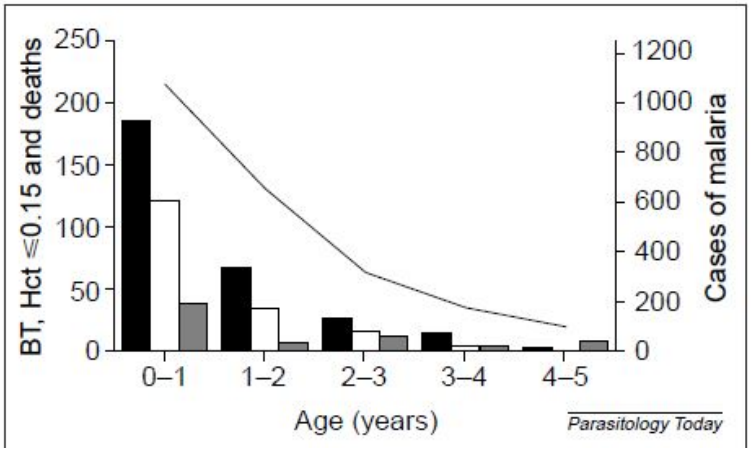


Figure 12. Histogram indicated number of cases of malaria by age from (Menendez et al., 2000b).

Searching for malaria anemia-provoking mechanisms other than schizont rupture, the loss of non-parasitized RBC (npRBC) was described. The implication of npBC can explain the lack of correlation between parasitemia and the severity of anemia seen in many studies (Phillips and Pasvol, 1992, Slutsker et al., 1994) (Dondorp et al., 1999).

Host defense mechanisms to infection may contribute to RBC loss. The immune system responds promptly to parasites, initially by phagocytosis and oxidative burst as first-line defense mechanisms mediated by spleen and blood phagocytes and later by humoral and cellular specific responses to avoid the increase in parasitemia and consequently an excessive loss of RBCs. Obviously, this is sometimes not sufficient to prevent the onset of anemia.

The pathogenesis of malarial anemia remains not completely understood yet but several studies have revealed anomalies that could contribute to or aggravate the anemia.

Following, possible mechanisms described in literature to be involved in the onset of malarial anemia are summarized.

Rupture of pRBC

The lysis of pRBCs represents reduces drastically the number of circulating RBC. On completion of the parasite growth and proliferation inside the RBC daughter merozoites egress from the host cell, that lyses, and invade new RBC, restarting the cycle.

This repetitive rupture of pRBC each 48 h contributes to a first phase of anemic situation.
The lysis of the host cell is 'carefully prepared' by the growing parasite that exerts oxidative stress to its host RBC. During its growth, the parasite elicits the oxidation of band 3, the accumulation of oxidized hemoglobin and subsequently the phosphorylation of Syk and Lynn (Src-like nonreceptor tyrosine kinases) in the inner side of the RBC membrane and detachment of the cytoskeleton from the membrane. The consequent microparticle shedding eliminates modified membrane components like band 3 visible to immune cells. The shedding of microparticles weakens the membrane stability which facilitates the merozoite egress after schizogony. As explained later it helps also to bypass a premature phagocytosis of the pRBC as 'eat me' tags (CD47) can be shed with the microparticles.

Phagocytosis of pRBC and npRBC

After avid phagocytosis of mature parasites and expelled RB which is accompanied by a violent oxidative burst in primary human phagocytes cultured *in vitro*, both phagocytosis and burst are switched off irreversibly after approximately 2 hours in the otherwise vital cells. (Schwarzer et al., 1992b). Up to ten pRBC or residual bodies (the latter in terms of heme content) were phagocytosed by plated human monocytes after a phagocytosis time of 2-3 hours. In case of *ex vivo* differentiated monocyte-derived macrophage this value even increased to 25 pRBC. Thus, phagocytes are potent first line defense cells and their functional impairment after the first phagocytic meal may be partly overcome by the release of new phagocytes from the bone marrow after chemokine release. Indeed, *in vivo* circulating HZ-containing monocytes are considered as proof for an acute or former malaria episode. The accumulation of HZ in the spleen of individuals living in a malaria -endemic region indicate the massive phagocytic activity of spleen macrophages.
As described above, parasites export antigens in the membrane of host RBCs. The exposed parasite antigens bind IgG of immune sera and parasitized cells are opsonized by IgG and complement factor 3 (C3). The resulting phagocytosis is mediated by Fc-receptors and CR1 of phagocytes contributing to anemia.
Several groups have reported the finding of IgG and complement-coated RBCs in patients with severe malarial anemia (Abdalla, 1988) (Facer et al., 1979), suggesting a role for complement-mediated damage of RBC.
However, the phagocytosis of pRBC opsonized with non-immune serum by human primary monocytes, suggests immune-serum independent mechanisms for pRBC recognition by phagocytes. The presence of both circulating and resident macrophages laden with HZ in non-immune *P. falciparum* malaria patients or in vitro malarial models (pRBC are phagocytosed by cultured macrophages) support the idea that pRBC can be removed in the absence of specific immunity of the host.

Giribaldi et al. (Giribaldi et al., 2001) demonstrated in vitro that increasing amounts of aggregated band 3 were associated with deposition of complement fragment C3c and autologous IgG, specific for aggregated band 3.

The involvement of oxidative events in the formation of the antigenic membrane aggregates of band 3 was supposed as the formation of aggregates could be prevented by reductants and oxidant scavengers. A causal role of hemichromes in provoking aggregation of and co-localization with band 3 has been shown in senescent and oxidatively damaged RBCs by several authors (Low et al., 1985) (Waugh and Low, 1985) (Schlüter and Drenckhahn, 1986) (Waugh et al., 1986), and suggested by direct and indirect evidence in malaria-parasitized RBCs (Tilley et al., 1990).

In a phagocytosis study with non-immune serum for opsonization, Turrini et al. (Turrini et al., 1992), demonstrated that ring-parasitized RBC (RpRBC) are phagocytosed similarly to senescent or oxidatively damaged RBC, where deposition of immunoglobulin G (IgG) and complement produce recognition signals for phagocytosis. For pRBC infected with mature forms of parasite (trophozoites or schizonts), phagocytosis is more intense followed by the phosphatidylserine PS exposition that increases recognition (Turrini et al., 1992).

In contrast to detailed studies on the phagocytosis of pRBC, far less mechanistic studies were performed for phagocytosis of npRBC and it is not trivial to explain why these RBC are recognized by phagocytes, particularly in non-immune malaria patients, likely the majority of malaria anemia patients.

The invasion of parasite in RBC is complex, and may be completed in only a small fraction of erythrocytes targeted for infection. Parasite antigens are shed during entry, and many of these parasite-encoded erythrocyte-adhesive proteins are present at high levels near to npRBC. Probably they adhere to uninfected erythrocytes and this would result in membrane modification to erythrocytes, leading to their clearance from circulation (Haldar and Mohandas, 2009).

Moreover, the role of lipoperoxidation in the poorly elucidated process of npRBC loss has been studied by Uyoga S. et al. in 2012. The main message from this innovative *in vitro* study was that rosettes, which are specific cellular structures formed by few npRBC that adhere to a HZ-containing pRBC at trophozoite stage allow the transfer of 4-HNE from pRBC to npRBC. HNE-was shown by fluorescence microscopy in the nuclei-containing trophozoite and importantly, in the adherent npRBC (Uyoga et al., 2012).

The transfer of 4-HNE may have a crucial role in the phagocytic removal of large numbers of npRBCs, the hallmark of severe malaria anemia as in vitro performed erythrophagocytosis increased proportionally with increasing 4-HNE-binding to npRBC and significantly higher levels of 4-HNE were observed in children with severe malaria anemia vs. non anemic malaria patients in a pilot study with low case numbers (Uyoga et al., 2012).

Based on these findings I asked the question whether 4-HNE-labeling of npRBC may be a hallmark in anemia and possibly a missing functional link for increased phagocytosis.

The role of the spleen, that eliminates altered RBC, is important for clinical manifestations of malaria, especially for anemia (Buffet et al., 2009). About each 100-200 minutes, quality control of all circulating RBCs occurs regarding deformability or immune signals for retention of RBC in the slow circulation of the spleen such as clustered band 3 with bound autoantibodies or flipped PS in the external leaflet of the membrane (Turrini et al., 1992, Lutz, 2004) (Rey et al., 2014). Decreased RBC deformability was found in malaria (Cooke et al., 2004). Deformability loss correlated well with the degree of anemia. (Dondorp et al., 1999).

Basing on this fact, Skorokhod et al. (2007), demonstrated *in vitro* that the presence of 4-HNE-protein adducts in RBC membranes causes a decreased in RBC deformability likely due to modifications in RBC cytoskeleton and membrane proteins as they are found in pRBC and co-cultured npRBC (Skorokhod et al., 2007a).

In vivo, the HZ-containing trophozoite as well as isolated HZ are phagocytic targets for monocytes/macrophages. The reduced deformability of circulating erythrocytes carrying 4-HNE adducts should increase their phagocytic elimination in spleen, liver and bone marrow. Increased 4-HNE production by pRBC may additionally modify non-parasitized erythrocytes, induce their phagocytosis, and contribute to malarial anemia.

Dyserythropoiesis

Erythropoiesis is a process in the bone marrow, by which erythroid progenitors proliferate and differentiate into non-nucleated reticulocytes which leave the marrow and enter the blood where they mature to erythrocytes for two days.

It is important to note that erythropoiesis occurs in specialized niches in the marrow termed “erythroblastic islands”, composed of erythroblasts surrounding a central macrophage and in which cells proliferate, differentiate and enucleate.

During malaria infection, an ineffective erythropoiesis exacerbates anemia. A lower and inadequate release of erythrocytes from the bone marrow despite the increased production in response to hemolysis, and a premature death of erythroid precursors before their entrance in circulation were observed *in vivo* (Casals-Pascual and Roberts, 2006) (Newton et al., 1997).

Dyserythropoiesis was described in malaria anemia, i.e. a defective development of erythrocytes and the occurrence of erythroid precursor cells with abnormal shape and size (Dörmer et al., 1983).

Malaria anemia is characterized by low reticulocyte response (Chang et al., 2004), normal or increased production of erythropoietin (EPO), (Burgmann et al., 1996) and altered morphology of erythroid precursors (Dörmer et al., 1983, Casals-Pascual et al., 2006). Several evidences, both in animals and humans, indicate that during malaria anemia, bone marrow shows an inability to replenish erythrocytes lost by hemolysis through inadequate erythroid response. In this process the reduction of erythropoietin is not the unique cause and an alternative mechanisms was introduced by Giribaldi et al. (Giribaldi et al., 2004) and Skorokhod et al. (Skorokhod et al., 2010)

demonstrating the effect of HZ and its lipoperoxidation product 4-HNE on erythroid cells grown *ex vivo*.

More than 70 years ago, reticulocytopenia was described in *P. vivax* and *P. falciparum* malaria infection followed by reticulocytosis after parasite clearance (Vryonis, 1939) and was later causally suppressed erythropoiesis (Casals-Pascual and Roberts, 2006). Nonetheless the molecular mechanisms behind dyserythropoiesis in malaria are not entirely known.

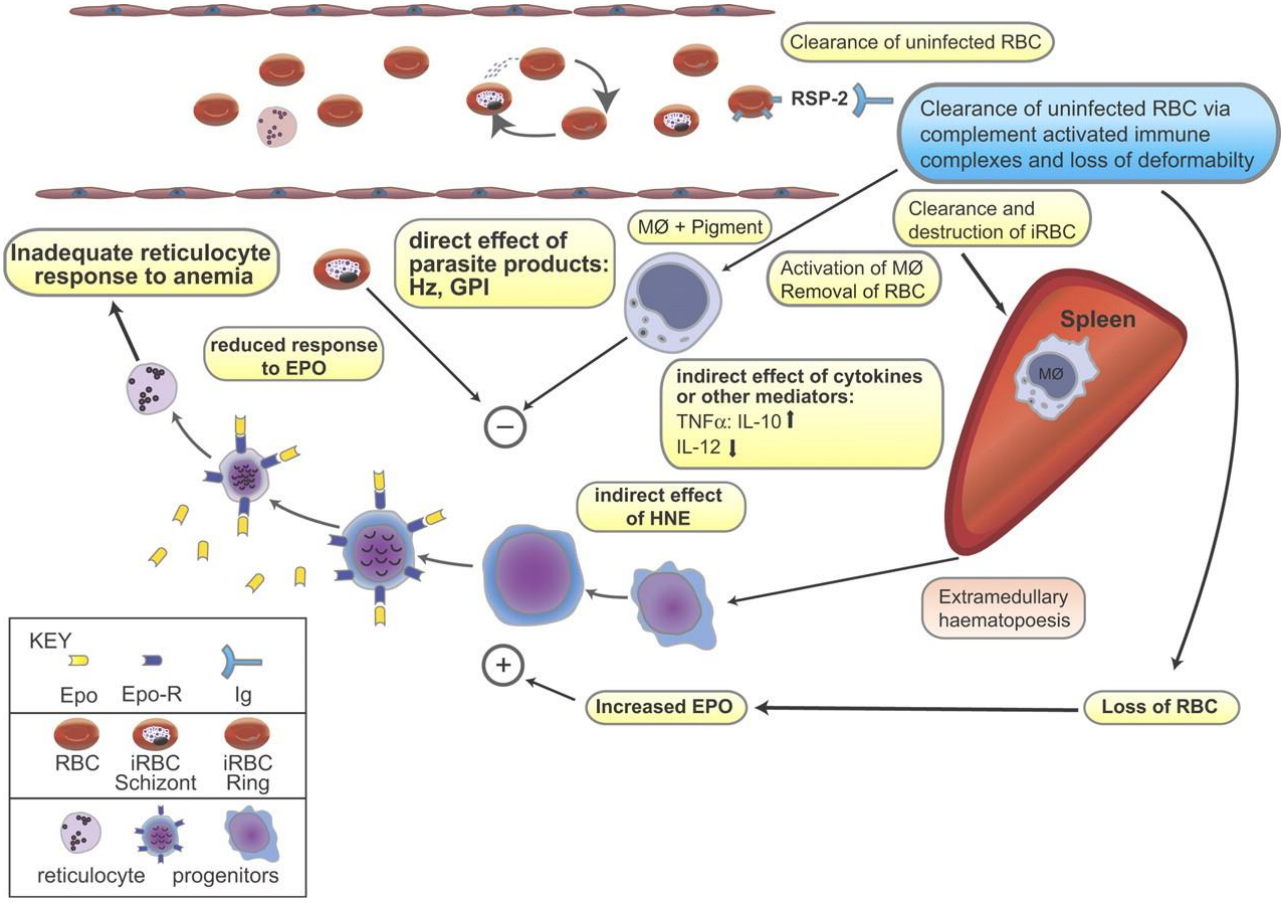


Figure 13. Direct and indirect effects of parasite on the development of malarial anemia (Lamikanra et al., 2007).

Only in 2010, it was shown that 4-HNE inhibits the erythroid cell proliferation during the whole period of erythropoiesis, involving the molecular regulators of the cell cycle, a series of controlled events that take place in cells leading to its division and differentiation (Skorokhod et al., 2010). Authors showed that both natural HZ and 4-HNE treatment of erythroid cells results in: (i)increased regulatory proteins, p53 (tumor suppressor protein 53) and p21 (cyclin-dependent kinase inhibitor 1); (ii)unbalanced phosphorylation of the retinoblastoma protein Rb and (iii)decreased levels of cyclins A and D. Importantly, neither HZ nor 4-HNE added at reasonable concentrations induced programmed cell death (apoptosis). Further, low micromolar 4-HNE delays the expression profile of differentiation markers: the functionally important membrane receptors for transferrin (TfR1, CD71), stem cell factor c-kit (CD117), interleukin-3 (CD123), and erythropoietin (Epo) as well as

the stem cell marker CD34, and the RBC markers glycophorin A and hemoglobin. GATA-1, the master transcription factor that controls the differentiation processes in erythropoiesis, was less expressed in HZ- and 4-HNE-treated cells. The strong conjugation of 4-HNE to the cells and the functional structural changes induced by 4-HNE lead to the description of molecular targets of 4-HNE in erythroid progenitors possibly involved in the inhibition of erythropoiesis in malaria anemia.

In patients with malaria, high concentrations of TNF-α have been associated with severe disease and TNF-α, has been thought to be the major cause of bone marrow dysfunction (Clark and Chaudhri, 1988). While severe disease in children is associated with elevated levels of proinflammatory and anti-inflammatory cytokines, the severity of anemia seems to be dependent on levels of TNF-α relative to the potent anti-inflammatory cytokine IL-10. Several clinical studies have demonstrated that a low ratio of plasma IL-10/TNF-α is associated with SMA in young children. Casals Pascal et al. demonstrated in vitro that TNF- α has a reversible effect on erythroid growth and development because its effect can be inhibited by using anti-TNF- α antibodies. The anti-TNF-alpha titer rises in malaria which makes the contribution of TNF to anemia questionable (Clark et al., 1989).

With regard to erythroid cell apoptosis controverse results were reported in different erythropoiesis models which does not allow yet to judge on a role of apoptosis *in vivo*. Lamikanra et al. demonstrated that HZ isolated from pRBC from *in vitro* cultures inhibited erythroid development independently of inflammatory mediators by inducing apoptotic pathways that involved the activation of caspase 8 and cleavage of caspase 3 and also the loss of mitochondrial potential (Lamikanra et al., 2009).

At the contrary, Skorokhod et al. could not confirm elevated apoptosis levels of erythroid progenitors and precursors in presence of DNA-free HZ-containing residual bodies which were naturally expelled from pRBC. Both laboratories concurred in the direct inhibition of erythropoiesis by HZ.

The lipoperoxidation mediated by HZ with consequent formation of 4-HNE in malarial anemia has a determinant role in diserythropoiesis as shown *in vitro* (Skorokhod et al., 2010). Cocultivation with HZ or treatment with low micromolar 4-HNE inhibited the erythroid cells growth, interfering with cell cycle without provoking apoptosis. P53 and p21 (2 crucial proteins in cell-cycle regulation), increased by HZ/HNE treatment and the retinoblastoma protein, central regulator of G1-to-S-phase transition, was consequently hypo phosphorylated, while expression of GATA-1, master transcription factor in erythropoiesis decreased. All These changes resulted in decreased expressions of cyclin A and D2 that retarded cell-cycle progression in ex-vivo differentiated erythroid cells and the K562 cell line. Additionally, an inhibition of critical receptors (R): transferrinR1, stem cell factor R, interleukin-3R, and erythropoietin R was observed in line with a delayed differentiation. The decreased receptor expression and the impaired cell-cycle activity decreased the expressing of glycophorin-A and hemoglobin in erythroid cells underlining the

inhibitory role of HZ and 4-HNE in erythropoiesis and probably malaria anemia (Skorokhod et al., 2010).

1.3.2 Non-malarial anemia forms in malaria regions

Despite evidence for a determinant role of malaria in anemia incidence, malaria is no longer considered the only reason for anemia (Moraleda et al., 2017) in malaria endemic regions, but may surely impact on anemia severity. Severe anemia is seen only in a small proportion of malaria cases and its pathogenesis is not clearly understood, but such factors as malnutrition, iron deficiency, bone marrow dysfunction, and the level of parasitemia are surely thought to contribute to its manifestation.

Sub-Saharan Africa and other African areas are poor areas where other infections than malaria, both bacterial and viral ones, are frequent and do aggravate the situation to develop anemia.

Furthermore, micronutrient malnutrition is usually highly prevalent in malaria endemic areas and the various vitamin deficiencies and therefore malnutrition in general contributes to anemia.

A good nutritional status is closely related to the development of an adequate immune response to infection, being on the one hand, an important determinant of the risk and prognosis of infectious diseases, and on the other hand, being directly influenced by infection.

When there is a synergic lack of specific nutrients, anemia development will become a significant burden (Chaparro and Suchdev, 2019).

Despite the seriousness of the consequences that anemia can cause, public health attention remains very limited. This is probably due to treating anemia as a by-product of processes of other diseases.

From a recent national survey conducted in Ghana on micronutrient deficiencies, malaria, hemoglobinopathies and malnutrition in young children and non-pregnant women (Wegmüller et al., 2020) concluded that in some areas of Ghana two deficiencies, namely, iron and vitamin A deficiency overlap to cause anemia. Anemia and several micronutrient deficiencies are highly present in Ghana, especially in the Northern Belt and in poor households (Wegmüller et al., 2020).

The scenario described for Ghana is common in almost all African countries, where poverty prevents very young children from having regular access to complete nutrition and the right supply of nutrients for an adequate development, is not guaranteed.

The most important micronutrient deficiency associated with anemia in malaria region regards iron. Iron is an important mineral needed to produce hemoglobin. It is involved in cell development and cell growth of the brain, muscle, and the immune system (Beard, 2001) and is a component of many enzymes. A relatively large amount of iron is required to produce RBCs (erythropoiesis) in the first few months after birth.

In healthy adults the daily nutritional need of iron is 10mg, of which approximately 1mg is absorbed. In new-born and growing children, the daily need per kg is much higher.

Ferritin and transferrin are the two proteins crucially important for storage and transport of iron in the human body. The iron status is commonly monitored by serum ferritin (Mei et al., 2005). Ferritin, in fact is the major iron store of mammals and is found in all tissues, but in particularly high

concentrations in the liver, spleen and bone marrow. It is present in most tissues as a cytosolic protein and its function is to store iron in a soluble form from which it can be readily mobilized. Hepatocytes, macrophages and Kupffer cells have been shown to secrete ferritin (Wesselius et al., 1994). The increase of serum ferritin is proportional to the baseline ferritin levels and available iron stores.

Transferrin is a glycoprotein produced in the liver, that circulates at high concentration in the blood where it is mainly responsible for the iron transport. Transferrin is a “shuttle protein” (Huebers et al., 1983). It has two iron binding sites, binds free iron in the serum and transports it to Tf-receptor (TfR) exposing cells. Once bound to the receptor the TfR-Tf-iron complex is endocytosed and free iron delivered to the cell, e.g., the bone marrow erythroid stem cells for erythropoiesis.

In general, infection and inflammation increase ferritin, which is an acute phase reactant. It decreases only slowly after the resolution of infection and remains elevated in the convalescent phases of infection. Thus, malaria-endemic countries it is difficult to interpret ferritin levels and their use as a biomarker of iron deficiency may underestimate the true prevalence of iron deficiency (Nyakeriga et al., 2004) (Zimmermann et al., 2005). Other biomarkers or combinations of biomarkers have been suggested for the assessment of iron deficiency in locations with a high prevalence of infection (Neuberger et al., 2016) such as the serum transferrin receptor, zinc protoporphyrin, transferrin saturation, and the ratio of serum transferrin receptor to serum ferritin (Lynch, 2011), as well as the adjustment of ferritin to C-reactive protein or alpha1-acid glycoprotein levels, or both (Mburu et al., 2008).

The debate whether iron supplementation during malaria infection could represent a beneficial treatment remains open since iron deficiency may be protective against malaria infection. In fact iron is widely used by pathogens for their own metabolism and killing ability (Beard, 2001) so iron deficiency might be a measure of the host to protect himself .

Many studies on the relationship between iron status and malaria lead to a fundamental hypothesis: poor iron and/or the sequestration of iron because of the physiologic actions of hepcidin in response to inflammation may protect against malaria and its associated morbidities by restricting the availability of iron to the parasite (Spottiswoode et al., 2014).

Glucose-6-Phosphate-Dehydrogenase (G6PD) deficiency

Glucose-6-phosphate dehydrogenase (G6PD) is the enzyme that maintains redox equilibrium in cells by catalyzing the first reaction in the pentose phosphate pathway. This pathway is essential to ensure the useful level of reduced nicotinamide adenine dinucleotide phosphate (NADPH) which confers primary oxidative defense in RBC (Lee et al., 2018).

G6PD deficiency is an X-linked enzyme disorder that leads to increased susceptibility to oxidative stress damage which is induced by food and drugs and causes hemolytic anemia. Inability to maintain normal redox status in G6PD deficient RBC results in oxidative stress that may be unfavorable to malaria parasites. This supports the protection hypothesis (Tishkoff et al., 2001) and

is likely based on preferential phagocytosis of early ring-stage pRBC, which does not compromise the host defense. This deficiency is globally distributed but presents a geographically prevalence with the historical distribution of malaria (Allison, 1963) (Howes et al., 2012).

α-Thalassemia

Among the genetic mutations that are accompanied by a survival advantage for malaria, is α-thalassemia. It represents a haemoglobinopathy associated with protection against malaria.

It is probably the most common monogenic gene disorder in the world and is especially frequent in Mediterranean countries, South-East Asia, Africa, the Middle East and in the Indian subcontinent.

Many studies carried out in areas where malaria is endemic, confirm that α-thalassemia is common and clearly associated with *P. falciparum* transmission intensity. Even if hemoglobinopathies are generally found in populations living or derived from malaria endemic areas, it is not clear why some polymorphisms are prevalent in some malaria endemic areas and absent in others. This is probably because of population migration or by introduction or a disturbing of an existing equilibrium (Enevold et al., 2007).

Alpha-thalassemia is characterized by abnormal or absent manufacturing of alpha-globin chains. This can lead to more than 15 different genetic mutations, each of which causes a dysfunction of the Hb. In sub-Saharan Africa, α^{+}-thalassemia affects up to 50% of the population, but protection against uncomplicated or severe malaria could not be demonstrated yet (Lell et al., 1999) (Mockenhaupt et al., 1999) (Allen et al., 1993).

Vitamin B12 deficiency

Together with vitamins A, B2, B6, and folic acid, vitamin B12 is implicated in the normal production of RBC. (Fishman et al., 2000). Vitamin B12 deficiency provokes pernicious anemia.

1.3.3 Oxidative modifications in RBC membranes from anemic patients. State of the art

This paragraph reviews the literature on oxidative-stress-elicited molecular modifications in the membrane of RBCs from patients with several anemia forms, focusing on modifications with a supposed pathomechanistic role in anemia. I refer to the lipoperoxidation–dependent conjugation of membrane proteins with 4-HNE and hemoglobin–oxidation-dependent phosphorylation of band 3.

Oxidative stress is considered to be a determinant of disease severity during Plasmodium falciparum malaria infection (Becker et al., 2004), (Atamna and Ginsburg, 1993). The large body of data from *in vitro* experimental human malaria models provides insights in possible pathways to pathological changes observed in patients and reveals plausible mechanistic roles of oxidative stress (Atamna and Ginsburg, 1993) (Giribaldi et al., 2001) (Schwarzer et al., 2008, Skorokhod et al., 2010) (Uyoga et al., 2012). By contrast, *in vivo* data on the pathogenic roles of oxidative stress are limited, though reports relate oxidative stress with modifications in RBC and plasma in clinical malaria (Das and Nanda, 1999) (Nanda and Das, 2000) (Kulkarni et al., 2003). Moreover, only a few studies have analyzed the role of oxidative stress in infant malaria (Griffiths et al., 2001) (Narsaria et al., 2012), and its impact on the development of malaria anemia (Uyoga et al., 2012).

Some authors suggest oxidative modifications and lipoperoxidation, usually measured as TBARS, just as symptomatic side effects of the immune response of the host to malaria infection and-at most- as marker for disease (Sohail et al., 2007) while others claim a relation to the physiopathology of the disease (Aguilar et al., 2014, Uyoga et al., 2012) suggesting the generation of ROS to be causally involved in the development of systemic complications of malaria.

Thus, Uyoga found a highly significant increase of 4-HNE-conjugates in npRBC of malaria patients from Kenia with severe anemia (hemoglobin <5g/dl) compared to patients with mild or no anemia (hemoglobin >5g/dl). She suggested that tagging RBC with 4-HNE might be a recognition signal for RBC removal in the spleen. Unfortunately, the study cohort was too small to draw definitive conclusions.

The second indication for a mechanistic role of the lipoperoxidation product 4-HNE in malaria anemia comes from a follow-up study with non-immune children in Mozambique (Aguilar et al., 2014). Children presenting with Hb <10 g/dl at their first acute malaria episode were grouped according to their capacity to recover from anemia by increasing Hb levels during convalescence, or to aggravate their condition by decreasing or sustained low Hb levels during convalescence (one month after acute malaria episode). 4-HNE-binding to the RBCs of children was determined at acute and convalescence states and compared between groups. Children with persisting anemia accumulated additionally 4-HNE-conjugates in their RBC membrane during the monthly convalescence period, while children that cleared their anemic state during convalescence lost 4-HNE-conjugates compared to the acute malaria state, respectively. Also, the group that did not

recover from anemia had significantly higher 4-HNE-conjugate levels on RBCs during convalescence compared to the recovery group.

There are indications that RBC in several anemia forms different from malaria infection show oxidative modifications and carry elevated levels of 4-HNE-conjugates.

4-HNE levels have been shown to correlate with the severity of anemia in chronic renal diseases (Siems et al., 2003) and increased not further specified carbonylation levels were also observed in patients with iron deficiency and beta thalassemia compared to non-anemic controls (Trombetta et al., 2006).

Further and larger studies are needed to establish the existence of a causal link between elevated levels of 4-HNE in the RBC membrane and anemia.

Modifications of RBC membrane protein band 3 protein are central in generation of removal signals in naturally senescent RBC for phagocytic recognition (Kay, 1984). Mannu et al. (1995) found that hemichrome binding to band 3 and hemichrome-mediated oxidation of band-3 cytoplasmic domains, generate high molecular weight band-3 aggregates and enhance opsonization by anti-band-3 antibodies. This sequence of events is supposed to be responsible for phagocytic removal of erythrocytes in thalassemia (Mannu et al., 1995).

Phosphorylation of band 3 due to hemichrome formation was found in RBCs obtained from patients with thalassemia, sickle cell disease, G6PD-deficiency and in *falciparum* pRBC, from in vitro cultures. In fact, in 2009, Pantaleo, Ferru, Turrini et al., founded that selective tyrosine phosphorylation of oxidized band 3 by Syk, may play a role in the recruitment of oxidized band 3 in large membrane aggregates that show a high affinity to natural Abs, leading to RBC removal from the circulation in both normal and G6PD-deficient red blood cells (Pantaleo et al., 2009).

In malaria patients increased anti-band 3 and anti-spectrin antibodies in Plasmodium vivax infection were associated with anemia (Mourão et al., 2018) which indicates that the above described model for a band 3 oxidation dependent aggregation of the protein with subsequent binding of auto-antibodies and complement factor 3 (CR3) might have a role *in vivo*.

Both modifications, 4-HNE-conjugation or hemichrome-elicited anti-band 3 binding may determine the life span of a RBC in blood circulation and increased levels may correlate with the incidence of anemia.

1.4 Objectives

The pathophysiology of malarial anemia is complex and multifactorial (Phillips and Pasvol, 1992). Oxidative stress elicited by the malaria parasite and its product HZ and by the host immune response to malaria infection is a hallmark of disease. Consequently, levels of bioactive lipoperoxidation product 4-HNE and hemoglobin oxidation product hemichrome increase in RBC membranes. Both parameters are promising pathomechanistic factors in anemia genesis.

The main question that I would like to answer is whether oxidative stress has a functional role in the onset of malaria anemia and in anemia of different etiologies. To reach the goal, functionally relevant oxidative stress parameters will be identified and evaluated on RBC membranes of 264 children regarding their possible association with the incidence of malaria anemia. Secondly, the parameters were proven for their relevance for non-malarial anemia forms. Thirdly, molecular mechanisms will be investigated by which oxidative modifications provoke anemia.

A combined *ex vivo* and *in vitro* approach was applied, assessing firstly, two promising oxidative RBC membrane-bound parameters (4-HNE membrane proteins conjugates and membrane-bound Hb and heme species) in blood samples from Mozambican children living in South-East African malaria endemic area and testing them for correlation with classical blood parameters of anemia and secondly, perform *ex vivo* studies on cell models to explore the molecular mechanism in the pathogenesis of anemia. As functionally relevant oxidative parameters I will focus: i) on the lipoperoxidation product 4-HNE, which forms covalent conjugates with RBC-membrane proteins and ii) on membrane-bound-Hb which binds to membrane protein band 3 due to oxidative challenge and membrane-bound heme species.

The approach included the exact quantification of 4-HNE-protein conjugates per RBC for all study participants by immunochemical techniques after protein extraction from isolated RBC membranes and their correlation with blood Hb. Similarly, membrane-bound heme species, mainly hemichromes, were detected by a luminescence method. The regression analysis between blood Hb and the above mentioned parameters, respectively, selected 4-HNE-conjugates as a promising modification as to the highly significant inverse correlation. To better investigate the involvement of lipoperoxidation in development of malaria anemia by the significant increases of 4-HNE-conjugation levels, RBC membrane proteins in moderate and severe anemic malaria patients were compared to non-anemic children. The degree of anemia moderate vs. severe is correlated with the degree of 4-HNE-conjugation. A similar increase of 4-HNE-conjugates is detectable in anemia patients of different etiologies.

Additionally, with the intent of investigating the impact of 4-HNE not only on malaria anemia but also on anemia of various etiologies, anemic children were divided in three groups based on the likely leading cause for anemia, namely, malaria, inflammation and others and their mean 4-HNE values compared to the values found in the respective non-anemic children group. As to the generalized impact of 4-HNE in anemia the search for 4-HNE sources during anemia genesis

became interesting. A series of regression curves were used to investigate correlation between 4-HNE conjugates in RBC membrane and different clinical parameters such as parasite count, inflammatory parameter CRP, nutritional deficiencies aimed to determine origin/causal effects of HNE to different pathophysiologic conditions. Children's cohort is very precious because they come from malaria-endemic areas and they are extremely susceptible to severe complications following malaria onset, due to concomitant infections and malnutrition/inflammatory state.

For the *in vitro* approach, both parameters, 4-HNE-protein conjugates and hemichromes, were formerly proven in *in vitro* cellular test systems to be functionally relevant for increased phagocytosis rates and/or inhibited erythropoiesis. So, in order to intensify the research on the possible causal effects of 4-HNE on anemia, labelling of 4-HNE protein conjugates has been assumed to increase by age of RBC leading to elimination of senescent ones in the spleen. For this, blood of healthy donors was used to analyze 4-HNE protein conjugates in RBC membranes of sub-cohorts of differently senescent RBC by flow cytometry, taking advantage of senescence-dependent differences of the physical parameters of cells, i.e., cell size and granularity. Then, a blood separation according to density was made with the idea that an increase in 4-HNE conjugates in the young RBC fraction mirrors stress erythropoiesis processes in the marrow and, elevated conjugation of senescent fractions indicates that 4-HNE may flag RBC for their phagocytosis. FACS and WB analysis were useful for the study of modifications in both the whole RBC and its membrane respectively. Specifically, 4-HNE-conjugation could become a hallmark of senescent cells with decreased anti-oxidative capacity and increased phagocytosis rates. To find out which RBC membrane proteins might be most implicated in modifications by 4-HNE, further comparison of WB results obtained with study cohort children and with subfractions of RBC from healthy blood donors, have been conducted. and the spectrin as functionally relevant and well characterized cytoskeletal protein was studied for potential changes in labeling with 4-HNE. A spectrin-4-HNE-dependent rigidification of RBC could explain an increase of phagocytosis and finally anemia, which remains to be shown subsequently.

Preliminary data from our laboratory suggest cell age-dependent differences in RBC phagocytosis. Feeding primary human monocytes with RBC fractions of different 4-HNE-load revealed most avidly phagocytosis for highly 4-HNE conjugated RBC.

Phagocytosis assays with young RBC supplemented with exogenous 4-HNE as model for lipoperoxidation-dependent modifications in absence of alternative changes by oxidative challenge and RBC from anemia patients need further to confirm the hypothesis of 4-HNE-driven pathogenesis of anemia.

It remains to be studied whether the use of 4-HNE scavengers may be a useful tool for anemia therapy in future.

CHAPTER 2

Materials and methods

2.1 Analysis of RBC membrane modifications of anemic children

Anemic children were analyzed for membrane modifications in peripheral RBC with emphasis on lipoperoxidation processes such as protein conjugations with lipoperoxidation product 4-HNE.

2.1.1 Patients

4-HNE- conjugates in RBC membranes were quantified from 264 donors. Samples used in the present study were collected during a double-blind randomized placebo-controlled trial conducted on non-immune 349 children from 2·5 to 24 months of age in Centro de Investigação em Saúde de Manhiça (CISM), located in Manhiça, Maputo Province, southern Mozambique to study the rate of acquisition of naturally acquired immunity against malaria in different period of infancy (Guinovart et al., 2012). Aguilar et al. assessed oxidative markers including 4-HNE-conjugates and membrane-bound hemoglobin, in 349 non-immune Mozambican newborns (which from now will be called cohort 1) at first acute malaria episode and in convalescence. recruited from the previous double-blind placebo-controlled chemoprophylaxis trial. 4-HNE conjugates were here assessed on RBC surface by flow cytometric analysis while membrane-bound Hb and hemicromes were studied in isolated RBC membranes by the hem-dependent luminol-enhanced luminescence method (Aguilar et al., 2014).

In my studies, blood samples from 264 Mozambican children (cohort 2) living in the Manhiça District Maputo Province, a South-Est African malaria–endemic area, were selected and analyzed. Children included were from 1 to 68 months of age and each of them was specifically described by the following parameters age, gender, hematological indices (peripheral blood cell count, mean cell Hb, blood Hb, white blood cells), nutritional facts (iron deficiency, malnutrition and levels of vitamin A, vitamin B12), infectious and inflammatory parameters (bacterial and viral infection, CRP, fever, presence of *P. falciparum* infection (parasitemia by PCR). Patients samples which were positively tested for HIV were excluded from analysis. Clinical and paraclinical information was accessible in a data base received from the Menendez group (Barcelona) together with samples. RBC were collected and preserved in glycerolyte cryo-preservation solution in 1.5 ml cryo-vials immediately after blood sampling in EDTA-containing vials, buffy-coat removal and washing. They were stored in a biological bank of Barcelona in liquid nitrogen until shipment in dry ice. RBC samples were stored until processing under nitrogen at -80°C for 0-10 months.

2.1.2 Membrane protein analysis of RBC

During malaria anemia, RBC undergo numerous structural changes. Many of these changes take place at the membrane and they could represent important pathomechanistic factors, relevant for the onset of anemia. Protein modifications by oxidation and products of lipoperoxidation, were analyzed in both isolated RBC membranes and intact RBC.

2.1.2.1 Preparation of membranes from fresh and frozen RBC

To extract RBC membranes, a hypotonic ice-cold buffered solution was used. Any centrifugation step was performed at 4°C. RBC suspension was thawed under non-reducing conditions by adding hypotonic lysis buffer (10 mM KH_2PO_4, pH 8 supplemented with 1mM EDTA and Complete® protease inhibitor cocktail at standard concentration as indicated by manufacturer). The first step was RBC lysis by adding lysis buffer at ten-fold volume excess to the still frozen RBC-suspension and keeping it on ice for 5 min shaking several times. The lysate was distributed in 4 microtubes of 1.5 ml (to facilitate membranes extraction), centrifugated for 3 minutes at 25100 g, and supernatant was discarded. The membrane-containing pellet was washed at least 5 times and centrifuged at 25100 g for 1 minute at 4°C. When the supernatant appeared colorless (free of hemoglobin), the sedimented membrane pellets of the same sample were unified and resuspended. Three aliquots (two µl each) were taken for protein quantification and for membrane bound heme quantification, respectively. The remaining ghost pellet was solubilized with 5 times concentrated Laemmli sample buffer (Laemmli, 1973) for quantitative protein extraction and stored at -20°C. To test the suitability of the frozen blood samples for analysis of oxidative parameters, membrane-bound heme and Hb and 4-HNE-membrane protein conjugates were compared between ghosts prepared from washed RBC immediately after blood sampling and those prepared from stored (in RBC of the same blood sample. Freezing and storage was performed in glycerolyte at -80°C. RBC membranes were extracted by hypotonic lysis. After electrophoretic protein separation and Western blot by immune-chemistry (as detailed below), the differences between both were insignificant with regard to 4-HNE-membrane protein conjugates. Thus, the frozen blood from the Biobank can be considered representative for the blood at withdrawal.

Regarding membrane- bound heme (as detailed below), the total-ghost HEME of freshly analyzed RBC was higher than those from frozen RBC thus the obtained results might be biased by the freezing/thawing cycle with glycerolyte.

2.1.2.2 Protein quantification

RBC ghost proteins were quantified using the Bradford assay. BioRad Dye Reagent Concentrate (BioRad, Heidemannstrabe München, Germany) was added to the diluted aliquots of ghosts, vortexed and placed in the cuvette for the measurement of absorbance in a spectrophotometer at 595 nm, as indicated by the manufacturer. The standard curve with bovine serum albumin (BSA, BioRad 2mg/ml) was obtained by measuring solutions of 0.5, 1.0, 2.0 mg/ml in triplicate. The

protein concentration of samples was calculated based on the standard curve. Results were expressed in μg/μl and insert in the database.

2.1.2.3 SDS-PAGE

Twenty μg of solubilized ghost proteins were separated in a 8% polyacrylamide gel electrophoresis, containing 0.5% (w/v, final concentration) sodium dodecyl sulfate (8% SDS-PAGE) and transferred to nitrocellulose membranes. Prior loading samples on the top of the slab gels beta-mercapto-ethanol was added at 5% (v/v) final concentration to the solubilized protein samples to reduce sulfhydryl groups of cysteine residues, breaking down sulfur bridges and 0.01% bromophenol blue (to monitor the progress of electrophoretic separation). In parallel to ghost protein samples, molecular weights markers (Amersham Full-Range Rainbow 12-225 kDa), bovine serum albumin (BSA) as 4-HNE-negative control and 4-HNE-conjugated BSA as HNE-positive control were loaded.

Running gels were prepared at 8% of acrylamide by mixing H_2O, acrylamide/N', N'-methylene-bis-acrylamide (30%), 1.5 M Tris (pH 8.8), 10% sodium dodecyl sulfate (SDS), 10% ammonium persulfate (APS) (SIGMA,Japan), Tetramethyl ethylene-diamine (TEMED). After solidification of gels, stacking gel was prepared by mixing H_2O, acrylamide/ N', N'-methylene-bis-acrylamide mix (30%), 1.5 M Tris (pH 6.8), SDS, APS, TEMED, and layered on top of the running gel and a 10-well comb inserted before solidification. Electrophoretic run was performed in a Trizma base/ glycine buffer, containing 0.1% SDS. The electrophoretic separation was started at 50 V to align proteins and continued at 150V for separation, at room temperature (RT) until the end of the electrophoretic run.

2.1.2.4 4-hydroxynonenal conjugated protein analysis by western blot

After completed protein separation, the proteins were transferred from the gel to nitrocellulose membranes (previously activated in transfer Buffer (containing Trizma base, glycine, SDS and methanol 15% (v/v). Transfer conditions were 70V for at least 3 hours at 4°C. To visualize protein bands on the transfer membrane, I used Ponceau Red (trichloroacetic acid 6%, Ponceau Red 0.2%, H2O) that binds the amino groups of lysine and arginine while protein staining in the gel was performed with Coomassie Blue (Coomassie Blue R 0.2%, methanol 50% acetic acid 7%, H2O) to check transfer efficiency.

After Ponceau Red staining, membranes were washed with milli-Q water and blocked with bovine serum albumin (BSA 5% (w/v) in phosphate buffered saline (PBS), containing 0.1% (v/v) Tween (PBS-T) for 60 min to prevent non-specific antibody binding. Blot membranes were incubated with mouse monoclonal primary antibody anti-4-HNE conjugates HNEJ-2 (AbCam) at a final dilution of 1:2000 in PBS-T, supplemented with 1% BSA (w/v), at 4°C overnight. Thereafter, membranes were washed 3 times for 15 minutes with PBS-T and incubated for 1 hour at room temperature with a secondary goat anti-mouse horse radish peroxidase (HRP)-conjugated antibody, diluted 1:20000

(Santa Cruz Biotechnology) in PBS-T, supplemented with 1% (w/v) BSA. To detect bands, I used Enhanced Chemiluminescence (ECL) based on electron transfer from hydrogen peroxide to luminol catalyzed by HRP, which is conjugated to the secondary antibody. Reduced luminol is luminescent and visualizes in my case 4-HNE-protein conjugates. To collect and elaborate quantitative data of protein conjugates the luminescence intensity was transformed in optical density (OD) by the acquisition in a Chemidoc-touch apparatus (ChemiDoc™ Touch Gel Imaging System 1708370), and densitometry analysis was performed by Image Lab. The OD of any protein band was referred to the OD of the positive control band to obtain normalized values (expressed in Relative Optical Density) which allows the comparison between samples. Normalized OD values are referred to as relative 4-HNE-protein conjugates.

Positive control was obtained by modifying BSA with 4-HNE (adapted from Crabb, 2002). Briefly, 250 µL BSA dissolved in 100 mM sodium acetate at pH 4.5 were added to 25 µL of 10 µmole HNE) dissolved in ethanol and incubated for 15-min at 37°C, then frozen (Crabb et al., 2002).

When necessary, the membranes have been stripped with a stripping buffer (0.2 M glycine, 1% Tween, 0.1% SDS, pH 2.2). Anti-beta Actin antibody (ab8227) was used as control at a dilution of 1:2000 in PBS-Tween.

2.1.3 Measurement of hemoglobin (Hb) and heme bound to RBC membranes

Heme-dependent luminol-enhanced luminescence was utilized to quantify Hb or heme bound to the cytoplasmic surface of RBCs. Luminescence was measured exploiting the hem-associated peroxidase activity which catalyzes the electron transfer from t-butyl-hydroperoxide (Sigma Chemical Co.) to luminol (5-amino-2.3-dihydro-l,4-phthalazinedione, Sigma Chemical Co.) (Demehin et al., 2002). RBCs suspensions of known cell number served as heme standard, which allows the normalization of samples.

2.1.3.1 Solubilization of ghosts

Aliquots of 10 ul of ghosts were solubilized for at least 3 hours at 4°C in 0,5 ml of 0.1 N NaOH, containing 3 mM EDTA, 0.05% Triton X100 to quantify the total heme content. The total heme content of ghosts includes adherent hemoglobin and hemoglobin oxidation products, hemichromes. The latter are insoluble at physiologic pH. For this, other aliquots of 10 ul of ghosts were incubated for 45 minutes at RT in 1 ml of PBS containing 3 mM EDTA, 0.05% Triton X100 to quantify hemoglobin. Aliquots of 1 ul of standard were incubated with 1 and 0.5 ml of NaOH-EDTA-Triton and PBS-EDTA-Triton, respectively. Two replicates were solubilized and two aliquots of either used for luminescence measurement.

2.1.3.2 Luminescence assay

As to the velocity of the luminescence reaction, the used luminometer (Sirius, Berthold Technologies GmbH & Co.KG, Bad Wildbad, Germany) needs two pumps that inject specific

solutions in a luminometer tube wherein the sample was manually pipetted. The solution for the injector 1 contained 100 ml of 0,1 N of NaOH, EDTA 3 mM, 28 µl of luminol 3,5 mg/ml DMSO while the solution for the injector 2 contained 100 ml of 0,1 N of NaOH, EDTA 3 mM, 100 µl t-butyl-hydroperoxide. Samples solubilized at neutral pH in PBS, were centrifuged at 13000 rpm (21500 g) for 1 minute to pellet particles and aliquots of sample and standard supernatants were measured in the luminometer against blank (PBS, containing EDTA and triton X100). Similarly, samples solubilized in NaOH/ EDTA/ triton X100 were measured in the luminometer against blank (only NaOH, containing EDTA and triton X100). The instrument releases data of luminescence in a time interval in relative luminescence Units per second (RLU/s) which is proportional to the amount of heme in the measured sample. Assuming one RBC contains 2 fmole of heme, total heme concentration and RLU/fmole heme were calculated for standard cell-suspension to compare it to sample heme concentration.

Hemichrome amount in samples was expressed in pmole/mg (normalized with protein concentration mg/ml) and were obtained by subtracting the results of the first reading (PBS) from the second reading (NaOH). In this way, hemichrome values were obtained for 113 of 173 children cohort. The total ghost-HEME means all types of heme species, especially denatured and oxidized Hb and expressed as total ghost-HEME.

2.1.4 Statistical methods

To set several data of children, all 264 samples were divided in two main groups based on Hb levels (g/dl). Children having Hb values less than 11 g/dl (1.4 to 10.9 g/dl) were considered anemic (N=180) while children with Hb levels greater or equal to 11 (11 to 15.3 g/dl) were considered non-anemic (N= 84). The anemic group was further subdivided in severe anemic group (N= 27; Hb < 5 g/dl) and not severe anemic group (n= 153; 5 g/dl ≤ Hb levels < 11 g/dl).

Another classification of the whole cohort concerned the presence/absence of malaria parasite (proven by qPCR). In this way the anemia group was further divided in a malaria (N= 72) and a no malaria group (N= 108) and also the non-anemic group in a malaria (N= 7) and no malaria group (N= 77).

Additionally, the two main groups were divided also for the presence of inflammation according to CRP values above 1.0 mg/dL. In the anemia group 66 children had inflammation while in the non-anemia group 13. Measured parameter values were tested for significant differences between groups by t-Student test.

To determine the correlation of 4-HNE-conjugates with the level of blood Hb, the Spearman rank correlation test was applied, where *R* is the Spearman's correlation coefficient (values of -1 and +1 are maximum correlations). To evaluate the statistical significance of correlation, I used STATA Data Analysis and Statistical Software.

2.2 Lipoperoxidation as function of RBC senescence

To check whether lipoperoxidation and subsequent 4-HNE production is detectable in senescent RBC, RBCs were separated according to cell age and analyzed for 4-HNE-protein- conjugates.

2.2.1 Density separation of human RBC

Human blood from healthy donors (volunteers from Sant'Anna AVIS hospital) was collected in 6 ml heparin-coated vacutainer tubes and processed within 1 hour after collection to separate RBC according their densities after careful removal of leukocytes and platelets.

2.2.1.1 White blood cells and thrombocytes deprivation from whole blood by cellulose

To remove leukocytes and platelets from peripheral RBC, heparinized blood was passed through a cellulose column at room temperature (RT) (Venkatesan et al., 2012). WBC interact with cellulose and remain in the solid phase while RBC quickly move with the mobile phase to leave the column.

A 20 ml syringe cylinder was used as column and a small tap instead of the needle has been mounted on the outlet conus to allow the control of flow-through and elution. The filling slurry for 1ml of whole blood to be separated, was prepared from 1 g of alpha cellulose (Sigma Aldrich C-8002 9004-34-6) which was resuspended in 10 ml of phosphate buffered saline supplemented with 10 mM glucose (PBS-G).

Before filling the column, a circle-shaped Whatman paper was placed at the bottom of the syringe to cover the outlet. Subsequently the cellulose slurry was filled in and cellulose allowed to sediment. Another piece of paper was placed on top of the cellulose and the column was equilibrated with PBS-G. The column was first hydrated with about 10 ml PBS-G, then loaded with 3 ml of the diluted blood (for each ml of whole blood, 0.5 ml of PBS-G was added). During the passage of blood through the column, PBS-G was added on the top to prevent the column from running dry. PBS-G was added to wash the column and eluate was collected until the whole column became nearly white. The filtrated leukocyte- and thrombocyte-depleted blood was collected in a 50 ml tube, washed with PBS-G and centrifuged for 5 minutes at 2500 rpm in a Heraeus centrifuge at 15 °C.

2.2.1.2 Determination of hematocrit

Washed RBC suspension was used to measure the hematocrit in a microcapillary by centrifugation. The ratio between solid particle and whole sample volume is calculated and expressed as percentage. The hematocrit has been adjusted to 30% with PBS-G.

2.2.1.3 Senescence- dependent fractionation of RBC on percoll gradient

RBC suspensions at 30% hematocrit was gently overlaid on a continuous density gradient previously pre-formed from 7.5 ml of 75% Percoll colloidal suspension in 14X95 mm Beckman tubes by centrifugation at 15600 rpm and 10° C for 20 min. Percoll (GE Healthcare, Uppsala,

Sweden), 228 mM NaCl (Sigma Aldrich)**;** 5 mM glucose (Sigma Aldrich); 90 mM Na_2HPO_4 (Sigma Aldrich)**;** H_2O.

RBC were separated in a Beckman SW40 rotor, run at 15600 rpm for 20 minutes at 4°C. The collection of the observed different fractions was performed manually with pipettes from top to bottom.

RBC of each fraction (0.1-0.4ml) were washed twice with PBS-G in 15 ml tubes and sedimented at 2500 rpm for 5 minutes with brake. The passage on hyperosmotic Percoll temporarily modifies erythrocytes and for this reason the addition of PBS-G for washing occurs slowly (drop by drop to avoid any cellular swellings).

Before starting decoration with antibodies for FACS acquisition or membrane preparation (ghosts), samples in PBS-G were transferred into 1.5 ml micro tubes and kept in cell incubator at 37°C, in a 5% CO_2/95% air (v/v) atmosphere for 1 hour recovery.

2.2.2 Physical parameters of RBC fractions assessed by FACS

For each test sample a micro tube was prepared with 80 ul of PBS, supplemented with 5mM glucose (PBS-G5) and 2% (v/v) fetal calf serum (FCS) (PBS-G5-FCS 2%) in which 2 ul of packed RBC were resuspended. RBC suspension was incubated with the primary antibody directed against 4-HNE-protein conjugates (HNE11-S Alpha Diagnostics, San Antonio, Texas, USA) at a final dilution of 1:50 for 30 minutes at RT. Then, RBCs were washed 2 times with PBS-G5-FCS 2% and sedimented at 21500 g for 1 minute, resuspended in 50 ul of PBS-G-FCS 2% and incubated with anti-rabbit IgG whole molecule conjugated with fluorescein isothiocyanate (FITC) from Sigma at final dilution of 1:400 for 30 minutes in the dark. In parallel another tube with 2 ul of the same blood sample was similarly treated with only secondary antibody in absence of the primary antibody as negative control. Cells were washed to remove excess antibodies and resuspended in PBS-G-FCS 2% for flow cytometry analysis in a Millipore easy Cyte Guava cytofluorimeter. Green fluorescence of FITC was recorded in the green channel and data analyzed using Guava Software 3.1.1.

2.2.3 Senescence parameters of RBC fractions assessed by FACS

Antigens were assessed on the surface of RBC from each fraction.

Antibodies used were: anti transferrin receptor (CD71)-FITC Abcam (Ab 239251); anti Glycophorin A (CD235A) Abcam (Ab 233580) and anti-integrin associated protein (CD47; Biolegend, San Diego, California) with its own isotype Mouse IgG1, κ (Biolegend).

After half an hour of incubation in the dark and at room temperature (except for CD47 antibody whose incubation conditions were 15-20 minutes in the dark and on ice) samples were washed twice with PBS-G5-FCS 2%. Reading at FACS was performed in 400 ul of suspension green channel. For analysis of samples, Guava Software 3.1.1 was used.

2.2.4 Reticulocyte count in RBC fractions by Brilliant Cresyl Blue staining

Aqueous Brilliant Cresyl blue (Sigma) solution was prepared at 1% (w/v) supplemented with 3.8 % sodium citrate (w/v) and filtered before use. Whole blood and brilliant cresyl blue solution were mixed at 1:1 ratio in a 1.5 ml microtube, mixed thoroughly and kept at dark and RT for 30 minutes. Then a thin smear was prepared from a drop of blood which was swiped on a microscope slide (MARIENFELD 1000004) with the edge of another slide. Once dried, the slide was observed with an oil immersion objective of 100-fold magnification (Zeiss Primo Star). Brilliant Cresyl Blue stains nucleic acids which are present in reticulocytes but not in mature RBC allowing differential counting of the dark-blue spotted reticulocytes and the bright RBCs under the microscope.

This protocol was applied for the three RBC fractions obtained from a density gradient after ultracentrifugation of whole blood to compare the quantity of reticulocytes and to see differences especially between the old (1F) and the young (3F) cell fraction.

Slides prepared in this way were analyzed immediately.

2.2.5 4-hydroxynonenal conjugates in intact RBC of different senescence stages by FACS analysis

Previous studies demonstrated that RBC become smaller with increasing cell age. Exploiting this age-dependent feature, I analyzed RBC from 14 healthy donors for 2 physical parameters, cell size and cell granularity, which are usually assessed in FACS analysis.

Unwashed fresh blood of 14 healthy donors was used to assess 4-HNE protein conjugates on RBC surface by FACS analysis.

I subdivided the entire cohort of RBC which are distributed along the x- and y-axis according to cell size and granularity, respectively in several sub-cohorts in order to analyze 4-HNE-protein conjugates on RBC of a defined narrow range of size. with aim to determine 4-HNE-conjugates of RBCs of different size and to conclude on the dependency on cell age.

Analyzing the physical parameters, I realized that the incubation with the antibodies slightly altered cell granularity and size. For this reason, I decided to abandon this method and separate blood according to RBC density on Percoll. After separation of RBC, I obtained 3 different fractions, each one was characterized first for physical parameters, cell-age dependent surface antigens and finally for 4-HNE-protein conjugates.

2.2.6 4-hydroxynonenal conjugates in isolated RBC membranes assessed by immunochemistry

To extract RBC membranes from each fraction, an ice-cold hypo-osmolar lysis buffer (10 mM KH_2PO_4, pH 8 supplemented with 1mM EDTA and Complete® protease inhibitor cocktail at standard concentration as indicated by manufacturer) was used. Any centrifugation step was performed at 4°C. The first step was RBC lysis by adding lysis buffer at ten-fold volume excess to the ice-cold RBC-suspension and keeping it on ice for 5 min shaking several times. The lysate was

distributed in microtubes of 1.5 ml and centrifugated for 3 minutes at 25100 g. The supernatant was discarded and the membrane-containing pellet was washed at least 5 times with lysis buffer and centrifuged at 25100 g for 1 minute at 4°C at a time. When the supernatant appeared colorless (free of hemoglobin), the sedimented membrane pellet was resuspended. Three aliquots were taken for protein quantification. The remaining ghost pellet was solubilized with 5 times concentrated Laemmli sample buffer (Laemmli, 1973) for quantitative protein extraction and stored at -20°C.

2.2.6.1 Electrophoretic separation of ghost proteins

Twenty μg of solubilized ghost proteins were separated in a 8% polyacrylamide gel electrophoresis, containing sodium dodecyl sulfate (8% SDS-PAGE) under reducing conditions applying the same protocol as described in 2.1.2.3.

The transfer of separated proteins to nitrocellulose, immune-detection of 4-HNE-conjugated protein bands with a specific anti-4-HNE-conjugate antibody and HRP-conjugated secondary antibody and visualization by ECL were performed as outlined in 2.1.2.4.

2.2.6.2 Analysis of 4-HNE- conjugated membrane proteins by western blot

To obtain quantitative data for protein conjugates the luminescence intensity of protein bands was acquired and transformed in optical density (OD) by a ChemiDoc-touch apparatus (ChemiDoc™ Touch Gel Imaging System 1708370), and densitometry analysis was performed by Image Lab. The OD of any protein band was referred to the OD of the positive control band on the same blot membrane to obtain normalized values (expressed in Relative Optical Density) which allows the comparison between samples. Normalized OD values are referred to as relative 4-HNE-protein conjugates. The analysis of 4-HNE-membrane protein conjugates in RBC fractions quantified i) the total content of 4-HNE conjugates in the membrane protein fraction by summarizing the ODs of all labelled protein bands in a sample lane and ii) 4-HNE conjugates with specific proteins by assessing the OD of single protein bands, e.g., alpha- and beta-spectrins.

2.2.7 Statistical methods

To compare values obtained from different fractions, I used Test-T. To evaluate the statistical significance of correlation, I used STATA Data Analysis and Statistical Software.

CHAPTER 3

Results

3.1 Correlation of blood hemoglobin with membrane-bound heme and 4-hydroxynonenal-protein conjugates in RBC of infants

Few previous studies describe membrane-bound hemoglobin levels in RBC of several anemia forms and data on 4-HNE-conjugates in anemia are scarce.

3.1.1 Impact of RBC storage on membrane-bound heme and 4-hydroxynonenal-protein conjugates in RBC

The present study investigates membrane-bound Hb and 4-HNE-protein conjugates in RBC which were stored in glycerolyte in liquid nitrogen in a Bio-bank after sampling as explicitly described in the methods paragraph. Storage may rise doubts on cell integrity and potential storage artifacts. For this the validity of measured date had to be proven for both parameters, by comparing frozen samples with fresh ones from the same blood donation. I prepared membrane from RBCs immediately after blood sampling and after freezing and storage in glycerolyte at -80°C. Membrane- bound heme and 4-HNE-membrane protein conjugates were compared between both membrane preparations.

As Figure 14 shows, the pattern of 4-HNE-membrane protein conjugate bands is very similar between freshly analyzed and stored RBC (lane 1 and 3, respectively). No additional conjugates appear and the intensities of the single 4-HNE-protein conjugate bands are quite similar between freshly drawn and stored RBC, indicating that the 4-HNE-conjugation of membrane proteins does not increase for storage. Also, no loss of membrane conjugates by freezing RBC in glycerolyte is detectable. The supplementation of exogenous HNE to freshly drawn RBC results in a very similar increase of detectable conjugates in both freshly analyzed as well as glycerolyte -stored RBC. (Figure 14, lane 2 vs. lane 1). Thus, the assessment of 4-HNE-conjugates in bio-banked RBC samples will yield useful data on lipoperoxidation-dependent protein modifications in study participants.

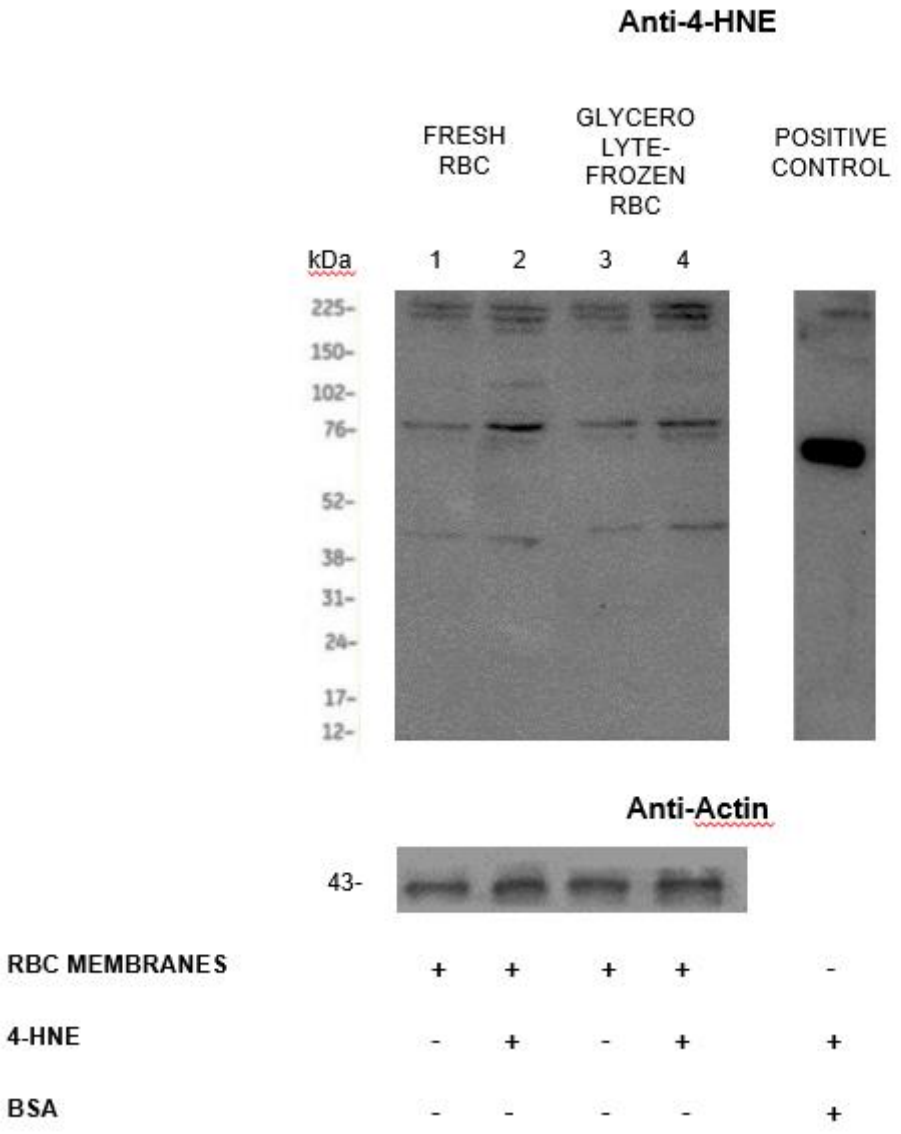


Figure 14. 4-HNE-membrane protein conjugate pattern in freshly drawn RBC and RBC after glycerolyte storage. The left two lanes (1 and 2) show conjugates of RBC membranes from freshly drawn blood, the middle lanes (3 and 4) from frozen, in glycerolyte stored RBC. Lanes 1 and 3 are not treated RBC, lanes 2 and 4 RBC incubated with 4-HNE. The positive control of lane 5 is BSA conjugated with 4-HNE (as detailed in methods).
The quantity of sample proteins transferred to the blotting membrane was checked by visualization of actin with anti-actin antibodies (bottom panel). Amersham Full Range molecular weight standards were mounted.

In contrast to membrane 4-HNE conjugates, membrane-bound heme levels detected by an heme-dependent luminol enhanced luminescence assay (luminometer Sirius, Berthold Technologies GmbH & Co.KG, Bad Wildbad, Germany), differed substantially between frozen-stored and freshly used RBC. Surprisingly, freshly analyzed RBC, had 2-3 times higher values of membrane bound heme than RBC which were frozen in glycerolyte before membrane preparation, the opposite I had expected in case of a storage-dependent oxidation (Figure 15) and I can rule out false high values due to storage. A lower intracellular hemoglobin concentration in frozen RBC is a less plausible reason for low Hb-membrane binding because the freezing solution glycerolyte protects RBCs against leakiness and no substantial Hb leakage occurs. A more plausible explanation for low Hb-membrane binding in frozen RBC is that the high glycerol concentration impedes binding of Hb to the inner membrane side at low ionic strength during cell lysis by the hypo-osmotic lysis buffer. Membrane-bound Hb measured in frozen-stored RBC might not mirror *in vivo* conditions.

Figure 15. Membrane-bound heme in membranes of freshly drawn RBC and RBC after glycerolyte storage. Mean of membrane-bound-Heme (nmole/mg) levels +/- SE of fresh RBC and glycerolite- frozen ones from 4 different donors Comparison is significative. Test T calculated a p value <0.001.

Heme was quantified in membranes prepared from RBCs either immediately after blood draw (FRESH) or after storage of an aliquot of the same RBC sample in glycerolyte at -80 C (GLYCEROLYTE) by heme-dependent luminol-enhanced luminescence. RBC membrane aliquots of known protein content were solubilized in Triton X100/ 0.1N NaOH (see methods for details) and assessed for heme-dependent luminescence. Luminescence was transformed in heme content by using a heme standard. The heme content is referred to protein content of membrane sample (nmoles/mg).

3.1.2 Correlation between RBC membrane protein-bound 4-hydroxynonenal and blood hemoglobin in study participants

In contrast to membrane bound-Heme, 4-HNE-conjugates were preserved and did not change under the applied storage and thawing conditions.

This allowed to collect 4-HNE data which are representative for the level of lipoperoxidation in freshly drawn RBC. 4-HNE-conjugates in RBC membranes were quantified from 264 donors and referred to a conjugate standard to eliminate errors by sensitivity variations of the antibody-based assay over a period of time (e.g., by antibody affinity changes, ECL-luminescence output differences). The obtained normalized values for 4-HNE-conjugates varied between donors from 0.1 to 2.5, which is due to the inter-individual variation of lipoperoxidation and the resulting 4-HNE level in RBC. Plotting the 4-HNE conjugate value paired with the individual blood Hb at the moment of bleed for all participating donors (N=264), the regression analysis allows the conclusion that blood Hb is inversely correlated with the level of 4-HNE-conjugates in RBC membranes (Figure 16; Spearman's rho was -0,483, with 262 degrees of freedom and $p=7,2 \times 10^{-17}$).

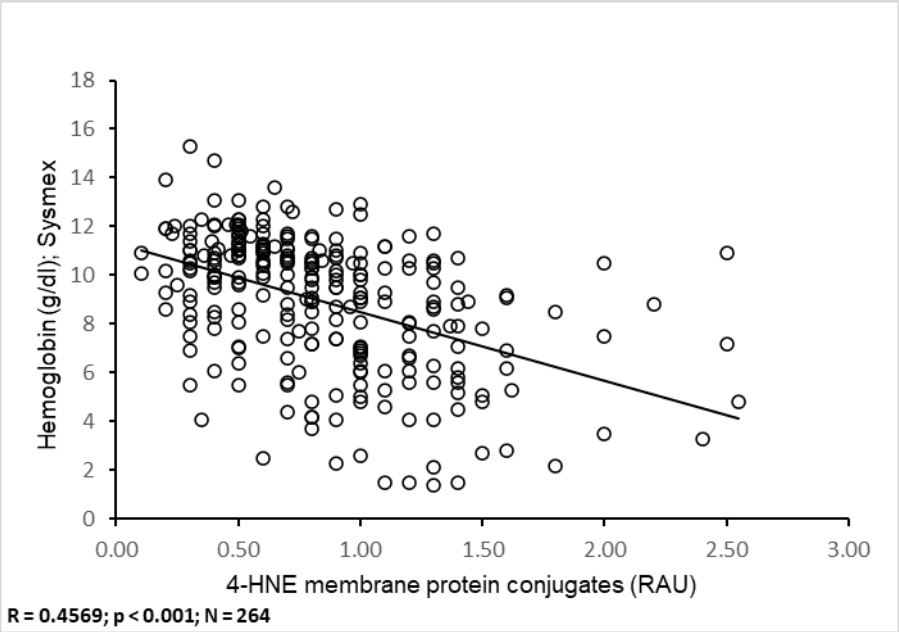


Figure 16. Correlation between 4-HNE membrane protein conjugates and blood Hb in study cohort (N = 264). Extracted RBC membrane proteins were used to perform SDS-PAGE and Western Blotting analysis with specific anti-HNE-conjugate antibodies. Each point in the diagram represents the individual data pair of the blood-Hb and the amount of 4-HNE conjugated to the RBC-membrane of each single child determined in the same blood sample. The Hb values of the y-axis were measured by semiautomatic cell counter (Sysmex F800 microcell counter, TOA Medical Electronics, Kobe, Japan). On the x-axis the level of 4-HNE-protein conjugates in the RBC membrane are plotted as Relative Arbitrary Units (RAU; see methods for details). The correlation of 4-HNE-protein conjugates with Hb was assessed using Spearman Rank correlation test, where R is Spearman's correlation coefficient. The correlation is significant at p-value equal to 7.2 X 10^{-17}.

Having obtained this result, means an inverse correlation between levels of blood Hb and 4-HNE membrane protein conjugates, I wanted to understand if this correlation was maintained in subgroups of anemia. For this reason, I divided the anemic group (N= 180) in 2 subgroups: the severe anemia group with children with blood Hb <5 g/dL (N= 27) and the not severe anemia group with children with blood Hb between 5 and 10.9 g/dL (N=153). The remaining children with blood Hb ≥11 g/dL formed the non-anemic group. Spearman's test was used to evaluate the significance of correlation. Only in case of the not severe anemia group I found a statistically significant inverse correlation between blood Hb and 4-HNE-conjugation in RBC with a p-value <0.05 (Spearman's rho was -0.19042 with 151 degree of freedom and p= 0.018388) (Figure 18), while values showed a weak negative linear relationship in the severe anemia group with p=0.069 (N=27) (Figure 17) and no correlation in the non anemic control group (p=0.16, N=68) (Figure 19).

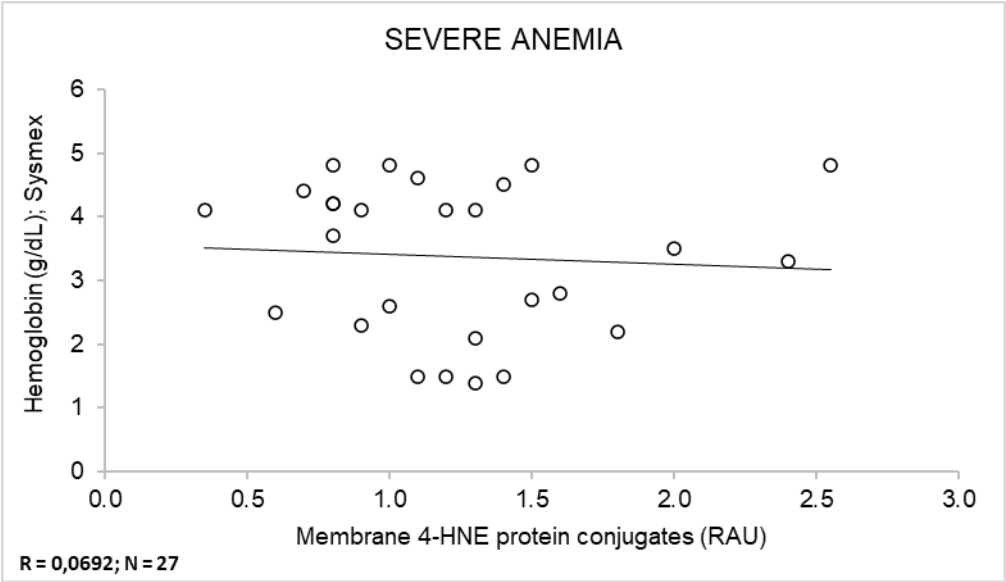


Figure 17. Correlation between 4-HNE membrane protein conjugates and blood in severe anemic subgroup. Regression curve between levels of blood Hb (g/dL and plotted on y axis) and levels of membrane 4-HNE protein conjugates (RAU and plotted on x axis). Each circle represents one child belonging to severe anemia group (Hb < 5 g/dL; N= 27). Using Spearman's test, no significative correlation was found (p-value: 0.4641).

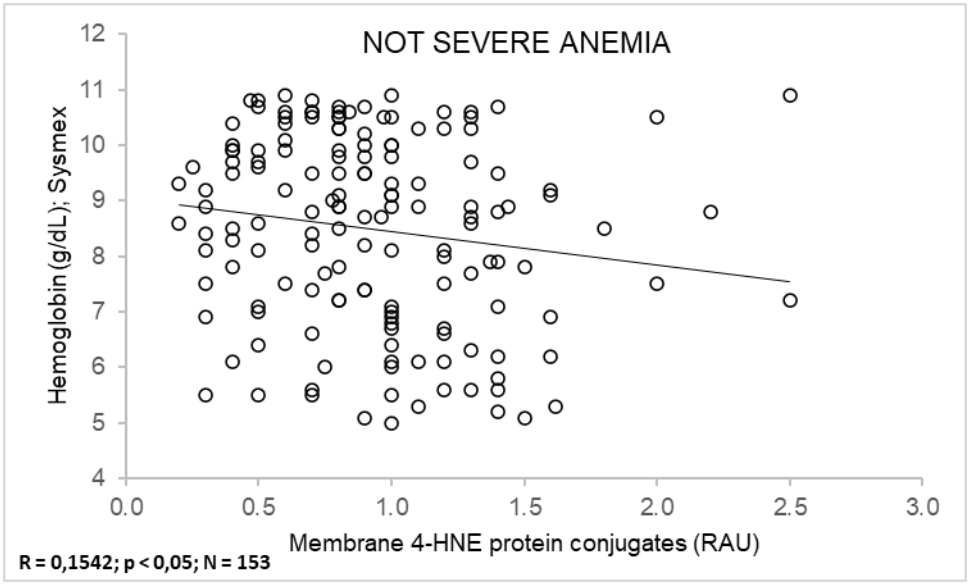


Figure 18. Correlation between 4-HNE membrane protein conjugates and blood in not severe anemic subgroup. Regression between levels of blood Hb (g/dL and plotted on y axis) and levels of membrane 4-HNE protein conjugates (OD and plotted on x axis). Each circle represents one child belonging to not severe anemia group (5 <Hb< 10.9 g/dL; N= 153). Using Spearman's test, significative correlation was found (p-value: 0.0183).

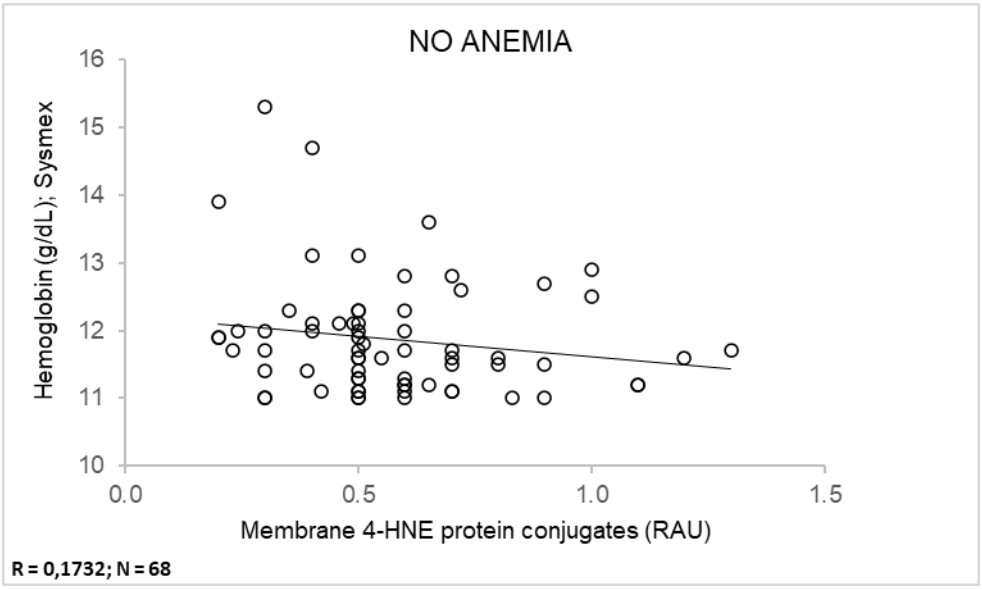


Figure 19. Correlation between 4-HNE membrane protein conjugates compared and blood Hb in non-anemic subgroup. Regression between levels of blood Hb (g/dL and plotted on y axis) and levels of membrane 4-HNE protein conjugates (OD and plotted on x axis). Each circle represents one child belonging to no anemia group (Hb ≥ 11 g/dL; N= 153). Using Spearman's test, no significative correlation was found (p-value: 0.1659).

3.1.3 Membrane-bound Hb and hemichromes in analyzed RBC from study participants

The oxidative parameter, membrane-bound heme in RBC, which includes any membrane standing heme-containing molecule, i.e. hemoglobin and hemichromes, significantly correlates with blood Hb of study participants (p=0.000248). The regression curve shows positive significantly between membrane-bound heme on blood Hb (Figure 20) in contrast to what I had expected (i.e. an inverse correlation between heme species and Hb levels). Consequently, significantly lower levels of membrane-bound heme were measured in the anemic as compared to the non-anemic cohort (Figure 21) and anemia patients show a significantly (p=0.016698) positive correlation of the parameter with blood Hb, too (Figure 22). Here, membrane-bound Hb, not showing a negative correlation with levels of blood Hb, but positively associated with it, appear to be unpromising in their contribution to anemia. Taking in mind the strong modification of this parameter by the freezing/thawing procedure of RBC, I could suppose that the obtained data may not mainly mirror changes due to the redox state in the study participants at the time of blood draw but depend on the Hb concentration in the lysate.

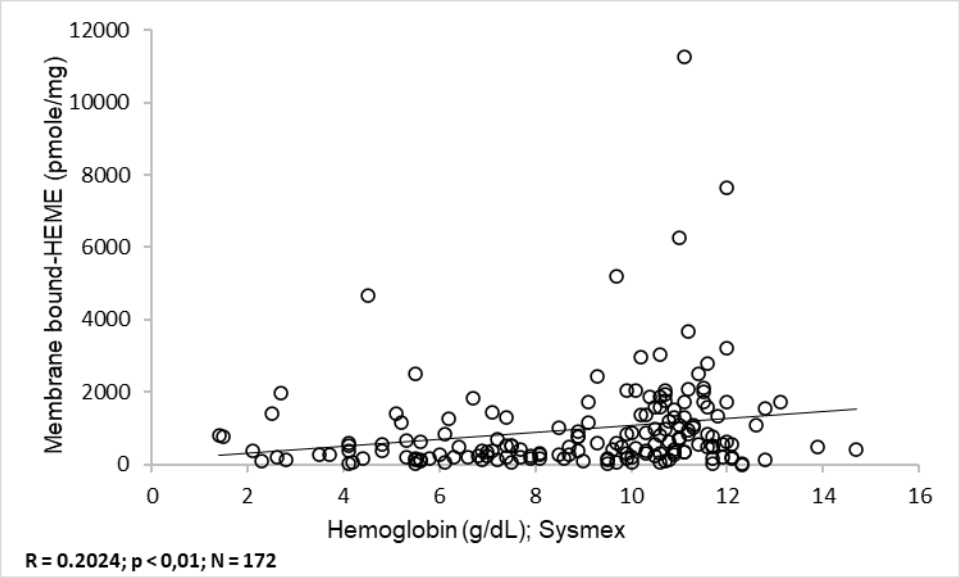


Figure 20. Correlation between membrane-bound HEME (pmole/mg) and levels of blood Hb (g/dL) in study cohort (N = 172). Correlation found was linear: low levels of membrane bound-HEME correspond to low levels of blood Hb and viceversa. P-value calculated by Spearman's test was equal to 0.000248.

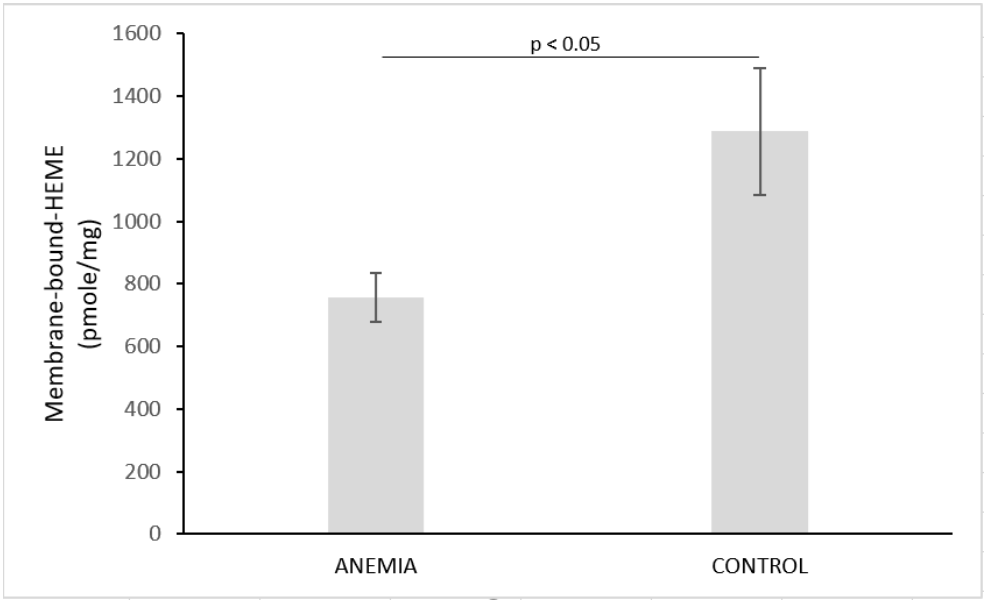


Figure 21. Comparison of levels of membrane-bound-HEME (pmole/mg) between anemic and control children group. Mean of membrane-bound-HEME (pmole/mg) levels +/- SE of anemic children (N= 122; Hb < 11 g/dl) and control (N= 51; Hb > 11 g/dl) was plotted. Comparison is significative (p value: 0.0180).

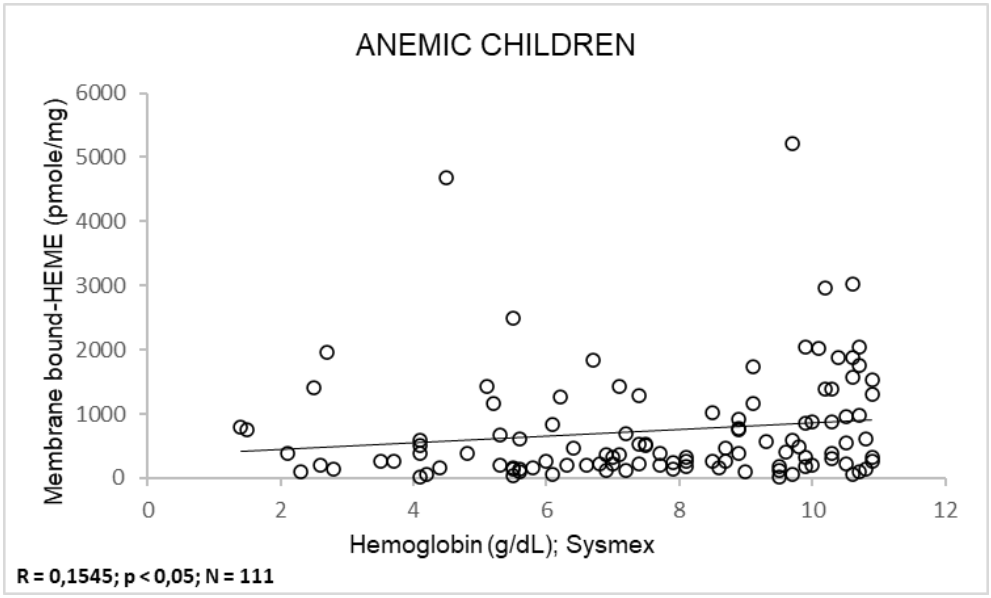


Figure 22. Correlation between levels of blood Hb and membrane-bound heme in anemic subgroup (N = 111). Membrane bound-HEME were matched with blood Hb levels. Correlation found was positive and linear (R= 0.1545): highest membrane bound-HEME values correspond to high Hb values. Spearman's test reveals a significant correlation with a p-value equal to 0.0166.

The lack of correspondence between measured membrane bound heme and oxidative stress is sustained by the inverse correlation between membrane-Heme and the oxidative stress marker 4-HNE membrane protein conjugates (Figure 23), that is the second marked used in the present study.

Spearman test found a significant reciprocal correlation between these two oxidative parameters (p-value: 0.000335).

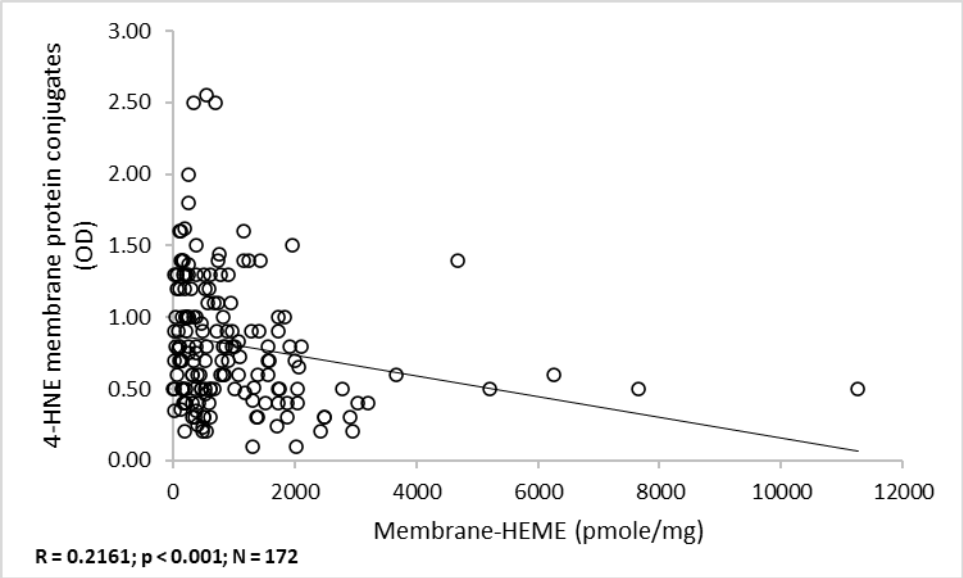
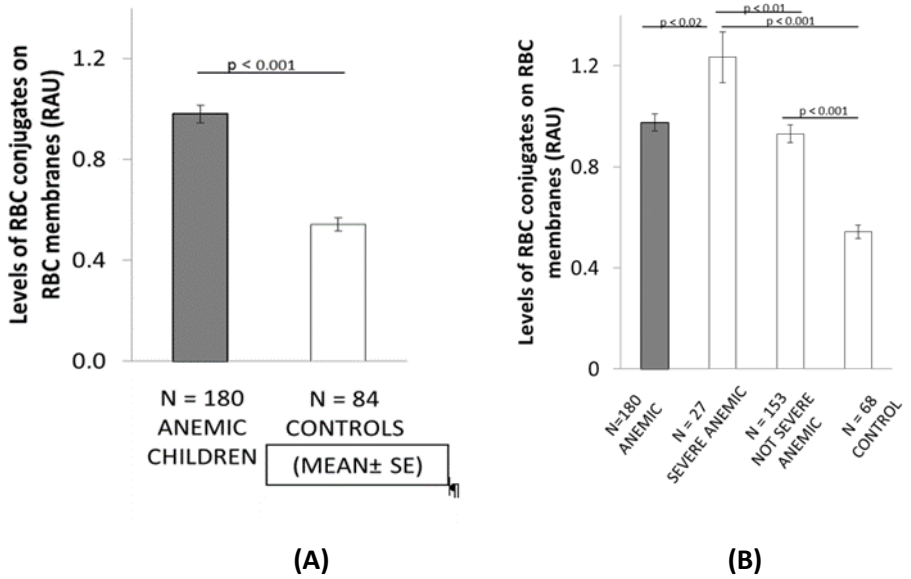


Figure 23. Correlation between membrane-Heme and 4-HNE membrane protein conjugates in study cohort (N = 172). Membrane bound-Heme were matched with 4-HNE membrane protein conjugates levels. Correlation found was inverse (R= 0.2161): lowest membrane bound-Heme values correspond to high 4-HNE membrane protein conjugates. Spearman's test reveals a significant correlation with a p-value equal to 0.000335.

3.2 Increased 4-hydroxynonenal-conjugate levels in moderate and severe anemia

In light of the regression curves plotted above, I compared the average values of 4-HNE membrane protein conjugates (found in all 264 RBC membranes of children) firstly, between the group of anemia patients and the control group and secondly, between anemia subgroups and the control group (Figure 24). As expected, RBC from anemic children carried nearly the double of 4-HNE conjugates compared to the non-anemic controls. RBC from children with severe, but also mild anemia had significantly higher 4-HNE-protein conjugate levels than cells from non-anemic study participants. Interestingly, the 4-HNE load of RBC seems to determine also anemia severity. Thus, cells from severe anemic children had significantly higher 4-HNE loads (approximately +25%) than mild anemia RBC.



(A) **(B)**

Figure 24. (A). Comparison of levels of membrane HNE-protein conjugates (RAU) between anemic and control children group. (N= 180; Hb < 11 g/dl) and control (N= 84; Hb > 11 g/dl). Mean HNE-conjugate levels +/- SE of anemic children (Hb < 11 g/dl; N= 180) and non-anemic children (Hb >/=11 g/dl; N= 84;) was plotted. Comparison is significative (p value < 0.001).

(B). Membrane HNE-protein conjugates (RAU) in dependency on severity of anemia.

The comparison of membrane HNE-protein conjugates (RAU) was performed between total anemic children (N=180; Hb < 11 g/dl), severe anemic children (N= 27; Hb < 5 g/dl), not severe anemic children (N= 153; Hb ≥ 5 g/dl) and control children group (N= 68; Hb ≥ 11 g/dl). Mean HNE-conjugate levels +/- SE of anemic children (Hb < 11 g/dl; N= 180); severe anemic children (N= 27); not severe anemic children (N= 153) and control (N= 68).

3.3 Increased 4-hydroxynonenal-conjugates in malaria anemia and non-malaria anemia

Looking for the origin of the increased 4-HNE conjugation in anemic children the etiology of anemia and the likely trigger for lipoperoxidation became important. First, I analyzed the contribution of malaria infection for anemia.

Table 2. Percentage and number of anemic children (N=180) and non-anemic one (N= 84) and malaria incidence.

ANEMIA N = 180		NON ANEMIA N = 84	
MALARIA	NON MALARIA	MALARIA	NON MALARIA
72 **(40%)**	108 (60%)	7 (8.3%)	77 (91.6%)

Nearly all malaria patients were anemic (approximatively 90%) as shown in Table 2, but malaria surprisingly turned out to be the cause for 40% of all anemia cases, only, and 60% of anemic children had etiologies different from malaria infection (Table 2). Hence, the significantly higher 4-HNE-conjugates in the whole cohort of anemic children in comparison with non-anemic ones (Figure 24A) cannot be explained by the occurrence of an acute malaria infection, alone. Thus, I analyzed the 4-HNE-conjugation in anemic vs. non-anemic children in sub cohorts formed according to anemia etiology. Dividing the children in 3 sub-groups: children with malaria, non-malaria inflammation, and other etiologies (mainly iron deficiency and malnutrition that means children presenting a number of nutritional deficiencies) a significant increase in 4-HNE conjugation accompanied anemia in any etiologic group. The mean of 4-HNE conjugates increased by 35-45% in anemia as compared to non-anemic controls in the malaria as well as the non-malaria inflammation sub-cohort, in the residual etiologies sub-cohort the increase was more modest with approximately 15%, though significant. The increase in malaria was highest if malaria was accompanied by inflammation, as usually the case in malaria. The increase of 4-HNE-conjugates in RBC membranes which was found in any anemia form regardless of malaria infection or increased inflammatory processes is a quite exciting result regarding a potential role of 4-HNE in anemia pathogenesis.

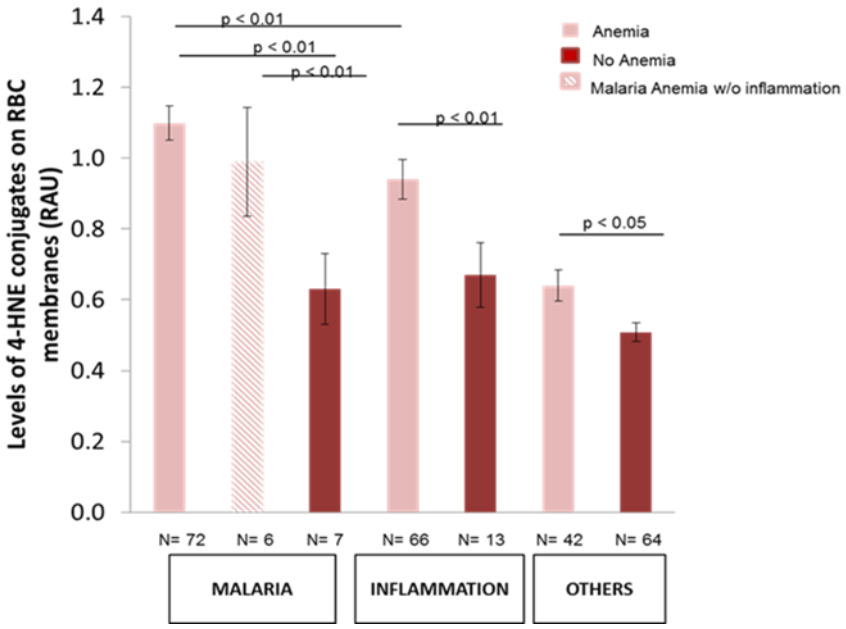


Figure 25. Levels of 4-HNE membrane-protein conjugates in sub-groups of anemic children. Comparison of mean values of 4-HNE membrane-protein conjugates (RAU) in anemic and parasite positive children with inflammation (N= 72) and without inflammation (N= 6) with the non-anemic children group (N= 7). The same comparison was done for anemic children with only inflammation (N= 66) and the non-anemic children group (N= 13) The last pair of columns shows the comparison between anemic children with etiologies different from malaria and inflammation (N= 42) and the respective non anemic children group (N= 64).

3.4 Correlation between 4-hydroxynonenal conjugation levels in RBC and blood parameters

In search for the cause of elevated 4-HNE production and conjugation I performed a series of correlation tests between 4-HNE-protein conjugates in RBC membranes and paraclinical parameters.

3.4.1 Correlation of 4-hydroxynonenal-protein conjugates with parasite count

I performed regression analysis between 4-HNE-conjugates and parasite count/µl as indicator of malaria infection to conclude on the direct effect of parasites on the 4-HNE load. In the database the entire cohort of malaria-positive tested children consists of qPCR-positive and/or microscopically positive individuals. Only qPCR assays offer reliable quantitative parasite counts/ul at low parasite load thus, not all 79 malaria positive children could be included in the regression analysis but only children with a positive qPCR (N=38), both, anemic and non-anemic.

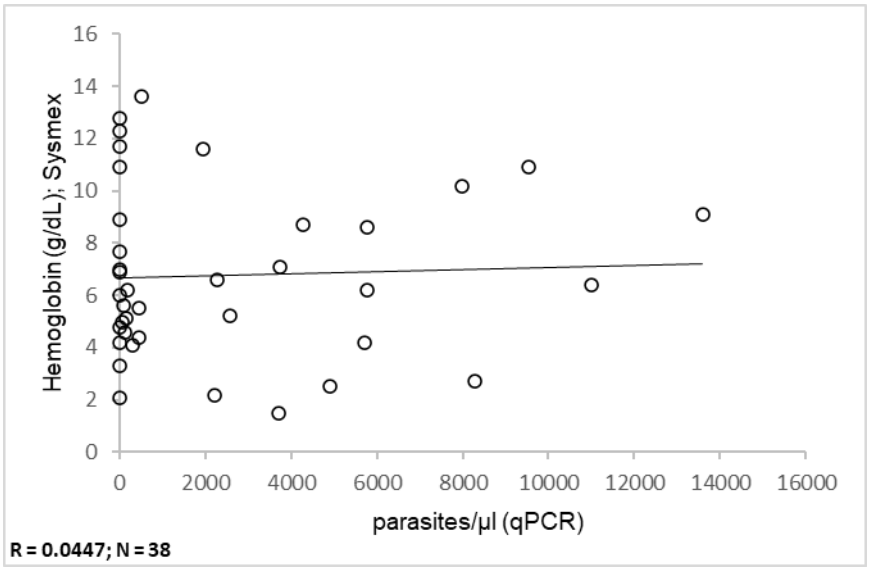


Figure 26. Correlation between levels of blood Hb and parasite count/µl. Regression was analyzed between values of blood Hb (g/dL) and parasites count (parasite/µl; measured with qPCR) in 38 children with or without anemia (specifically N= 33 anemic children and N = 5 non anemic children). A non-significant linear relationship was found (p-value = 0.9812).

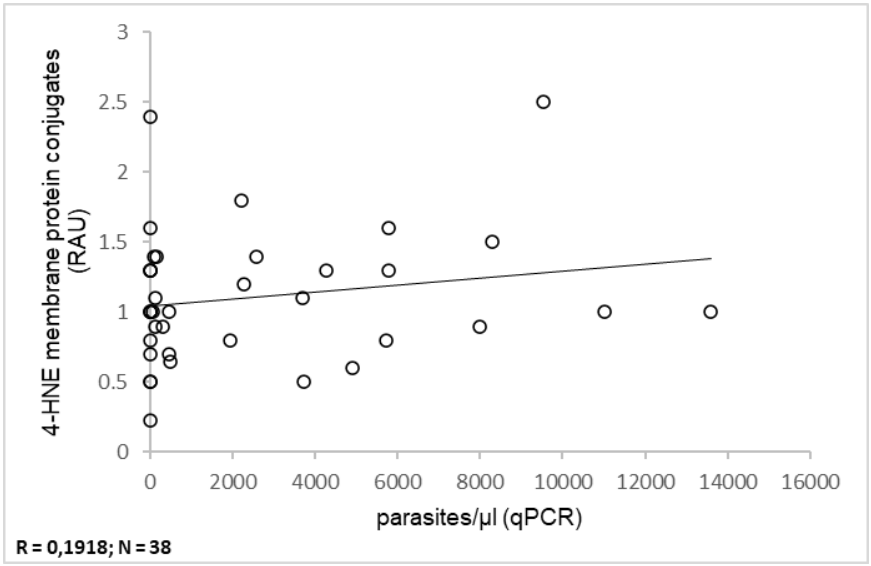


Figure 27. Correlation between 4-HNE membrane protein conjugates and parasite count /µl. Each circle represents the values for the parasite count (parasite/µl; measured with qPCR) and 4-HNE conjugates of a single study participant. The correlation between levels of 4-HNE membrane protein conjugates and parasites count was calculated for N = 38 children and is shown as linear regression curve. Correlation is weakly positive: In about half of the cases, high values of parasites/µl correspond to high values of 4-HNE membrane protein conjugates levels but there is also a small part where low levels of parasites/µl correspond to high values of 4-HNE membrane protein conjugates. Correlation wasn't significant (p-value = 0.7432).

By analyzing the relationship of both blood hemoglobin and levels of 4-HNE membrane protein conjugates with parasites/µl by regression analysis, I obtained a weak and insignificant positive relationship in both cases. High levels of hemoglobin corresponded to high levels of parasites/µl (R=0.047; *p-value* = 0.9812). The lacking negative correlation between parasite counts and blood-Hb (R=0.047, p= 0.9812, N=38) does not support the idea of a direct effect of parasites on anemia development at very low and low parasite count (ranging from 0.12-13596 parasites/µl blood which corresponds to a parasitemia of roughly 0.000011- 0.2%) in the small study cohort. Also, among non-anemic children with malaria, only 40% had very low parasites/µl, i.e. parasitemia (qPCR) of 1.25 to1.6 parasites/µl.

At such low parasitemia, the parasite itself does obviously not cause profound lipoperoxidations in the whole RBC cohort, as concluded from a very weak (R=0.1918; p-value =0.7432) positive correlation between 4-HNE-conjugation and parasitemia. Specifically, children (58%) with very low levels of parasites/µl had very variable levels of 4-HNE membrane protein conjugates ranging from 0.5 to 2.4 RAU. The relative high percentage of children with very low parasite counts that show a high level of 4-HNE conjugates make it more likely that additional factors induced by the parasite, but not dependent on parasite count determine the lipoperoxidation. These factors may depend on individual host responses to infection, e.g., immune reactions, and be not determined by parasite count at low parasitemia.

3.4.2 Correlation of 4-hydroxynonenal-protein conjugates with C-reactive protein

Inflammation was accompanied by increased 4-HNE conjugation in children infected or not with malaria (shown in Figure 25). For this reason, I checked the correlation between levels of 4-HNE membrane protein conjugates and the decisive indicator of inflammation, the C-reactive protein (CRP).

As Figure 28 shows, data reveal a positive linear correlation (R=0.3254): increasing levels of 4-HNE-membrane protein conjugates were significantly associated with increasing levels of CRP (*p-value*: 6.9×10^{-23}).

Interestingly, confining the regression analysis of 4-HNE membrane protein conjugates to low CRP (<1 mg/dL) cases which represent non-inflammatory states, a significant correlation with CRP between 0 to 1 mg/dL was seen, too (Figure 29). Note, that 4-HNE-values rarely rose above 0.8 RLU in children with such low CRP values in contrast to children with CRP above 1mg/dl.

Correlations of 4-HNE conjugates with CRP were seen in anemic as well as non-anemic children supporting the hypothesis of inflammation as the direct source of 4-HNE regardless of anemia and the accompanying hypoxic state (Figure 30).

Analyzing the anemic cohort (Hb values ranging from 1.4 to 10.9 g/dL) for correlation between 4-HNE membrane conjugates values and CRP levels the dependency found was positive as in the entire cohort and significant with a p-value of 5.3×10^{-11}. With regard to non-anemic children, both parameters were positively associated and 4-HNE membrane protein conjugates were significantly correlated with CRP levels in the non-anemic sub-group, too (R=0.4553; p-value=0.014).

Inflammation frequently causes iron deficiency and I verified whether the strong positive correlation between 4-HNE and CRP was maintained regardless of iron deficiency. Eighty children of the 264 had no detectable iron deficiency. As shown in Figure 29, 4-HNE membrane protein conjugates increase when CRP values increase in this sub-cohort of children without iron deficiency, too. I found a linear positive correlation (R=0.5926) that Spearman's test verified as significant, in fact p-value was <0.01. The correlation between 4-HNE conjugation and CRP in the anemic sub-cohort of children without iron deficiency (N=49) was similarly significant as in the entire cohort of not iron deficient children supporting the primary role of inflammation for lipoperoxidation and minimize the importance of iron deficiency for oxidative lipid modifications and 4-HNE production.

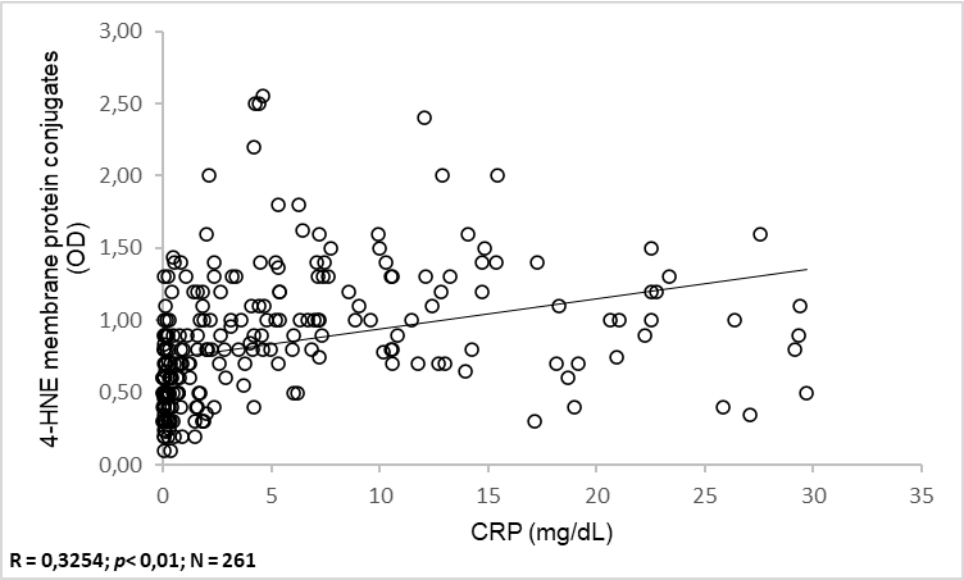


Figure 28. Correlation between 4-HNE-membrane protein conjugates and CRP levels. 4-HNE membrane protein conjugate values of 261 children were paired with the respective levels of CRP from 0.01 to 29.71 mg/dL. Correlation found (R: 0.3254) was significant. P-value by Spearman's test was equal to 6.92x 10^{-23}.

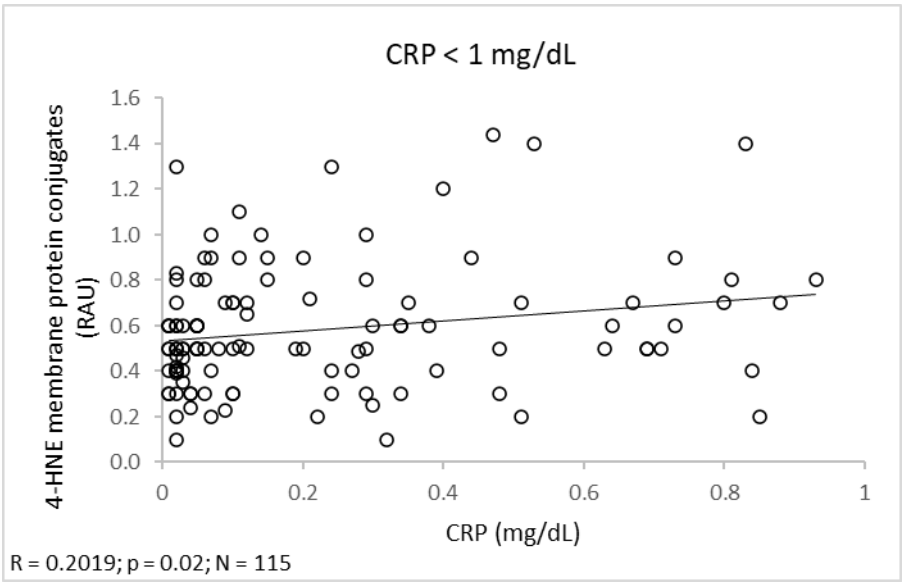


Figure 29. Correlation between 4-HNE membrane protein conjugates and selected (N= 115) levels of CRP (< 1 mg/dL). From this comparison high values of 4-HNE membrane protein conjugates overlap with CRP levels very close to 1. This linear positive correlation (R: 0.2019) is significative for Spearman's test (p-value: 0.0160).

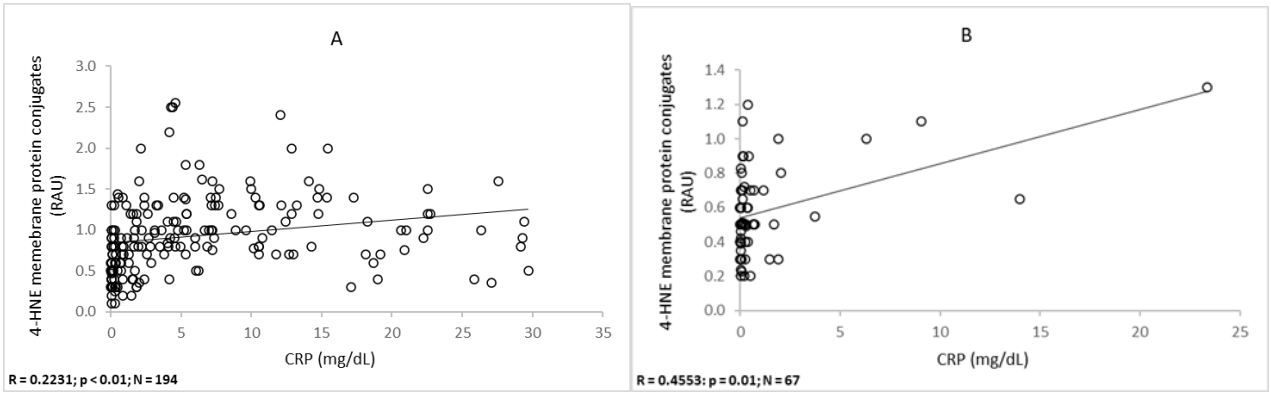


Figure 30. Correlation between 4-HNE membrane protein conjugates and levels of CRP in anemic children (A) and non-anemic controls (B). ***(A)*** *By pairing values of 4-HNE membrane protein conjugates (RAU) measured on RBC membranes of 194 anemic children with levels of CRP (mg/dL) turns out a linear positive correlation (R: 0.2231; p-value: 5.3x* 10^{-11}*).* ***(B)*** *By pairing values of 4-HNE membrane protein conjugates (RAU) of 67 children without anemia with levels of CRP (mg/dL) a linear positive correlation (R: 0.4553; p-value: 3.57x* 10^{-10}*) is observed.*

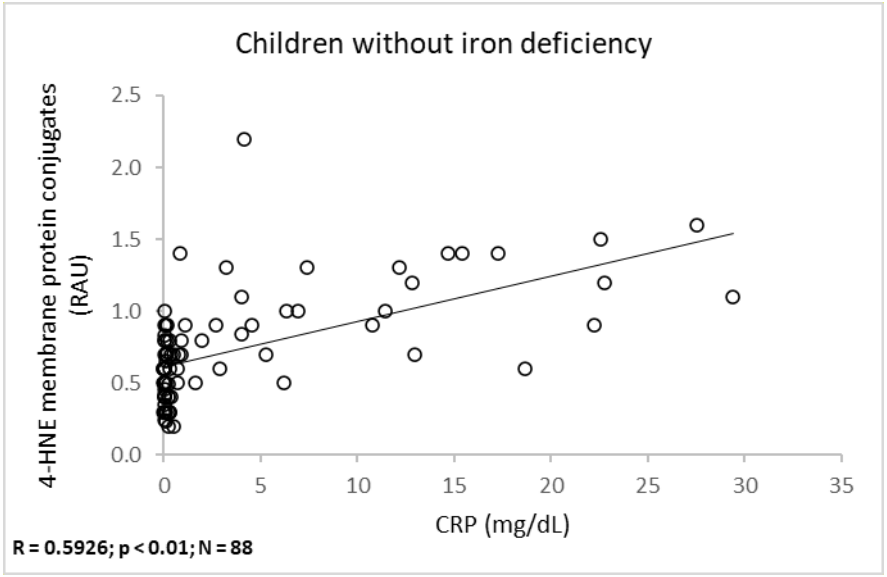


Figure 31. Correlation between 4-HNE-membrane protein conjugates and CRP levels in children without iron deficiency. 4-HNE membrane protein conjugate values of 88 children without iron deficiency were paired with the respective levels of CRP.

From this combination, increasing values of 4-HNE membrane protein conjugates (RAU) were accompanied by increasing levels of CRP (mg/dL). The significance of this linear positive correlation (R: 0.5926) was verified by Spearman's test that reveals a value equal to 8.77216x 10^{-10}.

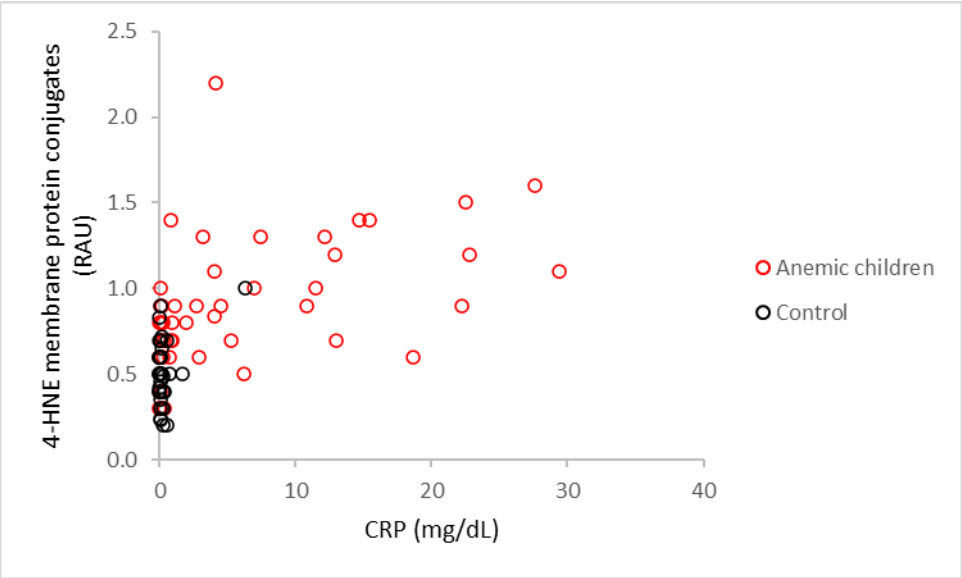


Figure 32. Correlation between 4-HNE-membrane protein conjugates and CRP levels in children with or without anemia. From this combination, high values of 4-HNE membrane protein conjugates (RAU) were accompanied by high levels of CRP (mg/dL). To distinguish anemic children from control, the first ones have been indicated with a red circle and the second with black circle like previous regressions.

3.4.3 Correlation of 4-hydroxynonenal-protein conjugates with plasma iron and soluble transferrin receptor

A widespread problem in sub-Saharan Africa and an important cause of anemia among children is iron deficiency. I used levels of plasma iron and of soluble transferrin receptor (sTfR) as well-established markers for iron deficiency anemia, and verified their correlation with 4-HNE-protein conjugates in RBC. Low levels of iron are accompanied by high levels of sTfR. I found that low levels of plasma iron, mirroring an anemic status, are accompanied by high levels of 4-HNE membrane protein conjugates. The inverse correlation (R=0.1676) was significant with p equal to 9.92E-07 according to Spearman's test (Figure 33). With regard to sTfR, I found that high levels of 4-HNE membrane protein conjugates coincide with high levels of sTfR (R=0.2315). The linear positive correlation was significant p= 0.003078) (Figure 34). Trying to relate iron to CRP levels, an inverse correlation has emerged (R: 0.2049; Figure 35). Spearman's test gives a significant result. P-value was equal to 9.01x 10-21 Based on the linear relationships between iron and 4-HNE conjugates and iron and CRP I hypothesize that inflammation that provokes 4-HNE increase is also responsible for the observed iron deficiency. Thus, the correlation between 4-HNE and iron might be or not causal. I checked whether the correlation between 4-HNE and iron was detectable in children without inflammation and found that this is not the case with p=0.9622 (Figure 36) and conclude that the iron deficiency alone does not determine the high 4-HNE-load. I cannot exclude however, that 4-HNE is a possible molecular mediator of inflammation-elicited iron deficiency, e.g., modifying transferrin or its receptor which was not investigated in this study.

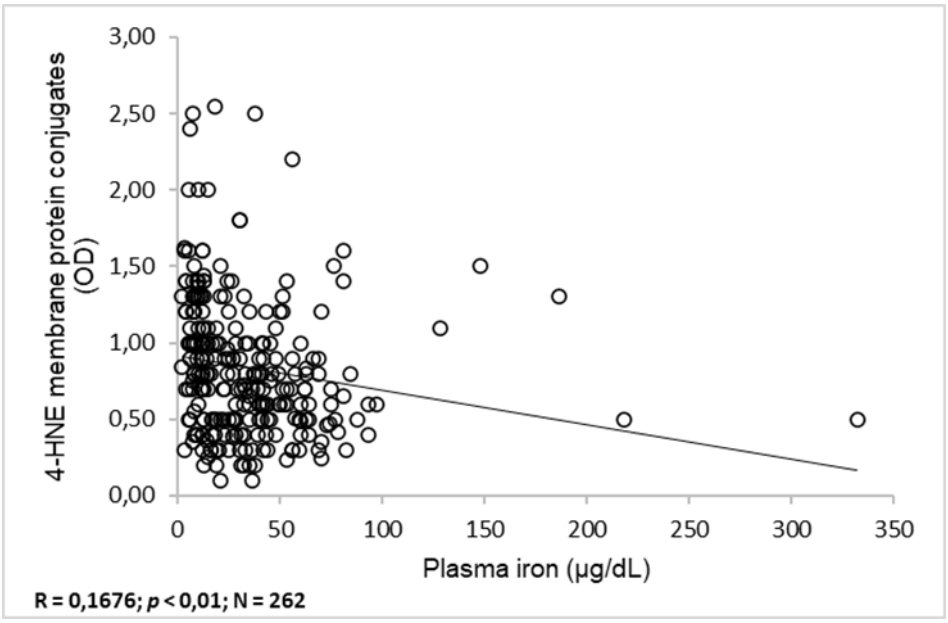


Figure 33. Correlation between 4-HNE membrane protein conjugates (OD) and levels of plasma iron (µg/dL) in the whole cohort of children (N = 262).

4-HNE membrane protein conjugates of 262 children (plotted on y axis) were coupled with levels of plasma iron (plotted on x axis). Correlation found was inverse (R = 0.1676): high levels of 4-HNE membrane protein conjugates coincide with low levels of plasma iron. This correlation was significant (p-value = 9.92x 10^{-7}).

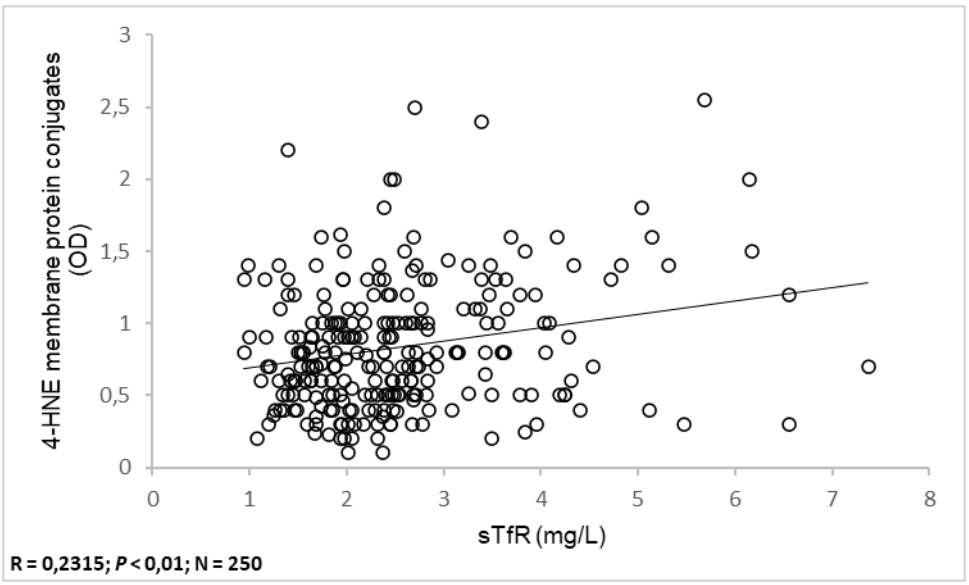


Figure 34. Correlation between 4-HNE membrane protein conjugates (RAU) and levels of sTfR (mg/L). RBC membranes of 250 children were paired with levels of sTfR (mg/L). Correlation found was positive: high levels of sTfR coincide with high levels of 4-HNE membrane protein conjugates (R = 0.2315). Correlation was significative with a p-value equal to 0.003078.

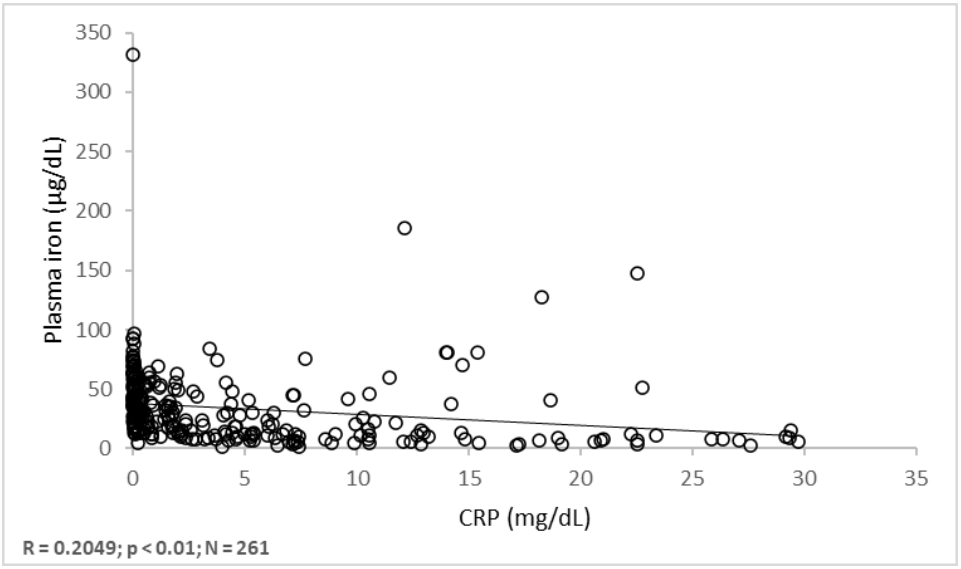


Figure 35. Correlation between levels of plasma iron (µg/dL) and CRP levels (mg/dL) in study cohort. 261 children were analyzed for both parameters of plasma iron and CRP levels. Correspondence found was inverse and p-value significative in fact Spearman's test calculated a value equal to 9.01 X 10^{-21}.

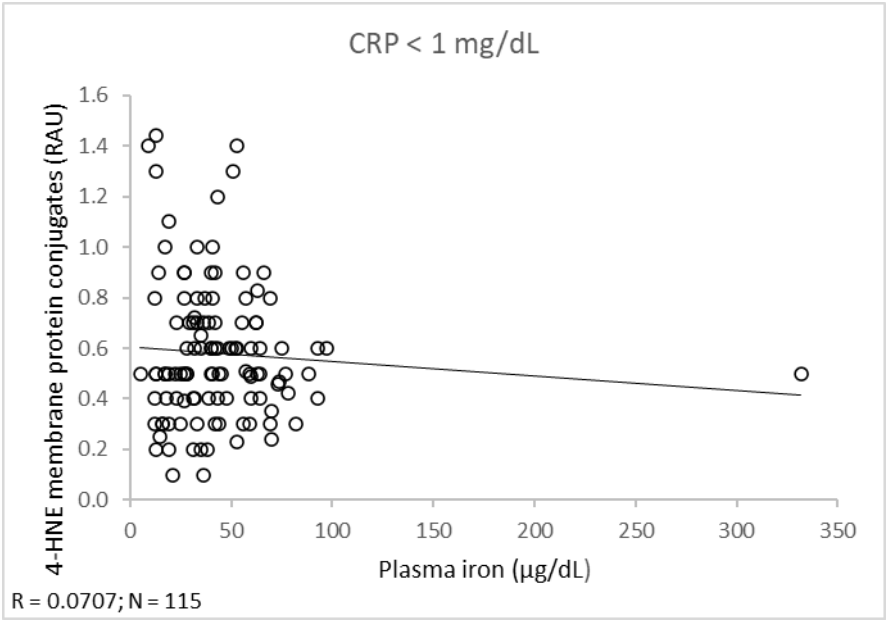


Figure 36. Correlation of 4-HNE membrane protein conjugates and plasma iron levels in children without inflammation (CRP < 1 mg/dL). 155 children without inflammation were tested for both 4-HNE membrane protein conjugates and plasma iron levels. From this pairing, correlation was inverse: high levels of 4-HNE membrane protein conjugates coincide with low levels of plasma iron even if p-value wasn't significative (0.9622).

3.4.4 Correlation of 4-hydroxynonenal-protein conjugates with Vitamin A

Levels of 4-HNE membrane protein conjugates were also analyzed for their possible relationship with levels of vitamin A that I have considered as an indicator of malnutrition. From this comparison, I found that high levels of 4-HNE membrane protein conjugates were associated with low levels of Vitamin A (Figure 37). The weak linear inverse correlation (R: 0.1232) was significant (Spearman's test p-value= 0.0511).

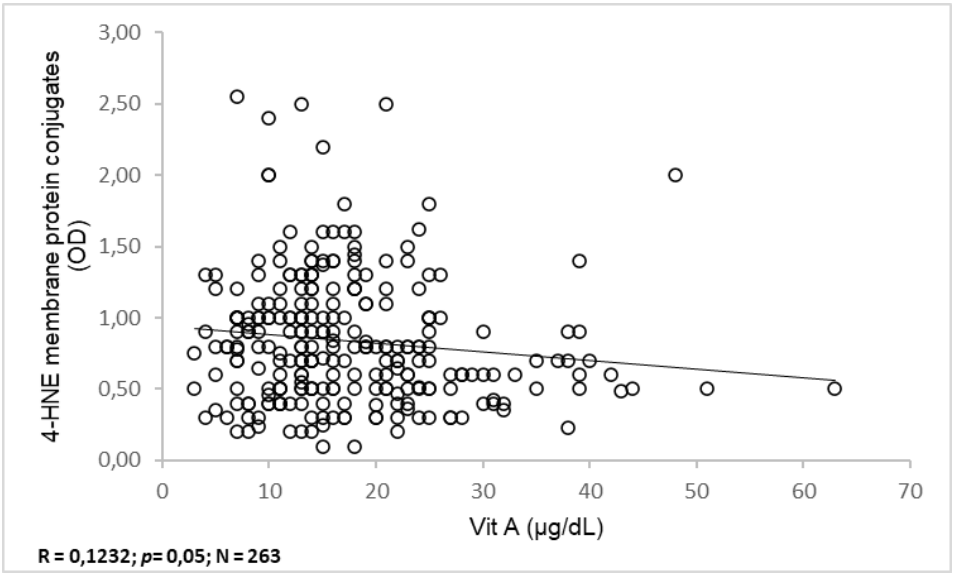


Figure 37. Correlation between 4-HNE membrane protein conjugates (OD) and levels of Vit A (µg/dL) in the whole cohort of children (N = 263)

Correlation found was inverse and Spearman's test reveals a significative p-value, equal to 0.0511.

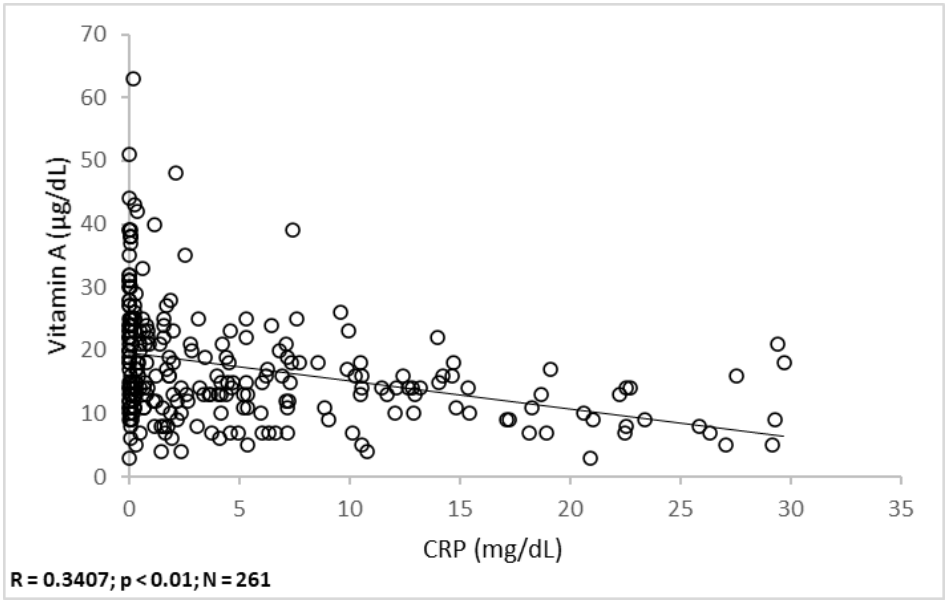


Figure 38. Correlation between levels of Vitamin A and levels of CRP in study cohort (N = 261). Comparison reveals an inverse and significant correlation in fact p-value obtained through Spearman's test was equal to 1.73x 10^{-11}, corresponding to p-value = 1.72X 10^{-11}.

One reason for Vitamin A deficiency is an inflammatory state and a significant linear inverse correlation between Vitamin A and CRP confirms the relationship of both parameters in the study group (Figure 38). To test whether vitamin A as regulator of hematopoiesis (RA/RAR cascade) may independently from inflammation contribute to an increase of 4-HNE conjugates, I excluded from the entire study group children with inflammation and performed regression analysis in the restricted cohort in absence of inflammation. Levels of 4-HNE membrane protein conjugates did no longer correlate with Vitamin A levels and Spearman's test calculated a p-value equal to 0.2 (Figure 39). This likely excludes Vitamin A as independent determinant for 4-HNE-conjugation.

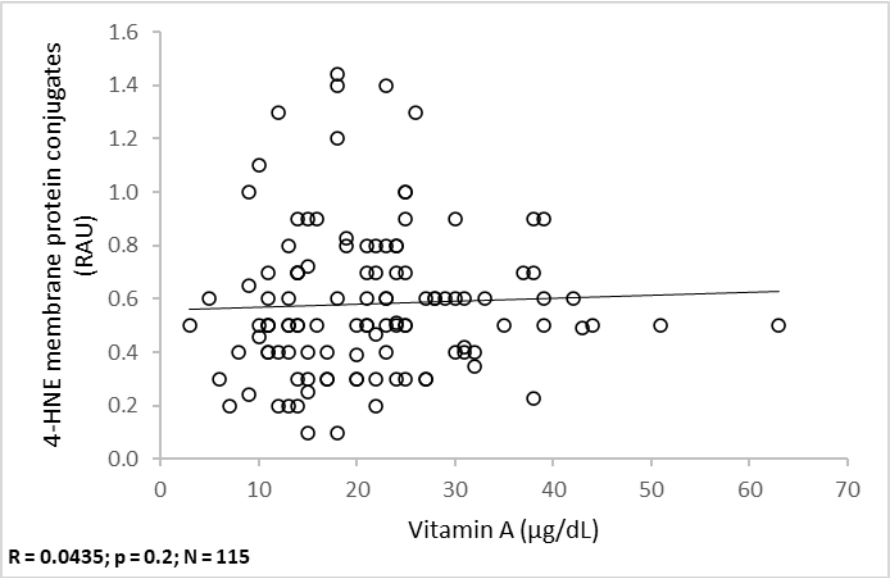


Figure 39. Correlation between 4-HNE membrane protein conjugates and levels of Vitamin A in children without inflammation.

4-HNE membrane protein conjugates (RAU, on y axis) were paired with levels of Vitamin A (µg/dL, on x axis) in 115 children selected for the absence of inflammation (CRP values ranging from 0 to 1 mg/dL). Spearman's test reveals a non-significative correlation with a p-value equal to 0.2.

3.4.5 Correlation of 4-hydroxynonenal-protein conjugates with erythropoietin

As expected, the erythropoietin level was inversely related to blood hemoglobin (Figure 42). For this it is not surprising that 4-HNE was significantly correlated with erythropoietin (Figure 40), a correlation which went lost in the inflammation free-cohort under study (Figure 41). The level of Epo is strongly related to the inflammatory state as concluded from the relationship of Epo with CRP (Figure 43).

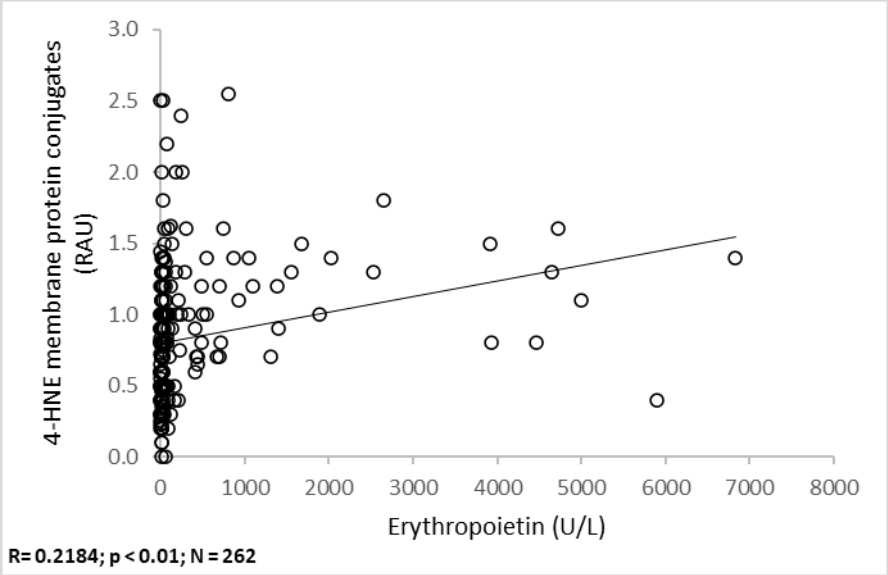


Figure 40. Correlation between 4-HNE membrane protein conjugates and levels of erythropoietin in study cohort (N = 262). Values of 4-HNE membrane protein conjugates (RAU on y axis) are significative correlated (R: 0-2184) with levels of erythropoietin (U/L on x axis). P-value calculated by Spearman's test was equal to 5.12x 10^{-6}.

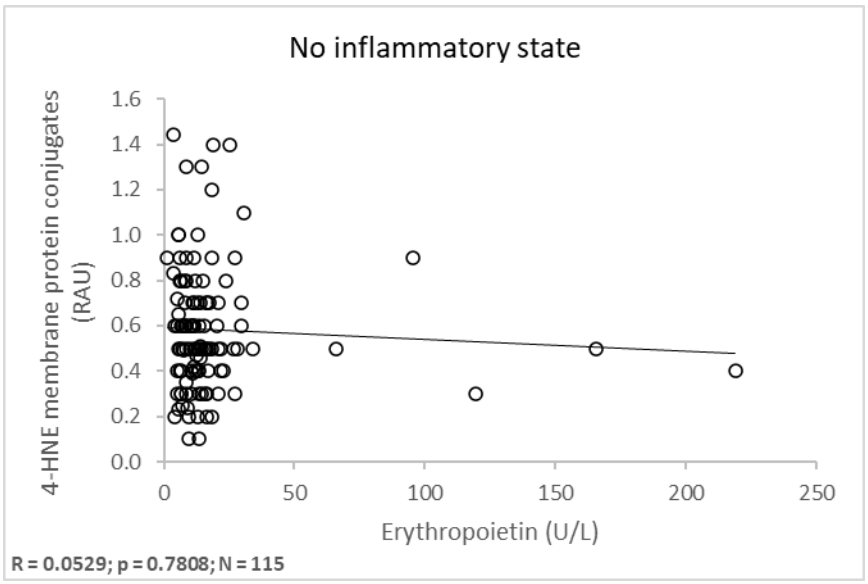


Figure 41. Correlation between 4-HNE membrane protein conjugates and levels of erythropoietin in 115 children without inflammation. 4-HNE membrane protein conjugates of only children without inflammation were correlated with levels of erythropoietin. Correlation was inverse (R: 0.0529) since low levels of erythropoietin are coincident with high 4-HNE membrane protein values. There is no significance as Spearman's test reveals a value equal to 0.788.

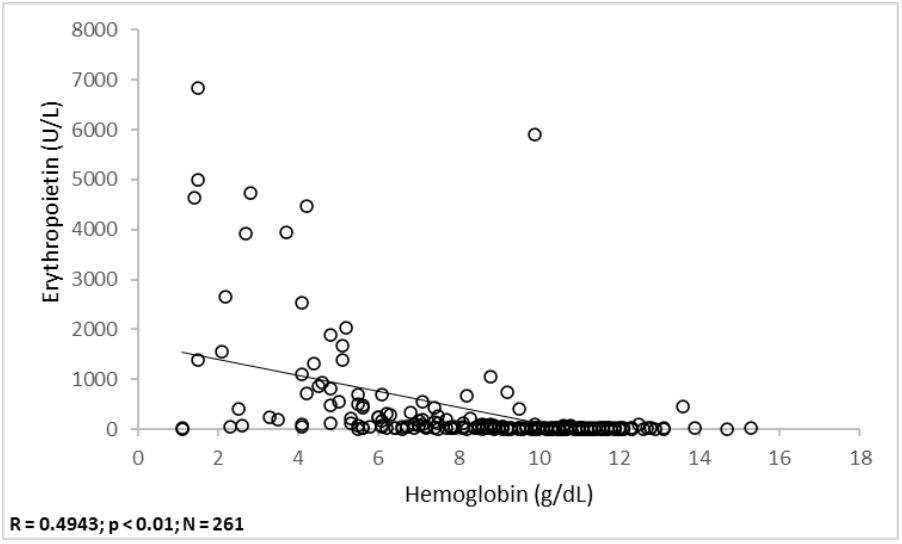


Figure 42. Correlation between hemoglobin values and levels of erythropoietin in study cohort (N = 261).

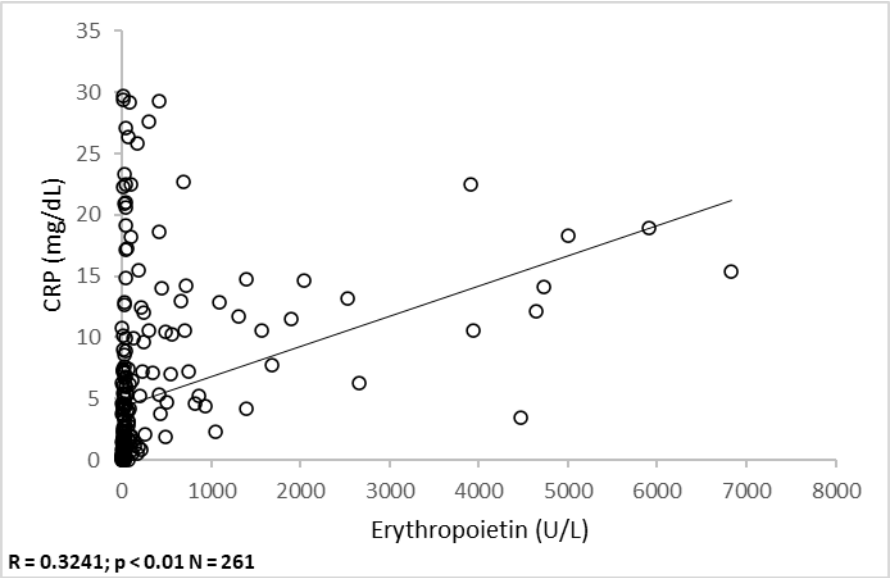


Figure 43. Correlation between CRP and erythropoietin in study cohort (N = 261).
Levels of CRP on y axis matched with levels of erythropoietin on x axis. Correlation found was inverse (R: 0.3241) and significant (Spearman's test found a p-value equal to 6.68 x 10^{-34}).

3.4.6 Correlation of 4-hydroxynonenal-protein conjugates with lactate dehydrogenase activity

To complete the relationship between anemic parameters and 4-HNE, latter was also paired with LDH, classical parameter of cellular damage, in our study of RBC damage. LDH is present in all tissues and cells, including RBC. In the case of anemia, the rise of LDH can be seen as an increment of hemolysis.

4-HNE membrane protein conjugates values show a linear correlation i.e., that high levels of LDH are accompanied by increasing levels of 4-HNE membrane protein conjugates (Figure 44). This correlation persists and is even stronger in the group of anemic children (Figure 45) and weak but still significant in the control group albeit (Figure 46). Haptoglobin and LDH are inversely corelated with each other as shown in Figure 47, which identifies hemolysis as cause of elevated serum LDH activity.

Finally, one can conclude that inflammation is a plausible source for 4-HNE by eliciting lipoperoxidation in the studied children. 4-HNE conjugates with RBC proteins. Exceeding the critical level of conjugates, RBC are recognized by phagocytes. Phagocytosis goes ahead with the release of a portion of cytosol of the target cell which we see as increase of LDH, but also decrease of haptoglobin due to released Hb.

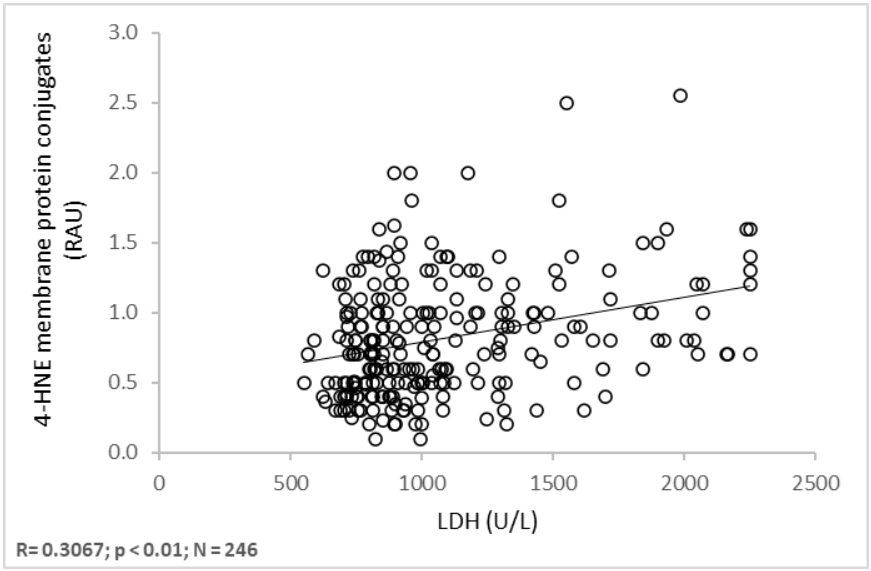


Figure 44. Correlation between 4-HNE membrane protein conjugates and levels of LDH (U/L) in study cohort (N = 246). 4-HNE membrane protein conjugates were compared with levels of LDH to verify a possible correlation. From this matching a linear positive correlation was found, in particular high levels of LDH correspond to high 4-HNE membrane protein conjugates values. Spearman's test calculated the significance of this correlation, corresponding to 5.12 x 10^{-06}.

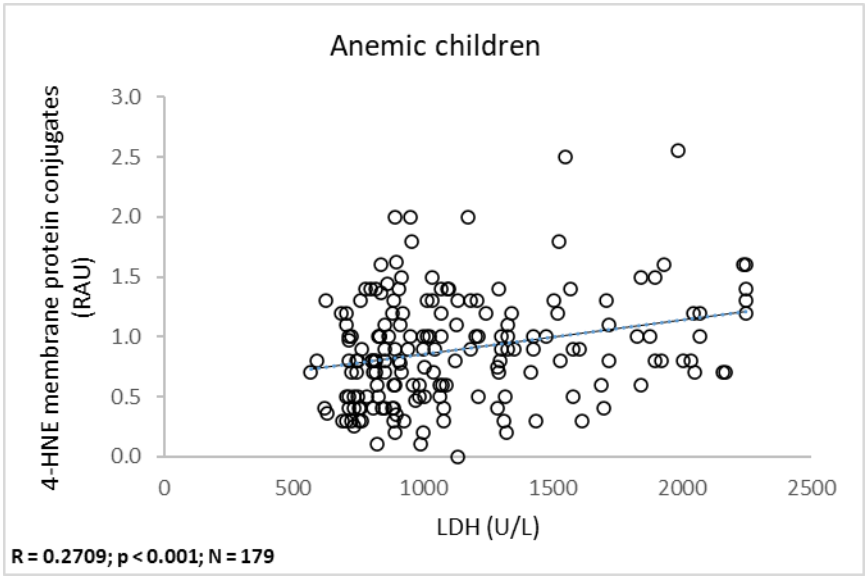


Figure 45. Correlation between 4-HNE membrane protein conjugates and LDH levels in anemic sub-cohort (Hb levels until 10.9 g/dL). Correlation between 4-HNE membrane protein conjugates (RAU) and LDH levels (U/L) was also checked in the anemic children group (N= 179). Result of matching was a linear positive correlation (R: 0.2709) and significative (p-value: 0.000188).

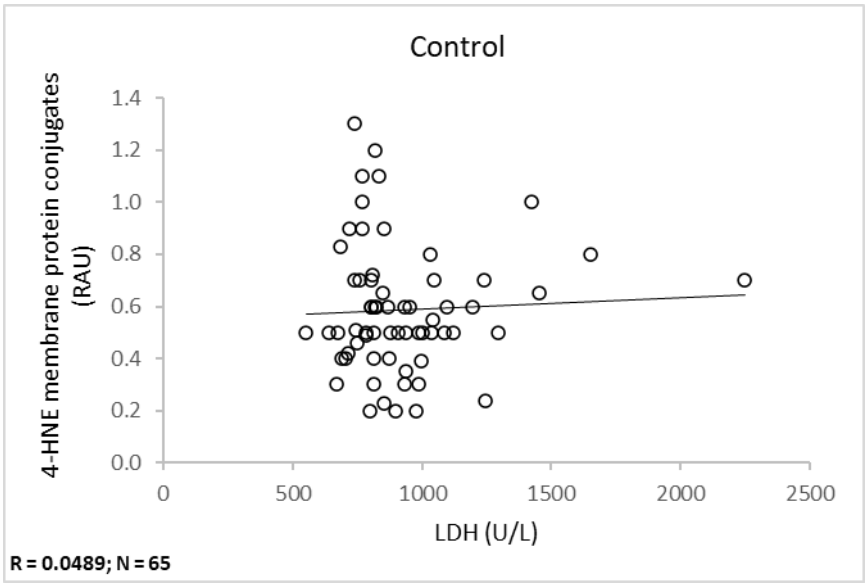


Figure 46. Correlation between 4-HNE membrane protein conjugates (RAU) and LDH levels (U/L) was also checked in the control children group (N= 65) (Hb levels from 11 g/dL). Result of matching was a mild linear positive correlation (R: 0.0489) and non-significative (p-value: 0.98996).

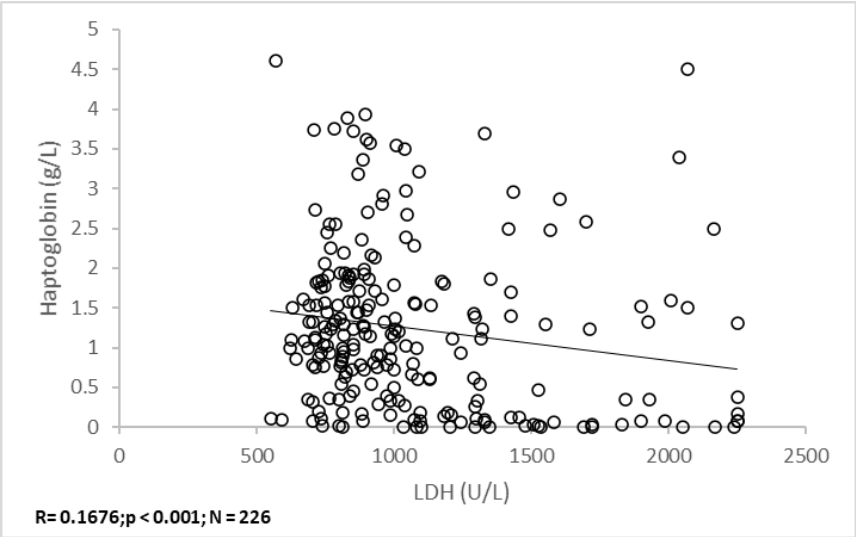


Figure 47. Correlation between levels of haptoglobin and LDH (on x axis) in 226 children. Levels of haptoglobin (g/L) compared with levels of LDH (U/L). Correlation found was inverse (R: 0.1676) and significative (p-value: 0.000999).

3.5 Mechanistic role of 4-hydroxynonenal-conjugation in anemia

The main result that emerges from the comparison between anemia of any genesis and age-matched non-anemic controls and the regression analysis of 4-HNE-conjugates with several blood parameters, is that 4-HNE conjugates accompany any anemic state and should be rather cause for than consequence of anemia. My intention was to cover the gap concerning the mechanism by which accumulation of 4-HNE causes anemia.

3.5.1 4-hydroxynonenal-membrane protein conjugates in RBC of different cell age analyzed by FACS without prior cell separation

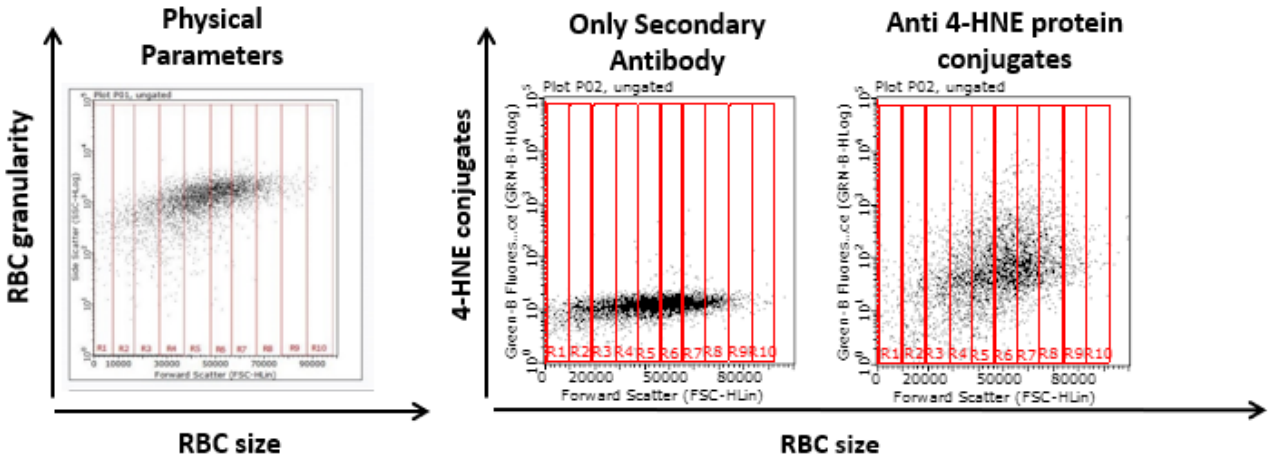
In the effort to find the role that the lipoperoxidation product 4-HNE might have in the pathogenesis of anemia, I have supposed that 4-HNE could be mechanistically linked to impaired erythropoiesis or enhanced erythro-phagocytosis, both leading to anemia. I have analyzed 4-HNE protein conjugates in RBC membranes of sub-cohorts of differently senescent RBCs by flow cytometry, taking advantage of senescence-dependent differences of the physical parameters of cells, i.e., cell size and granularity. The idea is, that an increase in 4-HNE conjugates in the young RBC fraction mirrors stress erythropoiesis processes in the marrow and, elevated conjugation of senescent fractions indicates that 4-HNE may flag RBC for phagocytosis.

I have illustrated the approach of flow cytometric analysis of RBCs by the results obtained from one donor, representative for independent experiments with 5 different blood donors. The x-axis shows Forward Scatter (FSC-H) which indicates the size of cells. The flow cytometric dot plot area was vertically divided along the x-axis into 10 sections, R1-R10. Each section includes a distinct RBC subpopulation characterized by a narrow cell size range, which increases from R1 to R10 (Figure 48A).

This subdivision allows to analyze RBC sub-cohorts according to cell size and to compare their 4-HNE-load.

From these preliminary experiments, I found that cells in the last regions, i.e., the larger RBC, had higher 4-HNE values on the surface than the smaller and therefore older RBC in the initial regions (Figure 48B). Results were confirmed by T-Test analysis where the comparison between R1 from R3 up to R9, gives significant differences ($p<0.05$), respectively.

(A)



(B)

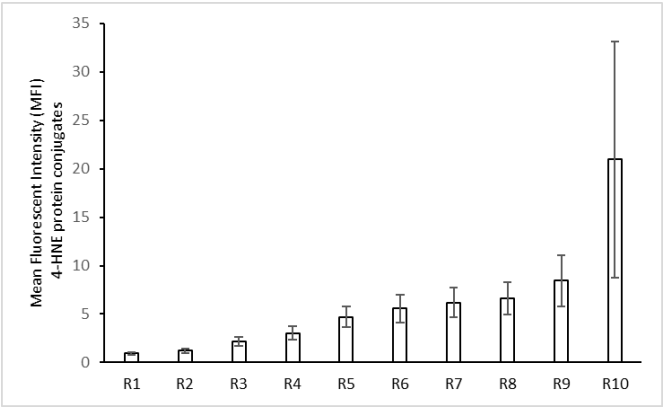


Figure 48. (A) 4-HNE protein conjugates on RBCs of different cell size by flow cytometry. The left plot represents physical parameters of RBCs population: On the x axis the forward scatter is indicated in linear mode (Lin) and represents the size of cells while on the y axis the Side Scatter Log indicates the granularity of cells in logarithmic mode. Each pixel plotted defines one cell regarding size and granularity. The other 2 plots show the distribution of cells regarding their green fluorescent intensity due to unspecific binding of FITC-labelled secondary antibody (middle panel) and the binding of the specific anti-4-HNE conjugate antibody visualized by the FITC-conjugated secondary antibody (right panel) against increasing cell size. One representative donor out of five.

(B) Comparison of mean fluorescence Intensity values (MFI) between ten sections (R1-R10) of RBC. Mean values of MFI ± SE of five distinct donors are plotted.

Carefully analyzing the physical parameters of cells, I suspected that antibody treatment affects the cell distribution and I decided to separate cells according to their density.

3.5.2 4-hydroxynonenal-membrane protein conjugates in RBC subpopulations of different cell age

To bypass modifications of cell-size distribution by antibodies used for conjugate analysis, the subdivision into sub-gates has been replaced by a more specific technique: the separation of RBC according to their density.

3.5.2.1 Physical parameters, reticulocyte count and surface antigen expression characterize cell age of density fractionated RBC

Physical cell parameters

After separation, RBC fractions were firstly analyzed for senescence-dependent differences of the physical parameters of cells, i.e. cell size and granularity.

The separation was performed on Percoll (see Methods for details), using Beckman ultracentrifuge. I obtained 3 different fractions (F1, F2, F3, from bottom to top, Figure 49A), each of them was analyzed for physical parameters and for levels of 4-HNE protein conjugates (as described in paragraph 3.5.2.2).

To quantify cells with different physical parameters in the three fractions, I divided the dot plot area with the whole RBC population in 4 quadrants, corresponding to different cell size and granularity ranges (Figure 49B). In the lower right quadrant (large cell volume and low granularity corresponding to young cells) percentages of cells and means of cell volume increase from fraction 1 (F1) to fraction 3 (F3) with 67.08 +/-13% (67.08+/- 2.46); 72.96 +/-3.5% (72.96 +/- 0.73) and 76.64+/-4.44% (76.64 +/- 0.98) of RBC in F1, F2 and F3, respectively, confirming that cells of the third fraction are bigger, have lower granularity (see lower position of cell cloud in F3) compared to cells of second and especially the first fraction. Senescence of RBC was considered to increase from F3 to F1.

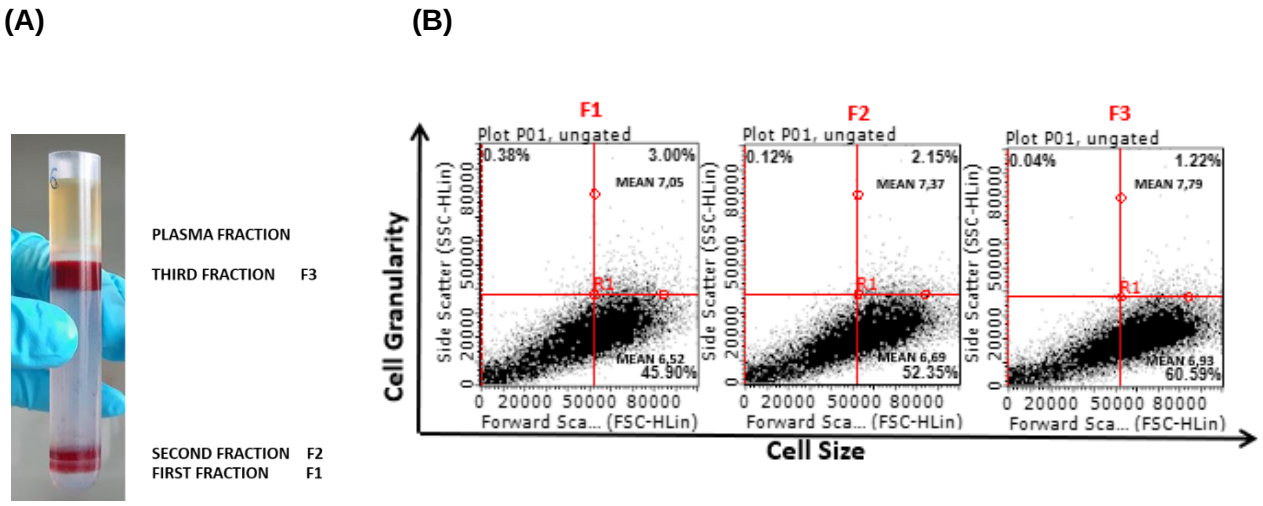


Figure 49. (A) Human blood after separation in a self-forming Percoll gradient. Three cell fractions (F1, F2, F3) were obtained after centrifugation of 2 ml blood at hematocrit of 30% stratified on a pre-formed Percoll gradient. (B) Analysis of qualitative difference between fractions by flow cytometry. Three dot plots to indicate qualitative differences among fraction 1 (F1), fraction 2 (F2) and fraction 3 (F3). On the x axis Forward Scatter indicates cell size in linear mode while cell granularity is represented by y axis as side scatter in linear mode.

Reticulocyte count

The presence of reticulocytes in 3 different fractions assessed by optical microscopy was used as criterion to judge the maturity of fractions. I report here an example of one of healthy donors. As expected, I observed a descending number of reticulocytes from the third fraction (F3) to the first one (F1).

Figure 50. Reticulocytes presence in RBC fractions F1, F2, F3 separated by density. Three representative images of discrete density fractions after separation on Percoll and stained with Cresyl Blue (see Methods for details). Images taken with a Zeiss Microscope equipped with 100-fold magnification emersion oil objective.

Surface antigen expression

In addition to reticulocyte content and separation according to cell density, the three obtained fractions were characterized by RBC-specific surface antigens expression. I focused on 3 specific clusters of differentiation (CDs): CD71 (transferrin receptor), CD235a (glycophorin A) and CD47 (integrin associated protein acting as a *don't eat me* signal to macrophages of the immune system). All three surface antigens decrease in mature RBC. Using FACS analysis, I monitored their presence on density fractionated RBC by anti CD71 FITC antibody, anti CD235a PE antibody and anti CD47 PE antibody.

For CD71, the differences between the three fractions were minimal, although in some experiments CD71 was higher in the third RBC fraction, the youngest one, compared to the relatively older first and second fractions (data not shown).

Also, in the case of CD235a, I haven't found differences among three fractions, while CD47 was clearly higher on the surface of younger RBC, as exemplified in Figure 51, MFI obtained from the use of anti CD47 antibody.

In general, is not easy to find typical age-specific markers for blood RBC. The antigens I have chosen, especially CD71 and CD235a, are mainly present in premature erythrocytic cells and early shed and invariant in circulating RBC. The quantity of these cells in blood is so low that I cannot detect them in my fractions.

CD47 however is continuously shed from peripheral RBC and reflects nicely the progress of senescence from F3 to F1, with the main loss between F3 and F2, characterizing F3 as young RBC population in comparison to F2 and F1. The difference of about 5% between F2 and F1 was less pronounced but still detectable.

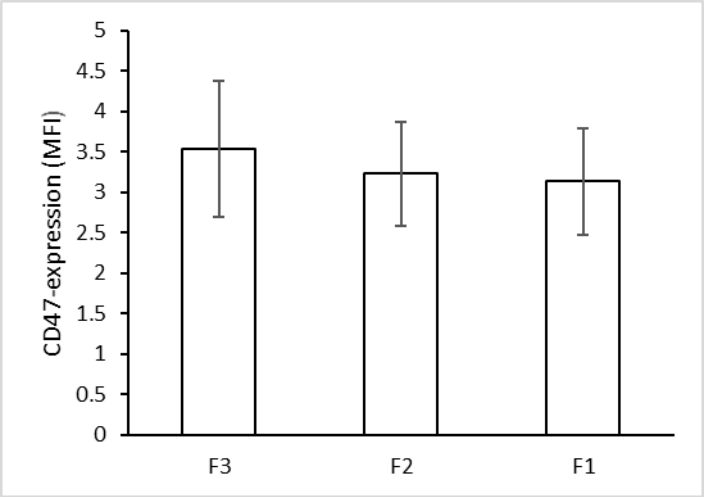


Figure 51. Mean values of MFI ± SE obtained using anti CD47 antibody in three distinct RBC fractions of three different donors. The figure shows an increase in mean value affecting the youngest F3 fraction.
In terms of percentage CD47 mean value of F3 is higher of 11% and 13% compared to F2 and F1 respectively.

3.5.2.2 Impact of cell age on 4-hydroxynonenal-binding to intact RBC surface proteins

After separation, RBC fractions were analyzed for 4-HNE membrane protein conjugates with a conjugate-specific antibody.

Contrary to what was obtained with FACS analysis of non-separated RBC (paragraph 3.5.1), RBC separated by centrifugation on a density gradient and analyzed by flow cytometry, show an age-dependent increase of HNE-conjugation, which supports the initial hypothesis that protein modifications with HNE of RBC increase by cell age, causing finally recognition by phagocytes and their removal. As shown in Figure 53 the both dense fractions F1 and F2 have a higher, though not significantly higher overall MFI value compared to F3.

I noticed that two differently 4-HNE positive subpopulations were formed as shown in Figure 53, panel B. These two subpopulations are referred to as the R2 and R3 region and were analyzed separately while the R1 region covers the entire cellular distribution (Figure 53, panel B).

In the R2 gate we see the signal of cells incubated with secondary antibody only but also a portion of RBC incubated with both anti 4-HNE-conjugate and secondary antibodies forms herein the first and less bright peak. Simultaneously in R3 a second fluorescence peak is formed by RBC treated with both anti 4-HNE-conjugates and secondary antibodies. These bright subpopulation of RBC carries more 4-HNE-conjugates. A short tail of RBC treated with only secondary antibody (negative control) was also detectable in R3. This overlapping could affect MFI result. In fact, there is a small percentage of negative control cells that give a signal in the R3 region.

Nonetheless, the mean MFI of RBC in R3 was 46.72, 89.32, 95.04 for fraction F3, F2, F1, i.e., the 4-HNE-conjugate load on the cell membrane increases by ell age. Similarly, the old fraction 1 had the highest share of 4-HNE-conjugate high positive cells with 50.69%, while F2 and F3 had 33.70% and 40.33 %. An example for the cellular distribution in the 3 fraction is given in Figure 54.

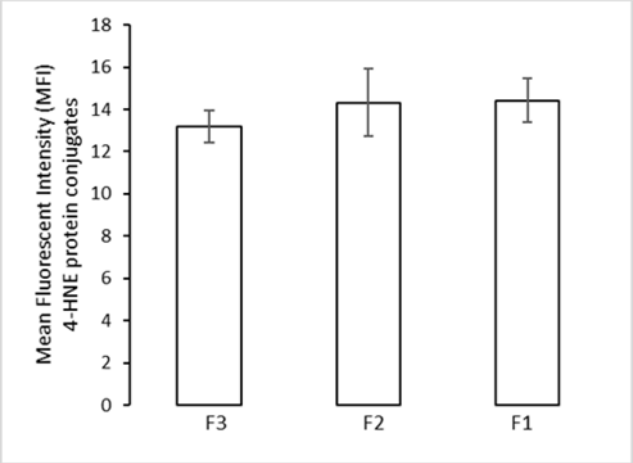


Figure 52. 4-HNE-conjugates of outer surface proteins of RBC density fractions. Comparison of mean values of 4-HNE surface protein conjugates (MFI) of three density fractions (F3, F2, F1) of cells from twelve different donors by FACS analysis. The observed differences were not significant p> 0.05.

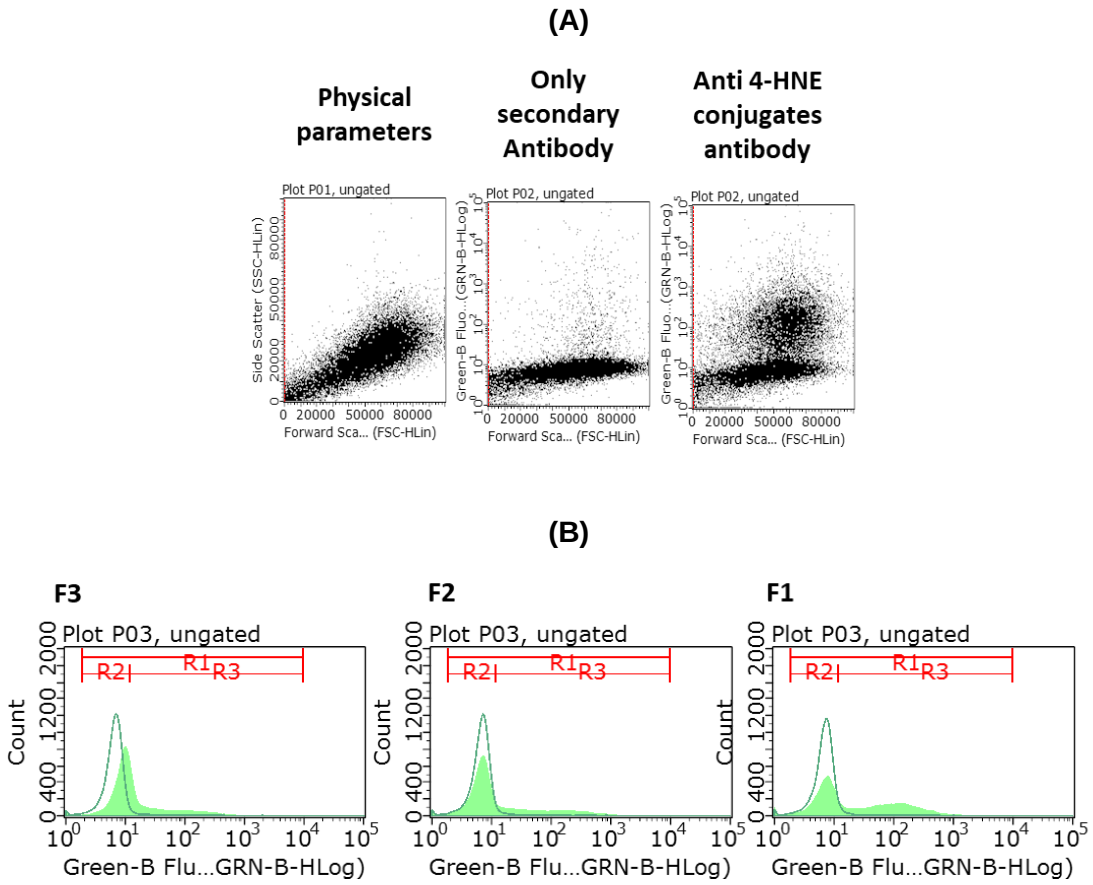


Figure 53. (A). Analysis of 4-HNE protein conjugates in intact RBCs of different cell age by flow cytometry. One representative flow cytometric dot plot of the first RBC fraction (F1) of a healthy donor after labelling with anti 4-HNE protein conjugates antibody. The first dot plot from the left shows the cell distribution in terms of cell size and granularity along the x-axis and y-axis, respectively. The other two dot plots represent cells after treatment with antibodies: Fluorescent dye FITC-conjugated secondary anti-rabbit antibody alone (central plot) and with the primary rabbit anti-4-HNE-conjugate antibody and subsequently with secondary anti-rabbit antibody to assess protein conjugation with 4-HNE. Forward Scatter on the x-axis in linear mode indicates cell size while the fluorescence on y-axis (Green-B Log) in logarithmic mode indicates 4-HNE-protein conjugates.

(B). Flow cytometric histograms of RBC fractions.

The two curves represent signals obtained with only secondary anti-IgG antibody (green line, empty histogram) and anti-4-HNE-conjugate antibody plus anti-IgG (green filled histogram). From left to right, the youngest RBC fraction F3, the older fraction F2 and the oldest one, F1 of the same donor shown in panel A, are plotted. On x- axis green fluorescence represents the HNE-carriage expressed in logarithmic mode while on y- axis the cell counts for distinct fluorescences are plotted.

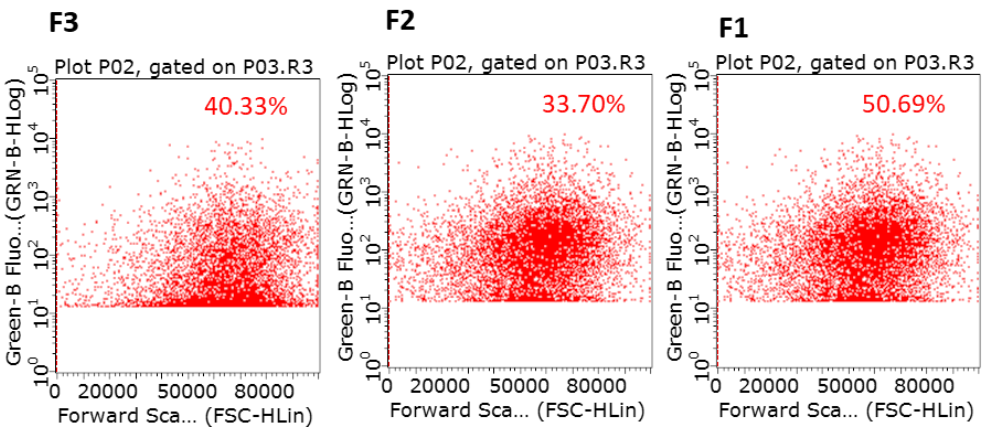


Figure 54. Percentage of 4-HNE-conjugate positive cells in the R3 gate region of the fractions F3, F2 and F1. of the donor analyzed in Figure 53. On the x axis Forward Scatter indicates cell size in linear mode while the fluorescence is indicated on y axis as Green-B Log in logarithmic mode. In this case Green-B Log is referred only to fluorescent signal obtained from the use of a couple of antibodies: the primary rabbit anti-4-HNE-conjugate antibody and fluorescent dye FITC-conjugated secondary anti-rabbit antibody.

3.5.2.3 Impact of cell age on 4-hydroxynonenal-membrane protein conjugates of RBC membrane extracts

In order to evaluate the course of protein modifications by 4-HNE, the values obtained from the three density fractions of RBC were compared. Modifications by 4-HNE on RBC membrane protein were analyzed in extracted RBC membrane proteins with a monoclonal mouse anti 4-HNE-protein conjugate antibody after electrophoretic separation and Western blotting with the aim to get information on the conjugation level of protein domains and such membrane proteins which are not accessible by antibody-labeling of intact cells as applied in the former described FACS analysis.

Figure 55 shows the typical pattern of 4-HNE-conjugated proteins from three fractions F1-3 of RBC separated by density exemplary from donors A, B and C. Prominent labeling was observed in the height of spectrin, band 4.1, 4.2, below 4.2 and actin.

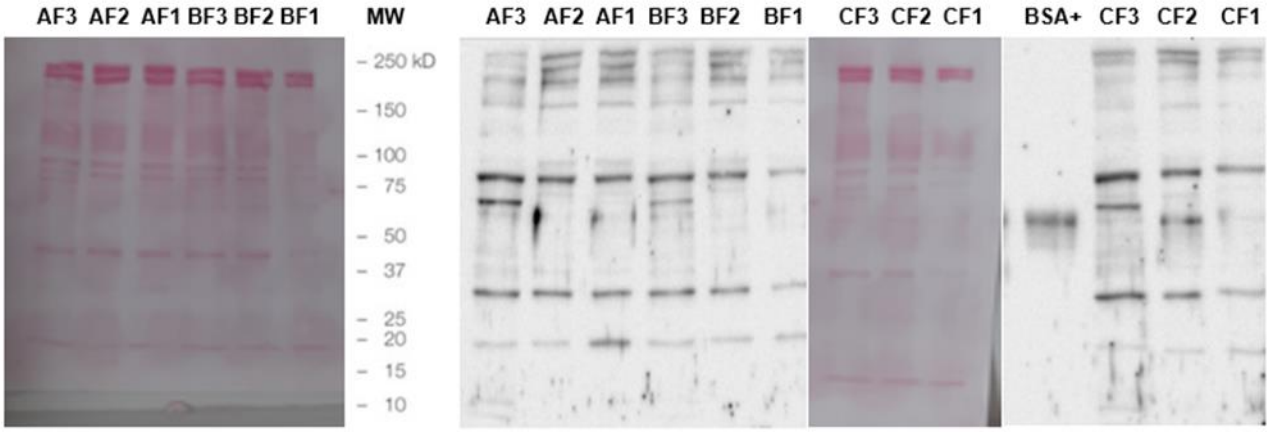


Figure 55. RBC membrane proteins obtained from different RBC fractions.
Two representative Western blot nitrocellulose membranes show electrophoretically separated RBC membrane proteins of three density fractions (F1, F2, F3) of cells from three different donors (A, B, C) after Ponceau Red staining (first and third panel from left) and after labelling of the same membrane with anti 4-HNE- antibodies (second and fourth panel; for details see chapter 2 Materials and Methods).

The mean values obtained for the three fractions of the 14 healthy donors were compared as seen in the histogram in Figure 56.

The sum of luminescence originating from 4-HNE-conjugated separated membrane proteins (as shown in Figure 55) was assessed for comparison between fractions. The mean 4-HNE-membrane protein conjugation levels for each of fractions F1-F3 were calculated from 14 donors and are shown in Figure 56. It was approximately 30% higher in the oldest fraction F1 as compared to F3 and F2 indicating a clear increase of 4-HNE-conjugation of membrane proteins during RBC senescence.

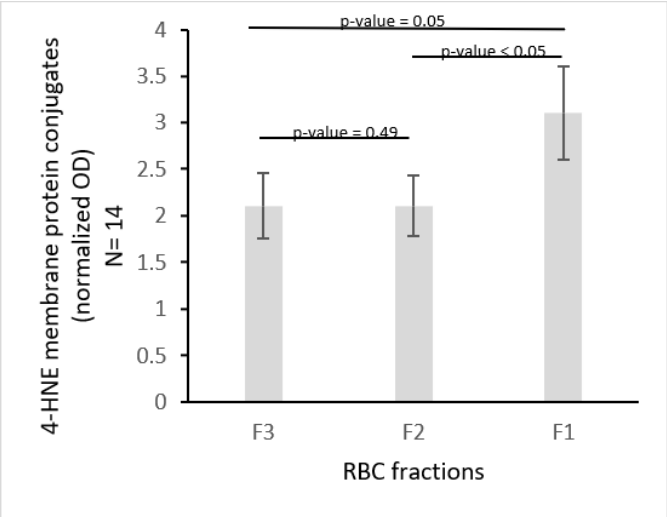


Figure 56. 4-HNE-conjugates of membrane proteins of RBC density fractions. Comparison of mean values of 4-HNE membrane protein conjugates (normalized OD) of 3 density fractions (F1, F2, F3) of cells from 14 different donors by Western Blot analysis. Normalized OD corresponds to luminescent signal obtained from anti 4-HNE protein conjugates antibody referred to both positive control (BSA+4-HNE) and Ponceau Red. The t-test reveals significative p-values equal to 0.05 and < of 0.05, respectively comparing F3 with F2 and F1 fractions. Only the t test between F2 and F1 fraction doesn't calculate a significative p-value (=0.49).

3.5.3 4-hydroxynonenal- conjugation of membrane skeleton protein spectrin increases in senescent RBC

Analyzing the pattern of 4-HNE-modified membrane proteins, it appears that differences between fractions mainly concern cytoskeleton proteins, especially on spectrin bands level.

Differences of 4-HNE modifications on skeletal components between the three fractions were verified with anti 4-HNE conjugate antibody.

The oldest fraction (F1) shows significantly more spectrin modifications by 4-HNE compared to RBC of both, intermediate and young age fractions, F2 and F3 (Figure 57).

In order to evaluate the contribution of spectrin modifications to the overall load of proteins with 4-HNE in the RBC membrane in the various fractions, the total protein load was determined and HNE-conjugated spectrin value was referred to it. Results show that total protein changes mainly affect the fraction F1 with the oldest RBC in contrast to spectrin-conjugation where RBC of intermediate fraction F2 demonstrated already elevated modifications vs. the young cells of F3 (Figure 58).

The ratio between spectrin and total protein conjugation identified RBC of intermediate age (F2) as cells in which conjugated spectrin has the highest share of approximately 30% in total protein conjugation as compared to both other fractions. Despite the absolute highest modification rate in spectrin in the oldest cells, additional protein modification with 4-HNE occurs and explains the lower ratio.

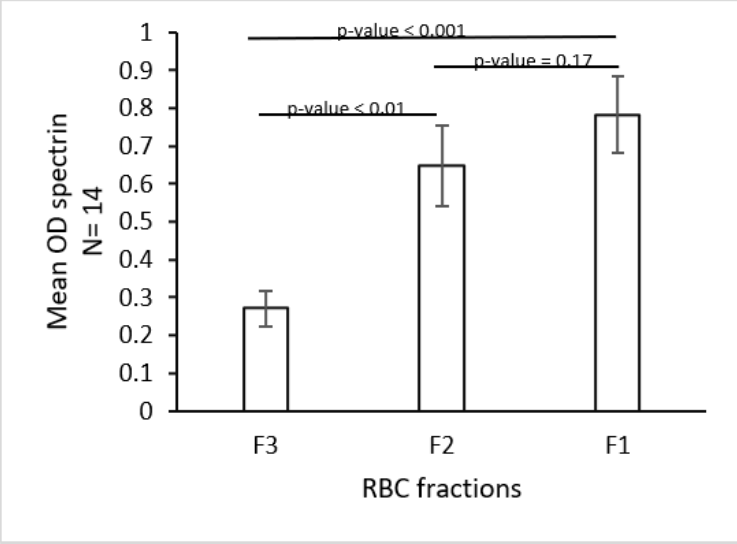


Figure 57. Membrane spectrin-conjugation with 4-HNE in RBC density fractions.

Mean values of membrane spectrin-HNE conjugates (mean OD spectrin) of 3 density fractions (F3, F2, F1) of RBC from 14 different donors were obtained by measuring specific anti-4-HNE-conjugate antibody binding to the electrophoretically separated and blotted spectrin bands. The t-test reveals significative p-values < of 0.01 and < of 0.001, respectively comparing F3 with F2 and F1 fractions. Only the t test between F2 and F1 fraction doesn't calculate a significative p-value (=0.17).

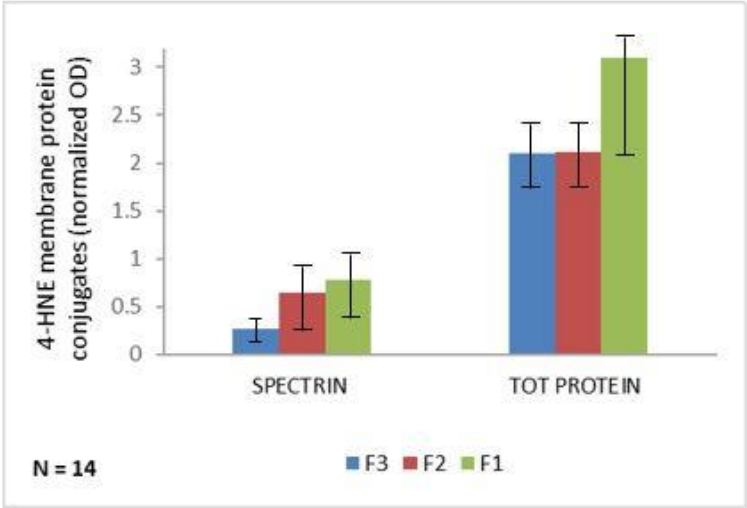


Figure 58. 4-HNE conjugation of spectrin and total membrane protein in three RBC density fractions. 4-HNE-spectrin conjugates and 4-HNE-membrane total protein conjugates were detected by specific 4-HNE-conjugate antibodies in the respective protein bands of electrophoretically separated membrane proteins from RBC fractions F1(green column), F2 (red column) and F3 (blue column). Columns are means of N=14 blood donors.

3.5.4 4-hydroxynonenal-conjugation of membrane skeleton protein spectrin in RBC of anemic children

Analysis of spectrin modifications by 4-HNE was conducted in RBC from 42 anemic children and on 21 non-anemic children. Differences between anemic children and age-matched non-anemic controls in their 4-HNE modifications on skeletal components were verified by anti 4-HNE conjugate antibodies.

As shown in the Figure 59, 4-HNE-spectrin and 4-HNE-total membrane protein-conjugates increased by 37% and 32%, in comparison to control.

Also the ratio between levels of 4-HNE spectrin modification and total protein modification reveals an increase in the group of anemic children, more than 33% of HNE-modifications of the RBC membrane is localized in spectrin, while in controls the share is 15% lower.

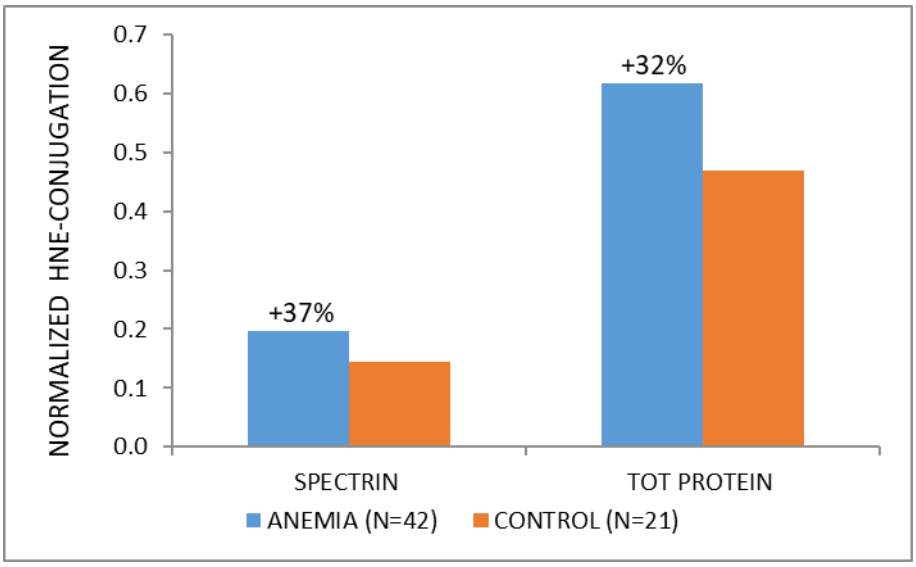


Figure 59. 4-HNE conjugates of spectrin and total membrane proteins in anemic and control children. 4-HNE-spectrin conjugates, 4-HNE-membrane total protein conjugates were quantified after electrophoretic separation of proteins and western blotting using specific anti-4-HNE-conjugate antibodies and ECL-based visualization. The luminescence intensities of the spectrin bands and of all protein bands were quantified and referred to the positive conjugate control run in parallel in any electrophoresis gel. The ratio between spectrin-conjugates and the sum of all protein conjugates in the membrane was calculated. Blue columns represent anemic children (N=42), orange columns control children (N=21). Percentages above the blue columns indicate the increase of the respective parameter in RBC of anemic children in comparison with non-anemic controls.

CHAPTER 4

Discussion

The purpose of my PhD thesis is to better understand the role of lipoperoxidation in the genesis of malaria anemia through the major end-product of n-6 PUFAs oxidation, 4-HNE. This nine-carbon atom long aldehyde has been largely studied *in vitro* because of its involvement in numerous pathologies as metabolic diseases, neurodegenerative diseases and cancers. This is due to its potent feature as modulator of numerous cell processes such as oxidative stress signaling, cell proliferation, transformation, cell death and cellular immune responses (Schwarzer et al., 2015).

In the case of malarial anemia, oxidative stress appears to be one of the cofactors that are associated with the onset of anemia (Percário et al., 2012). Although RBCs are able to counteract oxidative challenges with antioxidant mechanisms, high levels of oxidative processes may overwhelm the protection pathways and result in oxidative stress and cell damage. The peroxidation of membrane of polyunsaturated fatty acids is one of the consequences caused by excessive levels of oxidative stress in RBCs. The knowledge on the pathomechanistic role of lipoperoxidation and its products is limited and regards malondialdehyde-driven rather unspecific protein crosslinks (Narsaria et al., 2012) and phosphatidylserine redistribution between inner and outer leaflet of membrane in oxidatively stressed RBC (Freikman et al., 2008). Those studies use *in vitro* anemia models or are mainly performed in hereditary anemia forms. Data from studies in humans on the potential role of 4-HNE modifications are missing, despite their role in destabilizing normal RBC functioning, harming lipids, proteins and DNA.

In case of an elevated 4-HNE-level in RBC membranes this might be of particular pathomechanistic relevance for anemia and not to be just a marker molecule for an overwhelming pro-oxidant state. Under pathological conditions, the tissue and plasma membrane concentration of 4-HNE increases significantly and can reach concentrations much higher than physiological ones in lipid-rich cell structures the lipid peroxidation sites (Grune et al., 1997). One of my central results was the strong inverse correlation between levels of blood Hb (thus indicative of an anaemic state) and 4-HNE protein conjugates loaded on RBC membranes in children. This result, is the first one which demonstrated the dependency of blood Hb on 4-HNE-membrane protein conjugates of RBC and which was based on a sufficiently high number of study participants. Frozen blood from 264 African children that lived in Maniça, a region of Mozambique in which malaria results endemic was used for the study. The study included children between 1 and 68 months of age with blood Hb values between 1.4 and 15.3 g/dL. The children were enrolled in an anemia trial and the cohort can be considered representative for the local population of respective

age. Independently of the anemic or not anemic state, whose cutoff was set at 11g/dl according WHO guidelines, I observed a linear decrease of blood Hb with increasing 4-HNE conjugation. Though, the significance of correlation went lost in the control group when non-anemic children were analyzed separately, in contrast to the significant correlation in mild and severe anemia groups. This made me curious to understand the role 4-HNE could play in areas where malaria anemia is widespread and contributes substantially to mortality among children up to 5 years of age (DeMaeyer and Adiels-Tegman, 1985). The significance of the strong correlation was statistically confirmed by a p-value below 0.001.

I suppose that obtained values for 4-HNE-conjugates mirror the in vivo situation of the study participants at the moment of blood delivery as preliminary experiments validated the techniques and storage conditions applied to the samples after blood drawing. An artificial change of conjugate load could be excluded as demonstrated in Figure 14. I decided to isolate membranes from RBC and consequently extract proteins to study 4-HNE modifications of membrane proteins by specific antibodies using the Western blot technique as this approach allows to judge on the whole spectrum of membrane proteins and their modification by 4-HNE. Flow cytometry technique instead would certainly have allowed me to study changes at the surface of intact RBC, avoiding cell lysis and the risk to create artifacts, but on intact cells I had no access to modifications of proteins and protein domains placed at the cytosolic side of the membrane and inside the bilayer.

To avoid artefacts, membrane preparation was strictly performed under protease inhibitor control, on ice and each step was timely standardized. Thanks to these measures, replicates of membrane preparations had nearly identical 4-HNE-load and protein-4-HNE pattern (not shown) which underpins the reliability of results from study participants.

As expected from the regression study, explained above, anemic children had a significantly higher load of 4-HNE conjugates as compared to non-anemic controls. In fact, I found a difference in the mean value for 4-HNE-protein conjugates between anemic and non-anemic control children of almost 40%. (as shown in Figure 24A). Significantly higher mean HNE-load was also detectable comparing severe anemia children to non-severe anemic children which might support the idea of a mechanistic role of 4-HNE in anemia pathogenesis.

The advantage of my research also lies in the fact that I have been able to delve into the etiologies of anemia, constructing three different large groups of anemic children with malaria, anemic children with inflammation and anemic children who did not fit into the two previous groups and who I therefore referred to as "other etiologies". For each of these three groups there was its corresponding group of non-anemic children. Importantly, 4-HNE-conjugation of RBC membrane proteins was increased in all three anemia groups compared with the respective non-anemia group. The fact that significantly higher values of 4-HNE modifications were detected in subjects with anemia of different etiologies makes this molecule even more promising to play a role in anemia genesis independently of its etiology.

One of the closest studies to the one I conducted was done in 2012 by Uyoga et al. (Uyoga et al., 2012) where a significant difference was found between severe malaria anemia (Hb < 5 g/dL; N=5) and non- anemic children (N=33) in 4-HNE conjugation of RBC surface proteins measured by FACS in a small pilot study. Compared to this work the herein shown data are more conclusive as the number of anemic children tested for 4-HNE protein modifications was N=180 compared to only 5 children analyzed by Uyoga, also the higher number of non-anemic controls N=84 adds to more meaningful data. The main critical point in the Uyoga approach was, that the results represent ex-vivo data, in so far as 4-HNE analysis was performed after maturation of parasites ex vivo to trophozoite stage in washed RBC from the drawn blood, which introduces additional variables and data needed to be proven in vivo. Finally, my data confirm definitively the hypothesis that malaria anemia is accompanied by an increased 4-HNE conjugate level on RBC and extend the validity to any kind of anemia.

A second publication by Aguilar et al. (Aguilar et al., 2014) describes a concomitant moderate anaemia in those non-immune children recovering from an acute malaria attack with high (N=8) or increasing levels (N=4) of 4-HNE-RBC-surface conjugates. Embedded in a follow-up study, the number of anemia cases was too small to allow definitive conclusions on the impact of 4-HNE despite of statistical significance, but is now confirmed by my findings.

During malaria infection, phagocytosis represents one of the first defense mechanism against blood-stage malaria parasite (Schwarzer et al., 2015) and HZ is ingested *in vivo* by a large number of monocytes and spleen macrophages (Urban et al., 2005) with the aim to eliminate HZ-containing parasitized RBC and residual bodies from blood. It has been noted that HZ-load phagocytes are sources of huge quantities of 4-HNE (Schwarzer et al., 1996), resulting in membrane protein-4-HNE conjugates not only in the monocyte itself, but also in adjacent cells, e.g. shown in erythroid precursor cells in the erythropoietic iceland *in vitro* (Skorokhod et al., 2010).The same phenomenon of 4-HNE diffusion to neighbor cells was demonstrated (Uyoga et al., 2012) between trophozoites as source of 4-HNE and non-parasitized RBC.

Based on these former findings, my hypothesis was that 4-HNE could contribute, thanks to its chemical structure, to the elimination of RBC, especially non-parasitized ones.

In particular, I suppose 4-HNE to act as a kind of cellular aging mediator by tagging cell membrane proteins for cellular removal. I have revised previous publications regarding the importance of membrane modifications for RBC elimination from blood. Some alternative studies to the widely accepted model for the recognition of senescent or otherwise oxidatively stressed RBC by auto-antibody and complement binding to oxidation-elicited clusters of band 3 (Kay et al., 1994) focused on IgG-independent changes in either or both the carbohydrate or peptide moieties of glycoproteins, exposure of phosphatidylserine residues, or alterations in spectrin (Bratosin et al., 1998).

To understand how 4-HNE may elicit the removal of RBC from blood by phagocytes what is likely *in vivo* to occur, I studied the molecular role of 4-HNE-conjugates in senescent RBC as model cell for a physiologic phagocytosis target.

According to former publications (Piomelli and Seaman, 1993), density separation is one of the most useful techniques to study RBC aging. Thus, I separated RBC according to their density from blood of 12 healthy donors. The analyzed fractions were characterized in terms of cell age by density, cellular granularity, reticulocyte content and age-specific surface antigen expression of CD47, a don't eat me signal for young RBC.

The flow cytometric analysis of discrete density fractions of RBC from healthy donors, showed a cell age dependent accumulation of 4-HNE-protein conjugates on the cell surface. conjugates. As shown in Figure 52, both old cell fractions F1 and F2 have a higher, though not significantly higher overall MFI value for 4-HNE conjugates on the cell surface compared to F3 and a higher percentage of older cells is 4-HNE-conjugate-positive as compared to younger fractions 4-HNE-modified surface proteins are potentially recognized as non-self-structures and Damage-Associated Molecular Patterns (DAMP) by Toll-like receptors or humoral factors which results in an increased phagocytosis of the target cell.

In parallel to the 4-HNE conjugation with cell surface proteins, the total array of membrane proteins was analyzed to see modifications by 4-HNE in the integral and cytosolic portion of membrane proteins. For this, I probed membrane proteins with a monoclonal mouse anti 4-HNE-protein conjugate antibody after electrophoretic separation and Western blotting with the aim to get information on the conjugation level of protein domains and such membrane proteins which are not accessible by antibody-labeling of intact cells as applied in the former described FACS analysis.

Consistent with 4-HNE binding to surface proteins, conjugation of membrane proteins with 4-HNE was approximately 30% higher in the oldest fraction F1 as compared to F3 and F2 (Figure 56) indicating a clear increase of 4-HNE-conjugation of membrane proteins during RBC senescence. I noticed that the major conjugation was visible in spectrins in fact, making a detailed analysis for cytoskeleton proteins, I found that the most senescent RBC have significantly more spectrin modifications by 4-HNE compared to RBC of less senescent or young RBC as shown in Figure 57. So, I deduced that 4-HNE gets access and interacts with cytoskeleton proteins in senescent RBC. This modification of spectrin may result in modifications of physical properties of the cell and lead to improved recognition and engulfment by phagocytes.

Aldehyde-mediated cross-linking of various RBC membrane proteins certainly stiffens cells (Jain et al., 1983). A stiffer RBC is certainly an easier prey for monocytes, because myosin-II dimers assemble and contract actin filament at the phagocytic synapse in response to rigidity of human RBCs (https://dx.doi.org/10.1182%2Fblood-2014-06-585299). Thus, 4-HNE might contribute to stiffness in senescence of RBC.

In the presented study, RBC obtained from anemic patients interestingly, showed also an increased spectrin conjugation by 4-HNE as compared to cells from non-anemic.

It has been demonstrated in a silico model that 4-HNE modified residues of the ZU5-ANK ankyrin domain which subsequently forms complexes with β-spectrin. This results in local protein conformational changes, increased mobility in the modification sites, and localized structural changes between the positively charged patch of the ZU5-ANK domain. Carbonylation with 4-HNE on lysine residues decreased the affinity between ZU5-ANK and the 14-β-spectrin repeat by reducing electrostatic and van der Waals interactions. Proteins involved in the modification appear to have an increase in hydrophobicity, due to unfolding, with a consequent increase in aggregation (Møller et al., 2011) (Nyström, 2005) (Höhn et al., 2014).

The presented work provides further insight into understanding the loss of human erythrocyte deformation capacity under conditions of oxidative stress in different diseases (Alviz-Amador et al., 2019).

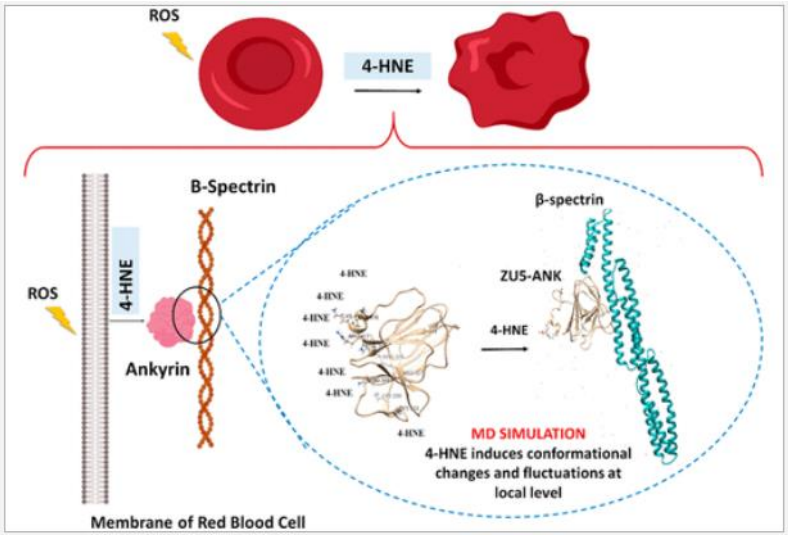


Figure 60. Functional role of 4-HNE in the membrane skeleton of RBC- an in-silico model. Reproduced from (Alviz-Amador et al., 2019).

Among the various functions performed by the complex network of cytoskeleton proteins there is also that of activating phagocytosis.

Within the complex system of membrane skeleton in which cytoskeleton and the plasma membrane are extremely and closely connected to each other, phosphatidylserine is an amino-phospholipid that is known to play a crucial role in mediating the recognition of senescent RBC, serving as an eat-me signal (Pretini et al., 2019).

The modification of spectrin by 4-HNE might easily add to an enhanced flip flop rate of PS and contribute to an enhanced recognition of RBC modified by the lipoperoxidation product., e.g. in anemia.

In an *in vivo* experiment, an increase in PS exposure is seen with RBC age, correlated to the RBC clearance from the circulation (Boas et al., 1998). For a long time it has been proposed that apoptotic cells that express PS can be cleared from circulation via macrophages by recognizing them through specific PS-receptors (Li et al., 2003). Yet recently, various receptors have been identified that can mediate binding and phagocytosis of apoptotic cells by the recognition of PS on these cells such as Tim1, Tim4 and Stabilin-2 (Kobayashi et al., 2007, Park et al., 2008). In addition, there are several bridging molecules such as the plasma proteins: lactadherin, Gas-6 and protein S, that have been described to bind to PS and direct PS to receptors on phagocytes, $\alpha_v\beta_{3/5}$ integrins and receptors of the TAM receptor family and mediate clearance of PS-positive cells (Raymond et al., 2009). Of these receptors, at least Axl, Tim4 and Stabilin-2 are expressed in red pulp macrophages. Thus, this opens the possibility that PS-exposing RBC are cleared in the spleen by phagocytosis by one or more of these PS or PS/ligand receptor pairs. As was already mentioned above, the loss of phospholipid asymmetry and the subsequent exposure of PS on the RBC surface may be a general trigger for RBC removal. It also seems that upon RBC storage the susceptibility to stress-induced PS exposure increases, thereby causing a considerable fraction of the RBC to be susceptible to removal after transfusion (Bosman et al., 2011).

Of interest, N-Acetyl-L-Cysteine (NAC) extents the half-life of circulating mouse erythrocytes *in vivo* RBC drawn from mice that were subsequently treated with NAC exhibited a significantly higher survival rate after the intravenous injection into the sibling mice than those RBC without an NAC treatment (Ghashghaeinia et al., 2012). NAC is an excellent 4-HNE scavenger and was shown to inhibit in vitro phagocytosis of RBC treated with 4-HNE by THP-1 phagocytes (Uyoga et al., 2012). These findings are encouraging to target the lipoperoxidation product 4-HNE in the therapy of anemia.

Recently, our research group set up an in vitro phagocytosis model and RBC of different cell age were fed to human primary monocyte. A significantly higher phagocytosis rate was observed when RBC that had a higher 4-HNE load were fed. This data are in line with the observation of Skorokhod of an increased stiffness and phagocytosis by immortalized monocytes of RBC which were treated with exogenous 4-HNE (Uyoga et al., 2012).

Future experiments with the addition of exogenous 4-HNE to RBC are necessary to further validate a mechanistic role to this aldehyde in the phagocytosis process.

Taking in mind that anemic children had 37% more 4-HNE-spectrin conjugates than the age-matched non-anemic and a 30-40% higher load of spectrin with 4-HNE which may result in an increased phagocytosis rate, 4-HNE can be said to be a very promising molecule in anemia genesis because it could play a role not only in RBCs recognition through modifications of surface proteins but could make them rigid thanks to the specific interactions it has with cytoskeleton proteins such as spectrin.

In addition to 4-HNE, membrane-bound-Heme species were assessed as second parameter that might increase due to an oxidative challenge. Heme species bound to the membrane include beside traces of free heme, hemichromes and mainly Hb. An increase of membrane hemichromes was described to occur during malaria infection of RBC in *in vitro* malaria models (Arese et al., 2005).

High levels of oxidative stress have a major impact on the main protein in the RBC, causing the oxidation of Hb. Oxidation causes Hb to have greater affinity for RBC membranes, causing the establishment of hemoglobin-membrane interactions and finally the generation of hemichromes. Hemichromes are well described structures in several anemia forms, like G6PD deficiency, but were also supposed to contribute to malaria anemia (Aguilar et al., 2014).

The results obtained for this oxidative parameter were different from those for 4-HNE protein conjugates. Paring blood Hb values of 173 children with their individual membrane-bound-Heme, I found a positive linear correlation which is not what I expected. The result does not mirror my initial hypothesis according to which membrane-bound-Heme could contribute to elevated elimination of RBC and decreasing blood Hb values. Instead, increasing blood Hb was accompanied by increasing Hb-binding to the isolated RBC membrane. The pathophysiological relevance of this correlation however is dubious as freezing of RBC in glycerolyte resulted in the loss of membrane bound Hb. Because membrane-bound Heme values might be biased by storage a future study with membranes from freshly drawn RBC must clarify whether membrane -bound Hb has a role in anemia genesis.

The very interesting result is certainly the significant association between high levels of 4-HNE and anemia.

Anemia is most prevalent in the regions where malaria is endemic: in Africa, between 31% and 90% of children suffer from anemia in malaria-endemic regions (Menendez et al., 2000a). The greatest mortality is associated with cerebral malaria and severe anemia, mostly in children less than five years of age, in malaria holoendemic areas (Guerra et al., 2008).

But in areas where malaria is still endemic and the cause of anemia is not restricted to this infection but multifactorial, finding parameters which contribute to the increase of the common link in anemia, such as 4-HNE-conjugates may have practical bearing for the treatment of anemia.

So, one of my purposes was to find associations between 4-HNE modifications in RBC membranes from 264 study participants and some causative factor inducing malaria anemia.

Many previous studies have underlined that the contribution of *P. falciparum* to the development of anemia is difficult to determine in malaria endemic area since a large proportion of children (often the majority) are parasitemic at any time and other causes of anemia, particularly iron deficiency and hemoglobinopathies, but also intestinal infections often coexist (Crawley, 2004). The parasite load in these children are low and additional individual factors obviously determine whether anemia occurs or not. From the 180 study participants that were anemic 40% had malaria (of which just

over 3% have malaria without inflammation), 37% only inflammation, 37% both. Just over 23% of the anemic group are children without either malaria or inflammation. Of these, the 50% is iron deficiency, 7% has nutritional deficiency and 12% not clearly defined factors. 12, 55 and 29% of anemic children had no one, one and more than one etiologic factor, respectively, that coincided. Similar results were obtained in a larger study (Moraleda et al., 2017). Importantly, blood Hb loss coincided with an increase of 4-HNE-conjugates independent of etiology. A very recent study (Nsiah et al., 2019), based on measurement of levels of oxidative stress through MDA, affirms that in children with complicated malaria, levels of MDA are significantly higher compared to control.

I expected that there was a correlation between the increase in 4-HNE and these factors.

Thus, at first, I checked if the presence of the malarial parasite correlated with low levels of Hb. In the non-immune children for which reliable parasite counts were available the expected negative correlation was not detectable (Figure 26). Children had mainly very low parasitemias (ranging from 0.12-13596 parasites/µl) and low blood Hb observed in some children did certainly depend on additional factors, provoked by the parasite, but not in a parasite count dependent fashion. Main candidate for such additional factor is the individual host immune response which varies substantially in its strength between individuals.

At such low parasitemia, the parasite itself does obviously not cause profound lipoperoxidations in the whole RBC cohort, as concluded from a very weak (R= 0.1918; p-value= 0.7432) positive correlation between 4-HNE-conjugation and parasitemia. Specifically, children (58%) with very low levels of parasites/µl had very variable levels of 4-HNE membrane protein conjugates ranging from 0.5 to 2.4 RAU. The relative high percentage of children with very low parasite counts that show a high level of 4-HNE conjugates make it more likely that additional factors induced by the parasite, but again not dependent on the parasite count determine the lipoperoxidation. These factors may most likely depend on individual host responses to infection, e.g. immune reactions, and be not determined by parasite count at low parasitemia. The importance of immune processes is underlined by the higher HNE-protein load in children whose malaria infection was accompanied by elevated inflammation parameters compared to malaria patients without inflammation as shown in Figure 25. Generally during an infection, and in malaria, too, a number of pro-inflammatory cytokines such as TNF, IL-1β, IL-8, IL-12, and IL-18 are secreted and stimulate cells of the immune system to produce ROS, that become an important source of 4-HNE. Specific for malaria are the HZ-laden PBMC. Strong experimental proofs exist, that HZ persists in mononuclear phagocytes, that are unable to digest the heme-rich crystals (Schwarzer et al., 2001). HZ-load does not induce apoptosis, but impairs many immune functions of the monocyte and is the source of substantial amounts of 4-HNE (Schwarzer et al., 1992b) (Skorokhod et al., 2004). The nine carbon atoms long aldehyde is not restricted to the phagocyte but able to cross the membrane and the immunologically silenced phagocytes function as shuttle and direct source of the bioactive

molecule which I able to modify membranes of other cells, e.g. RBC, when cells converge or interact.

Thus, the extent of the 4-HNE production and protein conjugation in malaria depends on the individual reactivity of the immune system rather than on the absolute parasite load.

After verifying that 4-HNE levels increased in anemia patients regardless of the presence of malaria (Figure 25), I decided to study first the contribution of inflammation to 4-HNE membrane protein conjugates. The regression curve between 4-HNE conjugates and CRP as representative markers for lipoperoxidation and inflammation, respectively, reveals that high levels of 4-HNE conjugates were significantly associated with high levels of CRP as shown in Figure 28-30. It is surprising and also exciting that the significant correlation persisted in the non-inflammatory state with CRP values below 1 mg/dL, when the majority of RBC samples had low 4-HNE-conjugate levels (below 0.7 RAU). There is no doubt to the fact that host's immune responses are an important source of lipid lipoperoxidation. This leads to an increasing accumulation of 4-HNE that is not "disposed of" by the potent anti-oxidative systems such as GSH that isn't able to implement an appropriate response or is unable to reach the site of conjugation, e.g. extracellular and intramembranous domains of membrane proteins or sites close to the bilayer. The significant correlations between CRP and 4-HNE protein conjugates in both anemic and non-anemic children, support the hypothesis of inflammation as the direct source of 4-HNE regardless of anemia and the accompanying hypoxic state.

Iron deficiency represents a common consequence of inflammation and could result in 4-HNE conjugation e.g. by hypoxia. To exclude iron deficiency as real cause of 4-HNE increase instead of inflammation, regression analysis was performed between levels of 4-HNE membrane protein conjugates and CRP levels in 88 children without iron deficiency. Results obtained for this group are in line with those of the previous correlation in the whole cohort. So, regardless of the iron deficiency, inflammation is strongly and significantly corelated with 4-HNE conjugation.

Focusing on the topic of iron deficiency, being a widespread problem in sub-Saharan Africa and an important cause of anemia among children, I choose levels of plasma iron and of sTfR as well-established markers for iron deficiency anemia, to verify their correlation with 4HNE-protein conjugates in RBC.

I first verified that low levels of iron were accompanied by high levels of sTfR, indicating an anemic state and was wondering whether iron deficiency may cause lipoperoxidation and 4-HNE conjugation in RBC. I Levels of plasma iron and 4-HNE membrane protein conjugates were significantly inversely correlated. Also, sTfR showed a significant correlation with 4-HNE, and increasing levels of sTfR were associated with increasing levels of 4-HNE. Further, I was wondering whether, the correlation between 4-HNE and plasma iron was causal or not. Thus, since iron deficiency is frequently accompanied by inflammation the real reason for a correlation with 4-HNE conjugates may not be iron by its own, but the inflammatory state in these patients. Indeed,

there was no correlation between the lipoperoxidation marker and plasma iron in those iron-deficient, children who had no signs of inflammation as measured by CRP below 1mg/dl. The absence of significance of this result led me to conclude that iron deficiency alone does not cause an increase in 4-HNE. The other way around, 4-HNE may modify proteins involved in iron homeostasis, such as transferrin or its receptor, playing an influential role in iron deficiency linked to inflammation.

Another aspect that I wanted to check for correlation with 4-HNE as it is usually highly prevalent in malaria endemic areas, is malnutrition.

Micronutrients are also involved in the pathogenesis of anemia and likely play a role in malarial anemia, but further work is needed to assess the contribution of micronutrient deficiencies to malarial anemia that is often underestimated (Nussenblatt and Semba, 2002).

I choose levels of vitamin A as deficiency is frequent in the area and vitamin A is involved in erythropoiesis by RA/RAR cascade and a significantly inverse correlation was seen between vitamin A and 4-HNE. The association between both parameters was not independent but strictly dependent on inflammation.

Due to its described role in proliferation and differentiation of erythroid cells (Skorokhod et al., 2010), I could not fail to correlate 4-HNE levels with erythropoietin levels in blood (EPO), also to get mechanistic information on the supposed role of 4-HNE in dyserythropoiesis.

In the work of Diez-Padrisa (Díez-Padrisa et al., 2011), authors find Hb to determine EPO expression in malaria just what I saw in the whole cohort I was investigating. In fact (Díez-Padrisa et al., 2011), I found an inverse correlation between levels of EPO and blood Hb along the whole range of blood Hb. My aim, however, was to prove the relationship between EPO and 4-HNE membrane protein conjugates. 4-HNE was significantly correlated with erythropoietin (Figure 40), a correlation which went lost in the inflammation-free sub-cohort under study (Figure 41). The level of EPO is strongly dependent on the inflammatory state as concluded from the relationship of EPO with CRP in my study (Figure 42). Recently, it has been published that in parallel to the classical regulation of EPO expression by hypoxia, infections also elicit an EPO increase, likely via pro-inflammatory cytokines and their signaling cascades. The increase of EPO in inflammatory context has been discussed as a cyto-protective mechanism in response to a pro-inflammatory cytokine storm (Jonker et al., 2013).

Cytoprotection by EPO should not play a role in mature RBC due to the lack of necessary signaling pathways but may be functional during erythropoiesis. Based on my data on mature RBC we may exclude EPO as a determinant parameter for 4-HNE generation as the observed correlation depends on the inflammatory state that frequently accompanied increased EPO levels.

The last parameter for which I verified a positive correlation with 4-HNE-conjugates is a typical indicator of cellular damage, serum lactate dehydrogenase (sLDH). sLDH increase is a hallmark of hemolytic anemia. sLDH is released from RBC during intravasal hemolysis and phagocytosis in the

spleen together with RBC cytosol. The most sensitive marker for hemolysis is the acute phase protein haptoglobin, which binds the released hemoglobin and consequently decreases during a hemolytic process. Haptoglobin and sLDH, inversely corelated with each other as shown in Figure 45, which identifies hemolysis as cause of elevated serum LDH activity in the study cohort. The positive correlation between 4-HNE conjugates and sLDH may mirror the elevated phagocytosis *in vivo* in donors with high 4-HNE load and we should bear in mind, that free Hb in blood plasma may contribute to lipoperoxidation as long it is not bound to haptoglobin.

In summary, I was able to identify inflammation as main source for 4-HNE-conjugates in RBC, which is quite plausible as activated immune cells fire ROS during their attack at any adjacent cell and lipoproteins. The consequence is the lipoperoxidation of membrane and lipoprotein lipids and resulting instable hydroperoxides of fatty acids spontaneously cleave 4-HNE. The bioreactive molecule moves well in the lipid layer due to its amphipathic character and reaches specific binding sites in proteins to form conjugates which were determined here. These covalent modifications change the protein properties and may result in functional loss not unlikely to hemolysis, changed surface antigen conformation and cell rigidity. All these changes favor the phagocytic removal, which may add another pro-oxidant factor plasma Hb. Plasma Hb as heme-carrying protein may add to above described lipoperoxidations by heme interaction with lipids in presence of oxygen, especially in states when haptoglobin is low and will not completely protect the cells from an oxidative insult by extracellular Hb.

Starting from the assumption that increased oxidative damage is one of the causes of enhanced removal of non-infected cell in malaria anemia (Greve et al., 1999), the results on 4-HNE modifications on RBC membranes explain the potential role of this compound in the pathomechanism of anemia of any etiology.

Changes in 4-HNE-conjugation were mainly observed in the two spectrins, with no particular differences between alpha and beta. Spectrin represents the principal membrane skeleton protein in RBC which, interacting with actin and the lipid bilayer, is crucial for RBC membrane stability (Manno et al., 2002). Thanks to its ability to bind to the bilayer, it regulates also RBC shape and surface characteristics (Snyder et al., 1985).

Arashiki et al. (2009) showed that spectrins are the primary targets of 4-HNE, and that 4-HNE-modified spectrins generate irreversibly cross-linked aggregates in human RBC under specific conditions (Arashiki et al., 2010).

Spectrin modifications at positions that are responsible for anchorage of the cytoskeleton to the lipid bilayer, via band 3-ankyrin and GPC-protein 4.1 may create stiffening and loss of resistance to stresses to which the RBC is normally subjected. This results in increased phagocytosis.

The main source of 4-HNE was shown here to be inflammation, hence an extracellular source for RBC in the blood. Also if in case of malaria, 4-HNE diffuses out from pRBC to modify npRBC and modifications will additionally occur during spleen passage, the initial site of interaction with the

target cell inevitably remains the outer surface before 4-HNE passes across the membrane to label spectrin and other membrane proteins.

For phagocytosis, RBC is transformed to be recognized by expert phagocytes, undergoing changes, at the surface level and at the structural level to become rigid.

To my knowledge this is the first large study available for 4-HNE-conjugate accumulation in peripheral RBC in anemia and blood hemoglobin had been correlated to serum levels of free 4HNE only in patients with renal anemia (Wiswedel et al., 2008).

Target proteins for 4-HNE were not identified yet in RBC of anemic patients and the herein found high load of spectrin with 4-HNE fits well with the above proposed molecular mechanism for rigidification of RBC and phagocytosis, but I found also increasing 4-HNE conjugation on the surface of the older RBC fractions from healthy blood donors. The modifications on surface proteins change the antigenic characteristics of the cell and may be recognized by phagocytes. Indeed, RBC with heavily modified surfaces were phagocytosed more avidly in most recently performed *in vitro* phagocytosis experiment (not shown). Without targeted modifications (Fang et al., 2013) in cell models for phagocytosis it is hard to decide whether a single process is limiting for phagocytosis, the rigidification or the antigen recognition by phagocytes or, more plausibly, both contribute. The available blood from patients did not allow to collect experimental data on cell surface modifications by 4-HNE, but I cannot exclude that, just as I found significantly greater changes in spectrins in anemia patients s than in controls, modifications are also increased on the cell surface and contribute to propagation of phagocytosis in anemics.

More elucidation is needed on the molecular target proteins but also on the specific amino acid residues involved in the covalent bond to deduce functional impairments.

Subsequent studies which include surface changes in RBC mediated by 4-HNE, are crucial to understand their impact on the development of any type of anemia that remains a major killer of young children in developing countries.

The profound modification by 4-HNE and its impact in anemic children suggests the idea of therapeutic use of drugs or plant extracts to combat the deleterious accumulation of 4-HNE in the RBC membrane. In adipose patients a successful reduction of RBC membrane modifications has been described (Wang et al., 2020). In addition, it has been demonstrated that in hypertriglyceridemic patients, after in vitro 4-hydroxytyrosol treatment, RBC membranes were protected from oxidative damage (Gallo et al., 2017).

No data are available yet for an application in anemia.

So, similarly to other diseases, the therapeutic administration of antioxidants or 4-HNE 'scavengers' might be useful to reduce the load with 4-HNE-protein conjugates with the aim to improve the anemic symptoms at least partially. For this reason, my results should be considered as an important basis for further studies.

References

ABDALLA, S. H. 1988. Peripheral blood and bone marrow leucocytes in Gambian children with malaria: numerical changes and evaluation of phagocytosis. *Ann Trop Paediatr,* 8**,** 250-8.

AGUILAR, R., MARROCCO, T., SKOROKHOD, O. A., BARBOSA, A., NHABOMBA, A., MANACA, M. N., GUINOVART, C., QUINTÓ, L., ARESE, P. & ALONSO, P. L. 2014. Blood oxidative stress markers and P lasmodium falciparum malaria in non-immune A frican children. *British journal of haematology,* 164**,** 438-450.

AHMED, M. & COX-SINGH, J. 2015. P lasmodium knowlesi–an emerging pathogen. *ISBT science series,* 10**,** 134-140.

ALLEN, S., ROWE, P., ALLSOPP, C., RILEY, E., JAKOBSEN, P., HILL, A. & GREENWOOD, B. 1993. A prospective study of the influence of α thalassaemia on morbidity from malaria and immune responses to defined Plasmodium falciparum antigens in Gambian children. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 87**,** 282-285.

ALLISON, A. 1963. Malaria and glucose-6-phosphate dehydrogenase deficiency. *Nature,* 197**,** 609-609.

ALVIZ-AMADOR, A., GALINDO-MURILLO, R., PÉREZ-GONZÁLEZ, H., RODRÍGUEZ-CAVALLO, E., VIVAS-REYES, R. & MÉNDEZ-CUADRO, D. 2019. Effect of 4-HNE Modification on ZU5-ANK Domain and the Formation of Their Complex with β-Spectrin: A Molecular Dynamics Simulation Study. *Journal of chemical information and modeling,* 60**,** 805-820.

AMBELE, M. A. & EGAN, T. J. 2012. Neutral lipids associated with haemozoin mediate efficient and rapid β-haematin formation at physiological pH, temperature and ionic composition. *Malaria journal,* 11**,** 337.

ARASHIKI, N., OTSUKA, Y., ITO, D., YANG, M., KOMATSU, T., SATO, K. & INABA, M. 2010. The covalent modification of spectrin in red cell membranes by the lipid peroxidation product 4-hydroxy-2-nonenal. *Biochemical and biophysical research communications,* 391**,** 1543-1547.

ARESE, P., TURRINI, F. & SCHWARZER, E. 2005. Band 3/complement-mediated recognition and removal of normally senescent and pathological human erythrocytes. *Cellular Physiology and Biochemistry,* 16**,** 133-146.

ATAMNA, H. & GINSBURG, H. 1993. Origin of reactive oxygen species in erythrocytes infected with Plasmodium falciparum. *Mol Biochem Parasitol,* 61**,** 231-41.

BARRERA, V., SKOROKHOD, O. A., BACI, D., GREMO, G., ARESE, P. & SCHWARZER, E. 2011. Host fibrinogen stably bound to hemozoin rapidly activates monocytes via TLR-4 and CD11b/CD18-integrin: a new paradigm of hemozoin action. *Blood,* 117**,** 5674-82.

BARUCH, D. I., PASLOSKE, B. L., SINGH, H. B., BI, X., MA, X. C., FELDMAN, M., TARASCHI, T. F. & HOWARD, R. J. 1995. Cloning the P. falciparum gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes. *Cell,* 82**,** 77-87.

BATTLE, K. E., GETHING, P. W., ELYAZAR, I. R., MOYES, C. L., SINKA, M. E., HOWES, R. E., GUERRA, C. A., PRICE, R. N., BAIRD, J. K. & HAY, S. I. 2012. The global public health significance of Plasmodium vivax. *Advances in parasitology,* 80**,** 1-111.

BEARD, J. L. 2001. Iron biology in immune function, muscle metabolism and neuronal functioning. *The Journal of nutrition,* 131**,** 568S-580S.

BECKER, K., TILLEY, L., VENNERSTROM, J. L., ROBERTS, D., ROGERSON, S. & GINSBURG, H. 2004. Oxidative stress in malaria parasite-infected erythrocytes: host-parasite interactions. *Int J Parasitol,* 34**,** 163-89.

BENEDETTI, A., COMPORTI, M. & ESTERBAUER, H. 1980. Identification of 4-hydroxynonenal as a cytotoxic product originating from the peroxidation of liver microsomal lipids. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism,* 620**,** 281-296.

BERHANE, K., WIDERSTEN, M., ENGSTRÖM, A., KOZARICH, J. W. & MANNERVIK, B. 1994. Detoxication of base propenals and other alpha, beta-unsaturated aldehyde products of radical reactions and lipid peroxidation by human glutathione transferases. *Proc Natl Acad Sci U S A,* 91**,** 1480-4.

BIEMBA, G., DOLMANS, D., THUMA, P. E., WEISS, G. & GORDEUK, V. R. 2000. Severe anaemia in Zambian children with Plasmodium falciparum malaria. *Trop Med Int Health,* 5**,** 9-16.

BITOH, T., FUEDA, K., OHMAE, H., WATANABE, M. & ISHIKAWA, H. 2011. Risk analysis of the re-emergence of Plasmodium vivax malaria in Japan using a stochastic transmission model. *Environmental health and preventive medicine,* 16**,** 171-177.

BOAS, F. E., FORMAN, L. & BEUTLER, E. 1998. Phosphatidylserine exposure and red cell viability in red cell aging and in hemolytic anemia. *Proceedings of the National Academy of Sciences,* 95**,** 3077-3081.

BOSMAN, G. J., CLUITMANS, J. C., GROENEN, Y. A., WERRE, J. M., WILLEKENS, F. L. & NOVOTNÝ, V. M. 2011. Susceptibility to hyperosmotic stress–induced phosphatidylserine exposure increases during red blood cell storage. *Transfusion,* 51**,** 1072-1078.

BRABIN, B. J., PREMJI, Z. & VERHOEFF, F. 2001. An analysis of anemia and child mortality. *J Nutr,* 131**,** 636S-645S; discussion 646S-648S.

BRATOSIN, D., MAZURIER, J., TISSIER, J., ESTAQUIER, J., HUART, J., AMEISEN, J., AMINOFF, D. & MONTREUIL, J. 1998. Cellular and molecular mechanisms of senescent erythrocyte phagocytosis by macrophages. A review. *Biochimie,* 80**,** 173-195.

BUCK, E. & FINNIGAN, N. A. 2019. History and Physical.

BUFFET, P. A., SAFEUKUI, I., MILON, G., MERCEREAU-PUIJALON, O. & DAVID, P. H. 2009. Retention of erythrocytes in the spleen: a double-edged process in human malaria. *Current opinion in hematology,* 16**,** 157-164.

BUFFINTON, G. D., HUNT, N., COWDEN, W. & CLARK, I. A. 1988. Detection of short-chain carbonyl products of lipid peroxidation from malaria-parasite (Plasmodium vinckei)-infected red blood cells exposed to oxidative stress. *Biochemical Journal,* 249**,** 63-68.

BURGMANN, H., LOOAREESUWAN, S., KAPIOTIS, S., VIRAVAN, C., VANIJANONTA, S., HOLLENSTEIN, U., WIESINGER, E., PRESTERL, E., WINKLER, S. & GRANINGER, W. 1996. Serum levels of erythropoietin in acute Plasmodium falciparum malaria. *Am J Trop Med Hyg,* 54**,** 280-3.

CASALS-PASCUAL, C., KAI, O., CHEUNG, J. O., WILLIAMS, S., LOWE, B., NYANOTI, M., WILLIAMS, T. N., MAITLAND, K., MOLYNEUX, M., NEWTON, C. R., PESHU, N., WATT, S. M. & ROBERTS, D. J. 2006. Suppression of erythropoiesis in malarial anemia is associated with hemozoin in vitro and in vivo. *Blood,* 108**,** 2569-77.

CASALS-PASCUAL, C. & ROBERTS, D. J. 2006. Severe malarial anaemia. *Curr Mol Med,* 6**,** 155-68.

CELADA, A., CRUCHAUD, A. & PERRIN, L. 1982. Opsonic activity of human immune serum on in vitro phagocytosis of Plasmodium falciparum infected red blood cells by monocytes. *Clinical and experimental immunology,* 47**,** 635.

CHANG, K. H., TAM, M. & STEVENSON, M. M. 2004. Inappropriately low reticulocytosis in severe malarial anemia correlates with suppression in the development of late erythroid precursors. *Blood,* 103**,** 3727-35.

CHAPARRO, C. M. & SUCHDEV, P. S. 2019. Anemia epidemiology, pathophysiology, and etiology in low- and middle-income countries. *Ann N Y Acad Sci,* 1450**,** 15-31.

CLARK, I., CHAUDHRI, G. & COWDEN, W. 1989. Roles of tumour necrosis factor in the illness and pathology of malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 83**,** 436-440.

CLARK, I. A. & CHAUDHRI, G. 1988. Tumour necrosis factor may contribute to the anaemia of malaria by causing dyserythropoiesis and erythrophagocytosis. *Br J Haematol,* 70**,** 99-103.

COLLABORATORS, G. B. O. D. S. 2015. Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet,* 386**,** 743-800.

COMPORTI, M. 1998. Lipid peroxidation and biogenic aldehydes: from the identification of 4-hydroxynonenal to further achievements in biopathology. *Free Radic Res,* 28**,** 623-35.

COOKE, B. M., MOHANDAS, N. & COPPEL, R. L. 2004. Malaria and the red blood cell membrane. *Semin Hematol,* 41**,** 173-88.

COWMAN, A. F., TONKIN, C. J., THAM, W.-H. & DURAISINGH, M. T. 2017. The molecular basis of erythrocyte invasion by malaria parasites. *Cell host & microbe,* 22**,** 232-245.

CRABB, J. W., O'NEIL, J., MIYAGI, M., WEST, K. & HOFF, H. F. 2002. Hydroxynonenal inactivates cathepsin B by forming Michael adducts with active site residues. *Protein Sci,* 11**,** 831-40.

CRAWLEY, J. 2004. Reducing the burden of anemia in infants and young children in malaria-endemic countries of Africa: from evidence to action. *The American journal of tropical medicine and hygiene,* 71**,** 25-34.

DAS, B. S. & NANDA, N. K. 1999. Evidence for erythrocyte lipid peroxidation in acute falciparum malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 93**,** 58-62.

DEMAEYER, E. & ADIELS-TEGMAN, M. 1985. The prevalence of anaemia in the world. *World health statistics quarterly 1985; 38 (3): 302-316;*.

DEMEHIN, A. A., ABUGO, O. O., JAYAKUMAR, R., LAKOWICZ, J. R. & RIFKIND, J. M. 2002. Binding of hemoglobin to red cell membranes with eosin-5-maleimide-labeled band 3: Analysis of centrifugation and fluorescence lifetime data. *Biochemistry,* 41**,** 8630-8637.

DÍEZ-PADRISA, N., AGUILAR, R., MACHEVO, S., MORAIS, L., NHAMPOSSA, T., O'CALLAGHAN-GORDO, C., NHALUNGO, D., MENÉNDEZ, C., ROCA, A. & ALONSO, P. L. 2011. Erythropoietin levels are not independently associated with malaria-attributable severe disease in Mozambican children. *PLoS One,* 6**,** e24090.

DONDORP, A. M., ANGUS, B. J., CHOTIVANICH, K., SILAMUT, K., RUANGVEERAYUTH, R., HARDEMAN, M. R., KAGER, P. A., VREEKEN, J. & WHITE, N. J. 1999. Red blood cell deformability as a predictor of anemia in severe falciparum malaria. *Am J Trop Med Hyg,* 60**,** 733-7.

DÖRMER, P., DIETRICH, M., KERN, P. & HORSTMANN, R. D. 1983. Ineffective erythropoiesis in acute human P. falciparum malaria. *Blut,* 46**,** 279-88.

ENEVOLD, A., ALIFRANGIS, M., SANCHEZ, J. J., CARNEIRO, I., ROPER, C., BØRSTING, C., LUSINGU, J., VESTERGAARD, L. S., LEMNGE, M. M., MORLING, N., RILEY, E. & DRAKELEY, C. J. 2007. Associations between alpha+-thalassemia and Plasmodium falciparum malarial infection in northeastern Tanzania. *J Infect Dis,* 196**,** 451-9.

FACER, C. A. 1980. Direct Coombs antiglobulin reactions in Gambian children with Plasmodium falciparum malaria. II. Specificity of erythrocyte-bound IgG. *Clin Exp Immunol,* 39**,** 279-88.

FACER, C. A., BRAY, R. S. & BROWN, J. 1979. Direct Coombs antiglobulin reactions in Gambian children with Plasmodium falciparum malaria. I. Incidence and class specificity. *Clin Exp Immunol,* 35**,** 119-27.

FANG, X., FU, Y., LONG, M. J., HAEGELE, J. A., GE, E. J., PARVEZ, S. & AYE, Y. 2013. Temporally controlled targeting of 4-hydroxynonenal to specific proteins in living cells. *Journal of the American Chemical Society,* 135**,** 14496-14499.

FIBACH, E., MANOR, D., OPPENHEIM, A. & RACHMILEWITZ, E. A. 1989. Proliferation and maturation of human erythroid progenitors in liquid culture. *Blood,* 73**,** 100-3.

FIORI, P. L., RAPPELLI, P., MIRKARIMI, S. N., GINSBURG, H., CAPPUCCINELLI, P. & TURRINI, F. 1993. Reduced microbicidal and anti-tumour activities of human monocytes after ingestion of Plasmodium falciparum-infected red blood cells. *Parasite Immunol,* 15**,** 647-55.

FISHMAN, S. M., CHRISTIAN, P. & WEST, K. P. 2000. The role of vitamins in the prevention and control of anaemia. *Public Health Nutr,* 3**,** 125-50.

FREIKMAN, I., AMER, J., COHEN, J. S., RINGEL, I. & FIBACH, E. 2008. Oxidative stress causes membrane phospholipid rearrangement and shedding from RBC membranes—an NMR study. *Biochimica et Biophysica Acta (BBA)-Biomembranes,* 1778**,** 2388-2394.

GALLO, G., BRUNO, R., TARANTO, A. & MARTINO, G. 2017. Are polyunsaturated fatty acid metabolites, the protective effect of 4-hydroxytyrosol on human red blood cell membranes and oxidative damage (4-hydroxyalkenals) compatible in hypertriglyceridemic patients? *Pharmacognosy magazine,* 13**,** S561.

GENTON, B., D'ACREMONT, V., RARE, L., BAEA, K., REEDER, J. C., ALPERS, M. P. & MÜLLER, I. 2008. Plasmodium vivax and mixed infections are associated with severe malaria in children: a prospective cohort study from Papua New Guinea. *PLoS Med,* 5**,** e127.

GETHING, P. W., ELYAZAR, I. R., MOYES, C. L., SMITH, D. L., BATTLE, K. E., GUERRA, C. A., PATIL, A. P., TATEM, A. J., HOWES, R. E. & MYERS, M. F. 2012. A long neglected world malaria map: Plasmodium vivax endemicity in 2010. *PLoS Negl Trop Dis,* 6**,** e1814.

GHASHGHAEINIA, M., CLUITMANS, J. C., AKEL, A., DREISCHER, P., TOULANY, M., KÖBERLE, M., SKABYTSKA, Y., SAKI, M., BIEDERMANN, T. & DUSZENKO, M. 2012. The impact of erythrocyte age on eryptosis. *British Journal of Haematology,* 157**,** 606-614.

GIARRATANA, M.-C., KOBARI, L., LAPILLONNE, H., CHALMERS, D., KIGER, L., CYNOBER, T., MARDEN, M. C., WAJCMAN, H. & DOUAY, L. 2005. Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells. *Nature biotechnology,* 23**,** 69-74.

GIRIBALDI, G., ULLIERS, D., MANNU, F., ARESE, P. & TURRINI, F. 2001. Growth of Plasmodium falciparum induces stage-dependent haemichrome formation, oxidative aggregation of band 3, membrane deposition of complement and antibodies, and phagocytosis of parasitized erythrocytes. *Br J Haematol,* 113**,** 492-9.

GIRIBALDI, G., ULLIERS, D., SCHWARZER, E., ROBERTS, I., PIACIBELLO, W. & ARESE, P. 2004. Hemozoin- and 4-hydroxynonenal-mediated inhibition of erythropoiesis. Possible role in malarial dyserythropoiesis and anemia. *Haematologica,* 89**,** 492-3.

GREVE, B., LEHMAN, L., LELL, B., LUCKNER, D., SCHMIDT-OTT, R. & KREMSNER, P. 1999. High oxygen radical production is associated with fast parasite clearance in children with Plasmodium falciparum malaria. *The Journal of infectious diseases,* 179**,** 1584-1586.

GRIFFITHS, M. J., NDUNGU, F., BAIRD, K. L., MULLER, D. P., MARSH, K. & NEWTON, C. R. 2001. Oxidative stress and erythrocyte damage in Kenyan children with severe Plasmodium falciparum malaria. *Br J Haematol,* 113**,** 486-91.

GRUNE, T., MICHEL, P., SITTE, N., EGGERT, W., ALBRECHT-NEBE, H., ESTERBAUER, H. & SIEMS, W. G. 1997. Increased levels of 4-hydroxynonenal modified proteins in plasma of children with autoimmune diseases. *Free radical biology and medicine,* 23**,** 357-360.

GUERRA, C. A., GIKANDI, P. W., TATEM, A. J., NOOR, A. M., SMITH, D. L., HAY, S. I. & SNOW, R. W. 2008. The limits and intensity of Plasmodium falciparum transmission: implications for malaria control and elimination worldwide. *PLoS Med,* 5**,** e38.

GUERRA, C. A., HOWES, R. E., PATIL, A. P., GETHING, P. W., VAN BOECKEL, T. P., TEMPERLEY, W. H., KABARIA, C. W., TATEM, A. J., MANH, B. H. & ELYAZAR, I. R. 2010. The international limits and population at risk of Plasmodium vivax transmission in 2009. *PLoS Negl Trop Dis,* 4**,** e774.

GUINOVART, C., DOBAÑO, C., BASSAT, Q., NHABOMBA, A., QUINTÓ, L., MANACA, M. N., AGUILAR, R., RODRÍGUEZ, M. H., BARBOSA, A. & APONTE, J. J. 2012. The role of age and exposure to Plasmodium falciparum in the rate of acquisition of naturally acquired immunity: a randomized controlled trial. *PLoS One,* 7**,** e32362.

HALDAR, K. & MOHANDAS, N. 2009. Malaria, erythrocytic infection, and anemia. *Hematology,* 2009**,** 87-93.

HARDING, C. L., VILLARINO, N. F., VALENTE, E., SCHWARZER, E. & SCHMIDT, N. W. 2020. Plasmodium Impairs Antibacterial Innate Immunity to Systemic Infections in Part Through Hemozoin-Bound Bioactive Molecules. *Front Cell Infect Microbiol,* 10**,** 328.

HAY, S. I., GUERRA, C. A., TATEM, A. J., ATKINSON, P. M. & SNOW, R. W. 2005. Urbanization, malaria transmission and disease burden in Africa. *Nature Reviews Microbiology,* 3**,** 81-90.

HÖHN, A., JUNG, T. & GRUNE, T. 2014. Pathophysiological importance of aggregated damaged proteins. *Free Radical Biology and Medicine,* 71**,** 70-89.

HOWES, R. E., PIEL, F. B., PATIL, A. P., NYANGIRI, O. A., GETHING, P. W., DEWI, M., HOGG, M. M., BATTLE, K. E., PADILLA, C. D. & BAIRD, J. K. 2012. G6PD deficiency prevalence and estimates of affected populations in malaria endemic countries: a geostatistical model-based map. *PLoS Med,* 9**,** e1001339.

HTTPS://WWW.WHO.INT/HEALTH-TOPICS/ANAEMIA#TAB=TAB_1. Available: https://www.who.int/health-topics/anaemia#tab=tab_1.

HUEBERS, H. A., HUEBERS, E., CSIBA, E., RUMMEL, W. & FINCH, C. A. 1983. The significance of transferrin for intestinal iron absorption.

JAIN, S. K., MOHANDAS, N., CLARK, M. R. & SHOHET, S. B. 1983. The effect of malonyldialdehyde, a product of lipid peroxidation, on the deformability, dehydration and 51Cr-survival of erythrocytes. *British Journal of Haematology,* 53**,** 247-255.

JONKER, F. A., CALIS, J. C., PHIRI, K., KRAAIJENHAGEN, R. J., BRABIN, B. J., FARAGHER, B., WIEGERINCK, E. T., TJALSMA, H., SWINKELS, D. W. & VAN HENSBROEK, M. B. 2013. Low hepcidin levels in severely anemic malawian children with high incidence of infectious diseases and bone marrow iron deficiency. *PloS one,* 8**,** e78964.

KANNAN, R., LABOTKA, R. & LOW, P. S. 1988. Isolation and characterization of the hemichrome-stabilized membrane protein aggregates from sickle erythrocytes. Major site of autologous antibody binding. *J Biol Chem,* 263**,** 13766-73.

KAPISHNIKOV, S., WEINER, A., SHIMONI, E., GUTTMANN, P., SCHNEIDER, G., DAHAN-PASTERNAK, N., DZIKOWSKI, R., LEISEROWITZ, L. & ELBAUM, M. 2012. Oriented nucleation of hemozoin at the digestive vacuole membrane in Plasmodium falciparum. *Proceedings of the National Academy of Sciences,* 109**,** 11188-11193.

KAY, M., WYANT, T. & GOODMAN, J. 1994. Autoantibodies to band 3 during aging and disease and aging interventions. *Annals of the New York Academy of Sciences,* 719**,** 419-447.

KAY, M. M. 1975. Mechanism of removal of senescent cells by human macrophages in situ. *Proc Natl Acad Sci U S A,* 72**,** 3521-5.

KAY, M. M. 1984. Localization of senescent cell antigen on band 3. *Proc Natl Acad Sci U S A,* 81**,** 5753-7.

KIM, S.-H., HWANG, S.-Y., SHIN, J. H., MOON, C.-S., KIM, D.-W. & KHO, W.-G. 2009. Molecular genetic characterization of the merozoite surface protein 1 Gene of Plasmodium vivax from reemerging Korean isolates. *Clinical and Vaccine Immunology,* 16**,** 733-738.

KIRSCHNER-ZILBER, I., RABIZADEH, E. & SHAKLAI, N. 1982. The interaction of hemin and bilirubin with the human red cell membrane. *Biochimica et Biophysica Acta (BBA)-Biomembranes,* 690**,** 20-30.

KOBAYASHI, N., KARISOLA, P., PEÑA-CRUZ, V., DORFMAN, D. M., JINUSHI, M., UMETSU, S. E., BUTTE, M. J., NAGUMO, H., CHERNOVA, I. & ZHU, B. 2007. TIM-1 and TIM-4 glycoproteins bind phosphatidylserine and mediate uptake of apoptotic cells. *Immunity,* 27**,** 927-940.

KRUGLIAK, M., ZHANG, J. & GINSBURG, H. 2002. Intraerythrocytic Plasmodium falciparum utilizes only a fraction of the amino acids derived from the digestion of host cell cytosol for the biosynthesis of its proteins. *Molecular and biochemical parasitology,* 119**,** 249-256.

KULKARNI, A. G., SURYAKAR, A. N., SARDESHMUKH, A. S. & RATHI, D. B. 2003. Studies on biochemical changes with special reference to oxidant and antioxidants in malaria patients. *Indian J Clin Biochem,* 18**,** 136-49.

LAEMMLI, U. 1973. Gel electrophoresis of proteins. *J. Mol. Biol.,* 80**,** 575-599.

LAMIKANRA, A. A., BROWN, D., POTOCNIK, A., CASALS-PASCUAL, C., LANGHORNE, J. & ROBERTS, D. J. 2007. Malarial anemia: of mice and men. *Blood, The Journal of the American Society of Hematology,* 110**,** 18-28.

LAMIKANRA, A. A., THERON, M., KOOIJ, T. W. & ROBERTS, D. J. 2009. Hemozoin (malarial pigment) directly promotes apoptosis of erythroid precursors. *PLoS One,* 4**,** e8446.

LECLERC, M. C., DURAND, P., GAUTHIER, C., PATOT, S., BILLOTTE, N., MENEGON, M., SEVERINI, C., AYALA, F. & RENAUD, F. 2004. Meager genetic variability of the human malaria agent Plasmodium vivax. *Proceedings of the National Academy of Sciences,* 101**,** 14455-14460.

LEE, J., KIM, T. I., KANG, J. M., JUN, H., LÊ, H. G., THÁI, T. L., SOHN, W. M., MYINT, M. K., LIN, K., KIM, T. S. & NA, B. K. 2018. Prevalence of glucose-6-phosphate dehydrogenase (G6PD) deficiency among malaria patients in Upper Myanmar. *BMC Infect Dis,* 18**,** 131.

LEECH, J. H., BARNWELL, J. W., MILLER, L. H. & HOWARD, R. J. 1984. Identification of a strain-specific malarial antigen exposed on the surface of Plasmodium falciparum-infected erythrocytes. *The Journal of experimental medicine,* 159**,** 1567-1575.

LELL, B., MAY, J., SCHMIDT-OTT, R. J., LEHMAN, L. G., LUCKNER, D., GREVE, B., MATOUSEK, P., SCHMID, D., HERBICH, K. & MOCKENHAUPT, F. P. 1999. The role of red blood cell polymorphisms in resistance and susceptibility to malaria. *Clinical Infectious Diseases,* 28**,** 794-799.

LI, M. O., SARKISIAN, M. R., MEHAL, W. Z., RAKIC, P. & FLAVELL, R. A. 2003. Phosphatidylserine receptor is required for clearance of apoptotic cells. *Science,* 302**,** 1560-1563.

LOW, P. S. 1986. Structure and function of the cytoplasmic domain of band 3: center of erythrocyte membrane-peripheral protein interactions. *Biochim Biophys Acta,* 864**,** 145-67.

LOW, P. S., WAUGH, S. M., ZINKE, K. & DRENCKHAHN, D. 1985. The role of hemoglobin denaturation and band 3 clustering in red blood cell aging. *Science,* 227**,** 531-533.

LUTZ, H. U. 2004. Innate immune and non-immune mediators of erythrocyte clearance. *Cell Mol Biol (Noisy-le-grand),* 50**,** 107-16.

LYNCH, S. 2011. Case studies: iron. *The American journal of clinical nutrition,* 94**,** 673S-678S.

MALAGUARNERA, L. & MUSUMECI, S. 2002. The immune response to Plasmodium falciparum malaria. *Lancet Infect Dis,* 2**,** 472-8.

MANNO, S., TAKAKUWA, Y. & MOHANDAS, N. 2002. Identification of a functional role for lipid asymmetry in biological membranes: Phosphatidylserine-skeletal protein interactions modulate membrane stability. *Proceedings of the National Academy of Sciences,* 99**,** 1943-1948.

MANNU, F., ARESE, P., CAPPELLINI, M. D., FIORELLI, G., CAPPADORO, M., GIRIBALDI, G. & TURRINI, F. 1995. Role of hemichrome binding to erythrocyte membrane in the generation of band-3 alterations in beta-thalassemia intermedia erythrocytes.

MBURU, A. S., THURNHAM, D. I., MWANIKI, D. L., MUNIU, E. M., ALUMASA, F. & DE WAGT, A. 2008. The influence and benefits of controlling for inflammation on plasma ferritin and hemoglobin responses following a multi-micronutrient supplement in apparently healthy, HIV+ Kenyan adults. *The Journal of nutrition,* 138**,** 613-619.

MEI, Z., COGSWELL, M. E., PARVANTA, I., LYNCH, S., BEARD, J. L., STOLTZFUS, R. J. & GRUMMER-STRAWN, L. M. 2005. Hemoglobin and ferritin are currently the most efficient indicators of population response to iron interventions: an analysis of nine randomized controlled trials. *The Journal of nutrition,* 135**,** 1974-1980.

MENDIS, K. N., DAVID, P. H., HOMMEL, M., CARTER, R. & MILLER, L. H. 1983. Immunity to malarial antigens on the surface of Plasmodium falciparum-infected erythrocytes. *The American Society of Tropical Medicine and Hygiene,* 32**,** 926-30.

MENENDEZ, C., FLEMING, A. & ALONSO, P. 2000a. Malaria-related anaemia. *Parasitology today,* 16**,** 469-476.

MENENDEZ, C., FLEMING, A. F. & ALONSO, P. L. 2000b. Malaria-related anaemia. *Parasitol Today,* 16**,** 469-76.

MESHNICK, S. R. 2002. Artemisinin: mechanisms of action, resistance and toxicity. *International journal for parasitology,* 32**,** 1655-1660.

MILLER, C. M. 2005. *Hemozoin mediated oxidation of polyunsaturated fatty acids*, Vanderbilt University.

MILLER, L. H. 1969. Distribution of mature trophozoites and schizonts of Plasmodium falciparum in the organs of Aotus trivirgatus, the night monkey. *The American journal of tropical medicine and hygiene,* 18**,** 860-865.

MOCKENHAUPT, F. P., BIENZLE, U., MAY, J., FALUSI, A. G., ADEMOWO, O. G., OLUMESE, P. E. & MEYER, C. G. 1999. Plasmodium falciparum infection: influence on hemoglobin levels in α-thalassemia and microcytosis. *The Journal of infectious diseases,* 180**,** 925-928.

MØLLER, I. M., ROGOWSKA-WRZESINSKA, A. & RAO, R. 2011. Protein carbonylation and metal-catalyzed protein oxidation in a cellular perspective. *Journal of proteomics,* 74**,** 2228-2242.

MORALEDA, C., AGUILAR, R., QUINTÓ, L., NHAMPOSSA, T., RENOM, M., NHABOMBA, A., ACÁCIO, S., APONTE, J. J., NHALUNGO, D. & ACHTMAN, A. H. 2017. Anaemia in hospitalised preschool children from a rural area in Mozambique: a case control study in search for aetiological agents. *BMC pediatrics,* 17**,** 1-10.

MOURÃO, L. C., DE PAULA BAPTISTA, R., DE ALMEIDA, Z. B., GRYNBERG, P., PUCCI, M. M., CASTRO-GOMES, T., FONTES, C. J. F., RATHORE, S., SHARMA, Y. D. & DA SILVA-PEREIRA, R. A. 2018. Anti-band 3 and anti-spectrin antibodies are increased in Plasmodium vivax infection and are associated with anemia. *Scientific reports,* 8**,** 1-12.

NANDA, N. K. & DAS, B. S. 2000. Presence of pro-oxidants in plasma of patients suffering from falciparum malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 94**,** 684-688.

NARSARIA, N., MOHANTY, C., DAS, B., MISHRA, S. & PRASAD, R. 2012. Oxidative stress in children with severe malaria. *Journal of tropical pediatrics,* 58**,** 147-150.

NEUBERGER, A., OKEBE, J., YAHAV, D. & PAUL, M. 2016. Oral iron supplements for children in malaria-endemic areas. *Cochrane database of systematic reviews*.

NEWTON, C. R., WARN, P. A., WINSTANLEY, P. A., PESHU, N., SNOW, R. W., PASVOL, G. & MARSH, K. 1997. Severe anaemia in children living in a malaria endemic area of Kenya. *Trop Med Int Health,* 2**,** 165-78.

NSIAH, K., BAHAAH, B., OPPONG AFRANIE, B., KOFFIE, S., AKOWUAH, E. & DONKOR, S. 2019. Oxidative stress and hemoglobin level of complicated and uncomplicated malaria cases among children: a cross-sectional study in kumasi metropolis, Ghana. *Journal of tropical medicine,* 2019.

NUSSENBLATT, V. & SEMBA, R. D. 2002. Micronutrient malnutrition and the pathogenesis of malarial anemia. *Acta Tropica,* 82**,** 321-337.

NYAKERIGA, A. M., TROYE-BLOMBERG, M., DORFMAN, J. R., ALEXANDER, N. D., BÄCK, R., KORTOK, M., CHEMTAI, A. K., MARSH, K. & WILLIAMS, T. N. 2004. Iron deficiency and malaria among children living on the coast of Kenya. *Journal of Infectious Diseases,* 190**,** 439-44.

NYSTRÖM, T. 2005. Role of oxidative carbonylation in protein quality control and senescence. *The EMBO journal,* 24**,** 1311-1317.

ORGANIZATION, W. H. 2015. *Guidelines for the treatment of malaria*, World Health Organization.

ORJIH, A. U., MATHEW, T. C. & CHERIAN, P. T. 2012. Erythrocyte membranes convert monomeric ferriprotoporphyrin IX to β-hematin in acidic environment at malarial fever temperature. *Experimental Biology and Medicine,* 237**,** 884-893.

PAGOLA, S., STEPHENS, P. W., BOHLE, D. S., KOSAR, A. D. & MADSEN, S. K. 2000. The structure of malaria pigment β-haematin. *Nature,* 404**,** 307-310.

PANTALEO, A., FERRU, E., GIRIBALDI, G., MANNU, F., CARTA, F., MATTE, A., DE FRANCESCHI, L. & TURRINI, F. 2009. Oxidized and poorly glycosylated band 3 is selectively phosphorylated by Syk kinase to form large membrane clusters in normal and G6PD-deficient red blood cells. *Biochemical Journal,* 418**,** 359-367.

PANTALEO, A., GIRIBALDI, G., MANNU, F., ARESE, P. & TURRINI, F. 2008. Naturally occurring anti-band 3 antibodies and red blood cell removal under physiological and pathological conditions. *Autoimmunity reviews,* 7**,** 457-462.

PARK, S., JUNG, M., KIM, H., LEE, S., KIM, S., LEE, B., KWON, T., PARK, R. & KIM, I.-S. 2008. Rapid cell corpse clearance by stabilin-2, a membrane phosphatidylserine receptor. *Cell Death & Differentiation,* 15**,** 192-201.

PAROLA, M., BELLOMO, G., ROBINO, G., BARRERA, G. & DIANZANI, M. U. 1999. 4-Hydroxynonenal as a biological signal: molecular basis and pathophysiological implications. *Antioxid Redox Signal,* 1**,** 255-84.

PERCÁRIO, S., MOREIRA, D. R., GOMES, B. A., FERREIRA, M. E., GONÇALVES, A. C. M., LAURINDO, P. S., VILHENA, T. C., DOLABELA, M. F. & GREEN, M. D. 2012. Oxidative stress in malaria. *International journal of molecular sciences,* 13**,** 16346-16372.

PHILLIPS, R. E. & PASVOL, G. 1992. Anaemia of Plasmodium falciparum malaria. *Baillieres Clin Haematol,* 5**,** 315-30.

PIOMELLI, S. & SEAMAN, C. 1993. Mechanism of red blood cell aging: relationship of cell density and cell age. *American journal of hematology,* 42**,** 46-52.

PISCIOTTA, J. M., COPPENS, I., TRIPATHI, A. K., SCHOLL, P. F., SHUMAN, J., BAJAD, S., SHULAEV, V. & SULLIVAN JR, D. J. 2007. The role of neutral lipid nanospheres in Plasmodium falciparum haem crystallization. *Biochemical Journal,* 402**,** 197-204.

POLI, G., BIASI, F. & LEONARDUZZI, G. 2008. 4-Hydroxynonenal-protein adducts: A reliable biomarker of lipid oxidation in liver diseases. *Mol Aspects Med,* 29**,** 67-71.

PRATO, M., GIRIBALDI, G., POLIMENI, M., GALLO, V. & ARESE, P. 2005. Phagocytosis of hemozoin enhances matrix metalloproteinase-9 activity and TNF-alpha production in human monocytes: role of matrix metalloproteinases in the pathogenesis of falciparum malaria. *Thr Journal of Immunology,* 175**,** 6436-42.

PRETINI, V., KOENEN, M. H., KAESTNER, L., FENS, M. H., SCHIFFELERS, R. M., BARTELS, M. & VAN WIJK, R. 2019. Red blood cells: chasing interactions. *Frontiers in physiology,* 10**,** 945.

PRICE, R. N., TJITRA, E., GUERRA, C. A., YEUNG, S., WHITE, N. J. & ANSTEY, N. M. 2007. Vivax malaria: neglected and not benign. *The American journal of tropical medicine and hygiene,* 77**,** 79-87.

RAYMOND, A., ENSSLIN, M. A. & SHUR, B. D. 2009. SED1/MFG-E8: a bi-motif protein that orchestrates diverse cellular interactions. *Journal of cellular biochemistry,* 106**,** 957-966.

REY, J., BUFFET, P. A., CICERON, L., MILON, G., MERCEREAU-PUIJALON, O. & SAFEUKUI, I. 2014. Reduced erythrocyte deformability associated with hypoargininemia during Plasmodium falciparum malaria. *Sci Rep,* 4**,** 3767.

RIFKIND, J. M., NAGABABU, E., RAMASAMY, S. & RAVI, L. B. 2003. Hemoglobin redox reactions and oxidative stress. *Redox Rep,* 8**,** 234-7.

RODRIGUEZ-MORALES, A. J., SANCHEZ, E., VARGAS, M., PICCOLO, C., COLINA, R., ARRIA, M. & FRANCO-PAREDES, C. 2006. Pregnancy outcomes associated with Plasmodium vivax malaria in northeastern Venezuela. *The American journal of tropical medicine and hygiene,* 74**,** 755-757.

SCACCABAROZZI, D., DEROOST, K., CORBETT, Y., LAYS, N., CORSETTO, P., SALÈ, F. O., VAN DEN STEEN, P. E. & TARAMELLI, D. 2018. Differential induction of malaria liver pathology in mice infected with Plasmodium chabaudi AS or Plasmodium berghei NK65. *Malaria journal,* 17**,** 1-9.

SCHAUENSTEIN, E., ESTERBAUER, H., JAAG, G. & TAUFER, M. 1964. The effect of aldehydes on normal and malignant cells. 1st report: hydroxyoctenal, a new fat aldehyde. *Monatshefte Fur Chemie,* 95**,** 180-183.

SCHLÜTER, K. & DRENCKHAHN, D. 1986. Co-clustering of denatured hemoglobin with band 3: its role in binding of autoantibodies against band 3 to abnormal and aged erythrocytes. *Proceedings of the National Academy of Sciences,* 83**,** 6137-6141.

SCHWARZER, E. & ARESE, P. 1996. Phagocytosis of malarial pigment hemozoin inhibits NADPH-oxidase activity in human monocyte-derived macrophages. *Biochim Biophys Acta,* 1316**,** 169-75.

SCHWARZER, E., ARESE, P. & SKOROKHOD, O. A. 2014. 4-Hydroxynonenal in the Physiology and Pathology of Malaria. *Encyclopedia of Malaria*.

SCHWARZER, E., ARESE, P. & SKOROKHOD, O. A. 2015. Role of the lipoperoxidation product 4-hydroxynonenal in the pathogenesis of severe malaria anemia and malaria immunodepression. *Oxidative medicine and cellular longevity,* 2015.

SCHWARZER, E., BELLOMO, G., GIRIBALDI, G., ULLIERS, D. & ARESE, P. 2001. Phagocytosis of malarial pigment haemozoin by human monocytes: a confocal microscopy study. *Parasitology,* 123**,** 125.

SCHWARZER, E., MÜLLER, O., ARESE, P., SIEMS, W. G. & GRUNE, T. 1996. Increased levels of 4-hydroxynonenal in human monocytes fed with malarial pigment hemozoin. A possible clue for hemozoin toxicity. *FEBS Lett,* 388**,** 119-22.

SCHWARZER, E., SKOROKHOD, O. A., BARRERA, V. & ARESE, P. 2008. Hemozoin and the human monocyte--a brief review of their interactions. *Parassitologia,* 50**,** 143-5.

SCHWARZER, E., TURRINI, F., GIRIBALDI, G., CAPPADORO, M. & ARESE, P. 1993. Phagocytosis of P. falciparum malarial pigment hemozoin by human monocytes inactivates monocyte protein kinase C. *Biochim Biophys Acta,* 1181**,** 51-54.

SCHWARZER, E., TURRINI, F., ULLIERS, D., GIRIBALDI, G., GINSBURG, H. & ARESE, P. 1992a. Impairment of macrophage functions after ingestion of Plasmodium falciparum-infected erythrocytes or isolated malarial pigment. *Journal of Experimental Medicine,* 176**,** 1033-41.

SCHWARZER, E., TURRINI, F., ULLIERS, D., GIRIBALDI, G., GINSBURG, H. & ARESE, P. 1992b. Impairment of macrophage functions after ingestion of Plasmodium falciparum-infected erythrocytes or isolated malarial pigment. *Journal of Experimental Medicine,* 176**,** 1033-1041.

SCORZA, T., MAGEZ, S., BRYS, L. & DE BAETSELIER, P. 1999. Hemozoin is a key factor in the induction of malaria-associated immunosuppression. *Parasite immunology,* 21**,** 545-554.

SEVERINI, C., MENEGON, M., DI LUCA, M., ABDULLAEV, I., MAJORI, G., RAZAKOV, S. & GRADONI, L. 2004. Risk of Plasmodium vivax malaria reintroduction in Uzbekistan: genetic characterization of parasites and status of potential malaria vectors in the Surkhandarya region. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 98**,** 585-592.

SHERLING, E. S. & VAN OOIJ, C. 2016. Host cell remodeling by pathogens: the exomembrane system in Plasmodium-infected erythrocytes. *FEMS microbiology reviews,* 40**,** 701-721.

SIEMS, W., QUAST, S., CARLUCCIO, F., WISWEDEL, I., HIRSCH, D., AUGUSTIN, W., KRAEMER, K., HAMPL, H. & SOMMERBURG, O. 2003. Oxidative stress in cardio renal anemia syndrome: correlations and therapeutic possibilities. *Clin Nephrol,* 60 Suppl 1**,** S22-30.

SIEMS, W. G., ZOLLNER, H., GRUNE, T. & ESTERBAUER, H. 1997. Metabolic fate of 4-hydroxynonenal in hepatocytes: 1,4-dihydroxynonene is not the main product. *J Lipid Res,* 38**,** 612-22.

SINGHAL, S. S., ZIMNIAK, P., AWASTHI, S., PIPER, J. T., HE, N. G., TENG, J. I., PETERSEN, D. R. & AWASTHI, Y. C. 1994. Several closely related glutathione S-transferase isozymes catalyzing conjugation of 4-hydroxynonenal are differentially expressed in human tissues. *Arch Biochem Biophys,* 311**,** 242-50.

SKOROKHOD, A., SCHWARZER, E., GREMO, G. & ARESE, P. 2007a. HNE produced by the malaria parasite Plasmodium falciparum generates HNE-protein adducts and decreases erythrocyte deformability. *Redox Rep,* 12**,** 73-5.

SKOROKHOD, O., SCHWARZER, E., GRUNE, T. & ARESE, P. 2005. Role of 4-hydroxynonenal in the hemozoin-mediated inhibition of differentiation of human monocytes to dendritic cells induced by GM-CSF/IL-4. *BioFactors,* 24**,** 283-289.

SKOROKHOD, O. A., ALESSIO, M., MORDMÜLLER, B., ARESE, P. & SCHWARZER, E. 2004. Hemozoin (malarial pigment) inhibits differentiation and maturation of human monocyte-derived dendritic cells: a peroxisome proliferator-activated receptor-γ-mediated effect. *The Journal of Immunology,* 173**,** 4066-4074.

SKOROKHOD, O. A., BARRERA, V., HELLER, R., CARTA, F., TURRINI, F., ARESE, P. & SCHWARZER, E. 2014. Malarial pigment hemozoin impairs chemotactic motility and transendothelial migration of monocytes via 4-hydroxynonenal. *Free Radical Biology and Medicine,* 75**,** 210-221.

SKOROKHOD, O. A., CAIONE, L., MARROCCO, T., MIGLIARDI, G., BARRERA, V., ARESE, P., PIACIBELLO, W. & SCHWARZER, E. 2010. Inhibition of erythropoiesis in malaria anemia: role of hemozoin and hemozoin-generated 4-hydroxynonenal. *Blood,* 116**,** 4328-37.

SKOROKHOD, O. A., SCHWARZER, E., CERETTO, M. & ARESE, P. 2007b. Malarial pigment haemozoin, IFN-gamma, TNF-alpha, IL-1beta and LPS do not stimulate expression of inducible nitric oxide synthase and production of nitric oxide in immuno-purified human monocytes. *Malaria Journal,* 6**,** 73.

SLUTSKER, L., TAYLOR, T. E., WIRIMA, J. J. & STEKETEE, R. W. 1994. In-hospital morbidity and mortality due to malaria-associated severe anaemia in two areas of Malawi with different patterns of malaria infection. *Trans R Soc Trop Med Hyg,* 88**,** 548-51.

SMITH, J. D., CHITNIS, C. E., CRAIG, A. G., ROBERTS, D. J., HUDSON-TAYLOR, D. E., PETERSON, D. S., PINCHES, R., NEWBOLD, C. I. & MILLER, L. H. 1995. Switches in expression of Plasmodium falciparum var genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. *Cell,* 82**,** 101.

SNYDER, L., FORTIER, N., TRAINOR, J., JACOBS, J., LEB, L., LUBIN, B., CHIU, D., SHOHET, S. & MOHANDAS, N. 1985. Effect of hydrogen peroxide exposure on normal human erythrocyte deformability, morphology, surface characteristics, and spectrin-hemoglobin cross-linking. *The Journal of clinical investigation,* 76**,** 1971-1977.

SOHAIL, M., KAUL, A., RAZIUDDIN, M. & ADAK, T. 2007. Decreased glutathione-S-transferase activity: diagnostic and protective role in vivax malaria. *Clin Biochem,* 40**,** 377-82.

SPOTTISWOODE, N., DUFFY, P. E. & DRAKESMITH, H. 2014. Iron, anemia and hepcidin in malaria. *Front Pharmacol,* 5**,** 125.

SPYCHER, S. E., TABATABA-VAKILI, S., O'DONNELL, V. B., PALOMBA, L. & AZZI, A. 1997. Aldose reductase induction: a novel response to oxidative stress of smooth muscle cells. *Faseb j,* 11**,** 181-8.

SRIVASTAVA, S. K., ANSARI, N. H., HAIR, G. A. & DAS, B. 1984. Aldose and aldehyde reductases in human tissues. *Biochim Biophys Acta,* 800**,** 220-7.

STANLEY, H. A., MAYES, J. T., COOPER, N. R. & REESE, R. T. 1984. Complement activation by the surface of Plasmodium falciparum infected erythrocytes. *Molecular Immunology,* 21**,** 145-50.

STEVENS, G. A., FINUCANE, M. M., DE-REGIL, L. M., PACIOREK, C. J., FLAXMAN, S. R., BRANCA, F., PEÑA-ROSAS, J. P., BHUTTA, Z. A. & EZZATI, M. 2013. Global, regional, and national trends in haemoglobin concentration and prevalence of total and severe anaemia in children and pregnant and non-

pregnant women for 1995-2011: a systematic analysis of population-representative data. *Lancet Glob Health,* 1**,** e16-25.

SU, X., HEATWOLE, V., WERTHEIMER, S., GUINET, F., HERRFELDT, J., PETERSON, D., RAVETCH, J. & WELLEMS, T. 1995. The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of Plasmodium falciparum-infected erythrocytes. *Cell,* 82**,** 89-100.

TILLEY, L., FOLEY, M., ANDERS, R. F., DLUZEWSKI, A. R., GRATZER, W. B., JONES, G. L. & SAWYER, W. H. 1990. Rotational dynamics of the integral membrane protein, band 3, as a probe of the membrane events associated with Plasmodium falciparum infections of human erythrocytes. *Biochimica et Biophysica Acta (BBA)-Biomembranes,* 1025**,** 135-142.

TILLEY, S. D., CORNUZ, M., SIVULA, K. & GRÄTZEL, M. 2010. Light-induced water splitting with hematite: improved nanostructure and iridium oxide catalysis. *Angewandte Chemie International Edition,* 49**,** 6405-6408.

TISHKOFF, S. A., VARKONYI, R., CAHINHINAN, N., ABBES, S., ARGYROPOULOS, G., DESTRO-BISOL, G., DROUSIOTOU, A., DANGERFIELD, B., LEFRANC, G., LOISELET, J., PIRO, A., STONEKING, M., TAGARELLI, A., TAGARELLI, G., TOUMA, E. H., WILLIAMS, S. M. & CLARK, A. G. 2001. Haplotype diversity and linkage disequilibrium at human G6PD: recent origin of alleles that confer malarial resistance. *Science,* 293**,** 455-62.

TJITRA, E., ANSTEY, N. M., SUGIARTO, P., WARIKAR, N., KENANGALEM, E., KARYANA, M., LAMPAH, D. A. & PRICE, R. N. 2008. Multidrug-resistant Plasmodium vivax associated with severe and fatal malaria: a prospective study in Papua, Indonesia. *PLoS Med,* 5**,** e128.

TROMBETTA, D., GANGEMI, S., SAIJA, A., MINCIULLO, P. L., CIMINO, F., CRISTANI, M., BRIUGLIA, S., PIRAINO, B., ISOLA, S. & SALPIETRO, C. D. 2006. Increased protein carbonyl groups in the serum of patients affected by thalassemia major. *Annals of hematology,* 85**,** 520-522.

TURRINI, F., GINSBURG, H., BUSSOLINO, F., PESCARMONA, G. P., SERRA, M. V. & ARESE, P. 1992. Phagocytosis of Plasmodium falciparum-infected human red blood cells by human monocytes: involvement of immune and nonimmune determinants and dependence on parasite developmental stage. *Blood,* 80**,** 801-8.

URBAN, B. C., HIEN, T. T., DAY, N. P., PHU, N. H., ROBERTS, R., PONGPONRATN, E., JONES, M., MAI, N. T., BETHELL, D. & TURNER, G. D. 2005. Fatal Plasmodium falciparum malaria causes specific patterns of splenic architectural disorganization. *Infection and immunity,* 73**,** 1986-1994.

UYOGA, S., SKOROKHOD, O. A., OPIYO, M., ORORI, E. N., WILLIAMS, T. N., ARESE, P. & SCHWARZER, E. 2012. Transfer of 4-hydroxynonenal from parasitized to non-parasitized erythrocytes in rosettes. Proposed role in severe malaria anemia. *British journal of haematology,* 157**,** 116-124.

VANDERESSE, R., COLOMBEAU, L., FROCHOT, C. & ACHERAR, S. 2016. Inactivation of malaria parasites in blood: PDT vs inhibition of hemozoin formation. *Current Topics in Malaria***,** 205.

VENKATESAN, M., AMARATUNGA, C., CAMPINO, S., AUBURN, S., KOCH, O., LIM, P., UK, S., SOCHEAT, D., KWIATKOWSKI, D. P. & FAIRHURST, R. M. 2012. Using CF11 cellulose columns to inexpensively and effectively remove human DNA from Plasmodium falciparum-infected whole blood samples. *Malaria journal,* 11**,** 1-7.

VINCENT, S. Oxidative effects of heme and porphyrins on proteins and lipids. Seminars in hematology, 1989. 105-113.

VRYONIS, G. 1939. Observations on the parasitization of erythrocytes by Plasmodium vivax, with special reference to reticulocytes. *American Journal of Hygiene,* 30.

WANG, F.-X., LI, H.-Y., LI, Y.-Q. & KONG, L.-D. 2020. Can Medicinal Plants and Bioactive Compounds Combat Lipid Peroxidation Product 4-HNE-Induced Deleterious Effects? *Biomolecules,* 10**,** 146.

WAUGH, S., WILLARDSON, B., KANNAN, R., LABOTKA, R. & LOW, P. 1986. Heinz bodies induce clustering of band 3, glycophorin, and ankyrin in sickle cell erythrocytes. *The Journal of clinical investigation,* 78**,** 1155-1160.

WAUGH, S. M. & LOW, P. S. 1985. Hemichrome binding to band 3: nucleation of Heinz bodies on the erythrocyte membrane. *Biochemistry,* 24**,** 34-39.

WEGMÜLLER, R., BENTIL, H., WIRTH, J. P., PETRY, N., TANUMIHARDJO, S. A., ALLEN, L., WILLIAMS, T. N., SELENJE, L., MAHAMA, A., AMOAFUL, E., STEINER-ASIEDU, M., ADU-AFARWUAH, S. & ROHNER, F.

2020. Anemia, micronutrient deficiencies, malaria, hemoglobinopathies and malnutrition in young children and non-pregnant women in Ghana: Findings from a national survey. *Plos One,* 15**,** e0228258.

WELBOURN, E. M., WILSON, M. T., YUSOF, A., METODIEV, M. V. & COOPER, C. E. 2017. The mechanism of formation, structure and physiological relevance of covalent hemoglobin attachment to the erythrocyte membrane. *Free Radic Biol Med,* 103**,** 95-106.

WESSELIUS, L. J., NELSON, M. E. & SKIKNE, B. S. 1994. Increased release of ferritin and iron by iron-loaded alveolar macrophages in cigarette smokers. *Am J Respir Crit Care Med,* 150**,** 690-5.

WHO, W. H. O. 2017. World malaria report.

WHO, W. H. O. 2018. World Malaria Report 2018. Geneva: World Health Organization. *Licence: CC BY-NC-SA,* 3.

WISWEDEL, I., PETER, D., GARDEMANN, A., CARLUCCIO, F., HAMPL, H. & SIEMS, W. 2008. Serum Concentrations of F 2-Isoprostanes and 4-Hydroxynonenal in Hemodialysis Patients in Relation to Inflammation and Renal Anemia. *Biomarker insights,* 3**,** BMI. S363.

ZIMMERMANN, M. B., CHAOUKI, N. & HURRELL, R. F. 2005. Iron deficiency due to consumption of a habitual diet low in bioavailable iron: a longitudinal cohort study in Moroccan children. *The American journal of clinical nutrition,* 81**,** 115-121.

Acknowledgements

Ho deciso di scrivere questa parte della tesi in italiano. Per me non è stato mai semplice "fare le conclusioni", dal tema delle elementari, ai ringraziamenti della tesi di laurea, fino a cercare di trovare qualche parola per dire grazie alla fine di questo mio lungo percorso di dottorato. Perciò ho pensato fosse più semplice esprimermi con la mia lingua madre.

Come per la maggior parte delle persone, questi ultimi mesi, sono stati per me non tanto facili perché mi sono trovata di fronte a situazioni che non erano state affatto contemplate, ma che soprattutto non potevano essere controllate da nessuno. Ciononostante, proprio grazie alle persone a me vicine, è uscita fuori una bella grinta in me e quella passione non si è mai spenta.

Premetto che l'ordine che ho utilizzato non è assolutamente una scala di importanza in quanto tutti voi davvero (qualcuno di sicuro l'avrò dimenticato), avete dato un senso a questo lungo percorso.

Grazie a Dr. Clara Menéndez e Dr. Ruth Aguilar e a tutti i campioni preziosi, sui quali il progetto è stato costruito.

Grazie alla mia mentore, Evelin, fonte di ispirazione e ingegno, è sempre riuscita a mantenere viva in me la passione per la scienza, per la vita di laboratorio, accompagnandomi dall'inizio con estrema pazienza in questo bellissimo progetto…Sono onorata di averti conosciuta!

Grazie ad Aleksei, che per primo mi ha fatto conoscere il 4-HNE! Anche se ad un certo punto non eri più presente in lab, per noi ci sei sempre stato e quel filo non si è mai interrotto.

Grazie anche al Professor Paolo Arese, esempio di grande scienziato.

Grazie ai revisori della tesi, dott.ssa Barrera Valentina e prof.ssa Pantaleo Antonella. Il vostro impegno è stato per me prezioso per migliorare il lavoro di tesi.

Elena e Dany, i miei due angeli custodi. Siete le sei del mattino davanti al microscopio, siete gli innumerevoli caffè, siete giornate intere nella camera sterile, mai pesate e sempre piacevoli, siete lo shopping online, siete ricordi che custodirò gelosamente nel mio cuore…E' stato bello per me lavorare con voi, e non solo. Grazie di tutto!

Grazie Cos, il tuo nome non è un caso….grazie per la tua presenza costante, pronto sempre a risolvere qualsiasi problema in lab ma soprattutto a strappare un sorriso dopo una dura giornata, magari non andata benissimo..Sei una persona fantastica e ti voglio bene davvero!

Grazie a tutto il piano di biochimica: la Prof.ssa Chiara Riganti, anche lei sempre disponibile, presente e pronta ad intervenire in qualsiasi esigenza. Carolina, Asia, Elena, Elisabetta, Giuliana, Loredana, i tantissimi studenti che ho avuto il piacere di incontrare nel mio percorso, grazie a tutti perché ad un certo punto quel piano era diventata la mia seconda casa!

Desidero ringraziare la mia famiglia perché rappresenta tutto ciò che sono diventata. Anche il modo in cui ho superato le ultime difficoltà lo devo a loro. Mamma e papà, credo siate i genitori che

ognuno di noi dovrebbe avere al proprio fianco: grazie per essermi stati sempre accanto, mi avete guardato con occhio sempre discreto, senza mai giudicare le mie scelte ed è così che mi avete insegnato quanto sia importante il valore della libertà…..A mio padre…che, nonostante lui non ci creda, è sempre presente nelle mie scelte. A mia madre, la persona che conosce qualsiasi sfumatura di me e la valorizza. A te non potrò nascondere mai niente! Grazie anche per avermi insegnato la gentilezza e per l'amore che ogni giorno ricevo da voi! Vi voglio bene

Un grazie speciale alle mie due sorelle, Margherita e Giusy. So che non amate tanto le parole, voi siete due persone concrete, da fatti, ma ognuna di voi due, a modo suo, ha davvero contribuito a farmi crescere proprio come due vere sorelle maggiori. Giusy è la sorella che, oltre alle a-mail, ti insegna come rialzarti, è la persona dalle mille idee e soluzioni, oltre che cacciatrice infallibile di refusi! Tu sei quella che ad un certo punto ti dice: "Adesso rialzati". Contagio per me di perseveranza e resilienza. Sei un esempio per me..con tutto il cuore, davvero, grazie! Marghe invece è diretta, schietta, sincerità fatta persona, ma dietro quelle parole so che c'è amore incondizionato e voglia di condividere con me le mie decisioni, nonostante i troppi km che ci separano. Sei sempre con me!

Michele ed Ettore, i miei due fratelli acquisiti. Ettore, (strano a dirsi lo so) ma a te devo la leggerezza con cui mi hai fatto affrontare questo ultimo periodo: ogni volta che tornavi da lavoro avevi sempre la battuta pronta per me. Grazie per avermi accolta e non avermi mai fatta sentire di troppo (non ti sarò mai sufficientemente grata) grazie per i vari insegnamenti tecnologici ma soprattutto grazie per la pazienza…due La Grotta in casa non sono di facile gestione! Michele, grazie per la tua costante presenza, anche a distanza riesci a trasmettermi serenità e affetto sincero.

Ai miei due splendidi nipotini, Rosy e Pier….quante cose mi sono persa di voi due ultimamente, lo so!!Ma grazie ai potenti mezzi della tecnologia, abbiamo condiviso quasi tutto, so che non è la stessa cosa ma credetemi tutte le ore passate su Meet erano per me motivo di gioia…Rappresentate la parte più bella e la ricchezza più grande che ho.

Luigi, sei il mio "zaino" preferito, senza il quale proprio non riesco ad uscire, all'interno del quale io potrei svuotarci di tutto perché tu puoi contenere tutto di me. Tu sei il complice indiscusso della mia vita per questo sei speciale!..Non esiste distanza che fortunatamente ci ha fatti allontanare, e sai che di momenti duri ce ne sono stati..mi ascolti per ore e mi dai consigli, mi sostieni e riesci a seguire i miei ragionamenti più contorti, mi sproni e mi abbracci. Hai una riservatezza disarmante, sei ogni giorno una bella scoperta. Anche se non riesco ad esprimere con le parole ciò che sei per me, so di essere fortunata ad avere accanto una persona così pura come te!

Mavi (in arte Mario)…galeotta fu Via Cibrario 39, gioie e dolori e momenti di assoluta spensieratezza, casa in cui sono avvenuti mille cambiamenti della nostra vita..in pratica tu hai condiviso questo percorso dall'inizio alla fine con me, anche a distanza mia piccola Steve Jobs! Sei diventata parte della mia vita e della mia famiglia e io della tua; potrei stare ore ed ore a

descrivere quello che insieme abbiamo vissuto e costruito..Un rapporto che difficilmente finirà perché il bene che ci vogliamo è fraterno. Molto spesso mi manca la nostra quotidianità ma basta rispolverare i ricordi, ancora nitidi, e mi torna il sorriso. Grazie anche ad Alessandro, per il coraggio che mi ha fatto conquistare dopo anni di scherzi organizzati ad hoc!

Fede, piccola grande donna. Con te è stato davvero un incontro bellissimo, da subito..Grazie perché in due siamo riuscite a costruire un piccolo team che funzionava perfettamente. Grazie per il sostegno e la fiducia che ogni giorno mi hai trasmesso. Grazie per l'aiuto che non è mai mancato da parte tua. A te auguro il meglio che la vita possa riservare. Te lo meriti!

Grazie alle mie fantastiche amiche del "Me ne frego", Giorgia, che, come una premurosa sorella ti accompagna silenziosamente senza mai essere di troppo; Ele, che di silenzioso ha poco, ma che ha una contagiosa energia. Grazie per i fantastici siparietti che riuscivamo a montare in un attimo. E infine Iris, la roccia che non molla mai…Grazie per la nostra piccola mascotte, il dono più grande che potessi farci! Tutte voi siete state complici di momenti in laboratorio indimenticabili, di sostegno reciproco ma anche di serate insieme, di momenti "in famiglia". Vi voglio bene!

Ringrazio anche la mia dolce Nicole, ti porto sempre con me!

Un grazie speciale ai miei amici di sempre, Francesco, Incoli, Satri, Vito, Timothy, Domenico, Vale, Luigi, Nick e Claudia, tutti testimoni di amicizie indistruttibili. Le videochiamate (anche quelle mancate a causa mia) mi hanno sempre riportata attorno al tavolino del nostro bar dove il tempo sembra non passare mai. Grazie ad ognuno di voi!

Grazie agli immancabili "Buongiorno" della mia dolce Valentina..Ti voglio bene!

Grazie alla mia terra, così lontana, dove però, ogniqualvolta io ritorni, riesco a riassaporare il valore della semplicità delle cose.

Grazie anche a chi non c'è più!

Vorrei concludere, dedicando a tutti voi una frase che ho trovato un bel po' di tempo fa in un biscotto della fortuna, che dice: "Presto attraverserai grandi acque"….Auguro a tutti voi di attraversare grandi acque, magari di inciampare, di sbagliare per conto vostro e scoprire così la bellezza della vita. Presto arriverà il momento in cui ci riabbracceremo ma fino ad allora spero che le mie semplici parole arrivino dritte al cuore di ognuno di voi….GRAZIE!!