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OXIDATIVE STRESS BIOMARKERS OF THE ERYTHROCYTE IN THE NEWBORN – A FOLLOW-UP STUDY

BIOMARKERY OXIDAČNÍHO STRESU ERYTROCYTŮ U NOVOROZENCE – FOLLOW-UP STUDIE

Master thesis/Diplomová práce

Supervisor: doc. PharmDr. Martina Čečková, Ph.D.

Consultants: Susana Rocha, Ph.D., prof. Alice Santos-Silva, Ph.D.

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“I hereby declare that this diploma thesis is my original author’s work. I also certify that I have acknowledged, cited and properly listed all the used literal sources and materials. The thesis has not been used to obtain different or same degree.”

„Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Tato práce nebyla použita k získání jiného či stejného titulu.“

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Hradec Králové, 2018 Karolína Zubatá

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ABSTRACT

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Title of diploma thesis: Oxidative stress biomarkers of the erythrocyte in the newborn – a follow-up study

Increased levels of oxidative stress (OS) have been described in healthy, full-term newborns as a consequence of the drastic changes introduced by birth and by the exposure to extrauterine environment. Our intention was to examine the OS levels in red blood cells (RBCs) of neonates and to further understand the changes that the newborn organism undergoes with its newly-acquired autonomy as this knowledge is limited and there are no reference values. Umbilical cord blood samples were collected from a small population of newborns ($n = 8$) and several hematological and biochemistry parameters were evaluated. Our experimental data consist of OS biomarkers measurements performed in different fractions of blood (RBC membrane, total RBCs and plasma): membrane bound hemoglobin (MBH), lipid peroxidation (LPO), quantification of catalase (CAT) and glutathione peroxidase (GPx) activities, determination of total antioxidant status (TAS) and quantification of total and oxidized glutathione; the same parameters were assessed after two months in the same subjects ($n = 8$) using peripheral blood samples, to obtain the follow-ups. Hematological and biochemistry data were in accordance with general knowledge and available reference values. Significant changes were recorded in five of our OS biomarkers: decrease in membrane LPO and MBH levels suggests that OS really fades with age. We recorded a significant decrease in GPx activity and an increase in CAT activity which probably reflects different specialization of the enzymes and the newborn´s increasing autonomy. With all the gathered data we could observe the process of the newborn´s adaptation to the outer world. The most significant contribution of this work are the new OS biomarkers reference values, which, to our knowledge, have not been published before. However, our population was small and only half of the selected biomarkers displayed a significant change in the follow-ups two months after birth. Further studies are warranted to extend the knowledge in the field of OS and its biomarkers because we believe that there is a great potential for them to be used in clinical monitoring and diagnosis.

keywords: oxidative stress, biomarker, erythrocyte, newborn

ABSTRAKT

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Oxidační stres (OS) bývá primárně spojován s patologickými stavy, byl však popsán i u zdravých, v termínu narozených dětí, a to v důsledku drastických změn, kterými organismus novorozence během a po porodu prochází. Cílem naší práce bylo pomocí vhodných biomarkerů proměřit hladiny OS v erytrocytech (RBC) novorozenců a přispět k porozumění toho, jak se organismus adaptuje na vnější prostředí. Tato problematika není plně prozkoumána a chybí oficiální referenční hodnoty. Vzorky krve byly získány od malého počtu subjektů ($n = 8$) a to dvakrát – v den narození (pupečníková krev) a po dvou měsících, jako follow-up vzorky (periferní krev). V nemocnici byla provedena základní hematologická a biochemická vyšetření, naše experimentální data zahrnují proměření několika biomarkerů OS v různých frakcích krve (RBC membrány, plasma a celé RBC): membránově vázaný hemoglobin (MBH), peroxidace lipidů (LPO), kvantifikace aktivity catalasy (CAT) a glutathion peroxidasy (GPx), stanovení celkové antioxidační kapacity (TAS) a celkového a oxidovaného glutathionu. Výsledky hematologických a biochemických vyšetření odpovídají znalostem o tomto tématu i referenčním hodnotám. Statisticky významné změny byly pozorovány u pěti z našich biomarkerů: pokles u membránové LPO a MBH poukazuje na to, že OS s věkem skutečně klesá. Zaznamenali jsme pokles aktivity GPx a nárůst u CAT, pravděpodobně v důsledku lehce odlišných cílů těchto enzymů a zvyšující se autonomie dětského organismu. Veškerá sesbíraná a naměřená dat poskytla dobré podklady pro studium vývoje a postupné adaptace dětského organismu na okolní svět a působení OS. Největším přínosem této práce jsou naměřené hodnoty OS biomarkerů, které mohou být orientačně použity jako referenční, jelikož, pokud je autorům známo, jiné zatím nebyly publikovány. Počet vzorků byl však omezený, a pouze u poloviny vybraných biomarkerů byla zaznamenána statisticky významná změna, novorozenec *versus* follow-up. Další výzkum je vyžadován pro prohloubení znalostí o OS a jeho biomarkerech, protože, jak věříme, tyto informace a hodnoty by mohly být s velkou výhodou používány v klinické praxi (monitorování, diagnostika).

klíčová slova: oxidační stres, biomarker, erytrocyt, novorozenec

CONTENT

1 INTRODUCTION

This project is a part of a long-standing research on oxidative stress (OS) in erythrocytes carried out by Prof. Alice Santos-Silva and Dr. Susana Rocha from the UCIBIO/ REQUIMTE, Laboratory of Biochemistry, Department of Biological Sciences, Faculty of Pharmacy, Porto University. The researchers from the Laboratory of Biochemistry cover a range of areas (Hematology, Immunology, Clinical Biochemistry and Molecular and Cell Biology). The major goal of their work being: "...the understanding of the cellular and molecular mechanisms underlying different inflammatory conditions, associated with physiological and pathological situations that can trigger serious or fatal events" (Anonymous c1996-2018).

In the present study we investigated the levels of OS in healthy full-term newborns. The birth itself has been proven to be a stressful event for the neonate since after leaving the womb it faces a relatively hyperoxic environment which results in an increased production of reactive oxygen species (ROS). The matter of interest is, how does the organism of the neonate copes and adapts to it (Friel et al. 2004, Muller 1987, Ochoa et al. 2003).

Although OS provokes a lot of discussion nowadays, the attention is mainly paid to its contributions to various disease processes. We, on the other hand, wanted to focus on physiological stress of the delivery and on the healthy newborns ability to adapt to it.

As we were at the very beginning of the research, our aim was to discover more about the topic, to test several basic methods, to gather data and to contribute to the current knowledge about the newborns biochemistry and antioxidant (AOX) defense mechanisms. This thesis should lay the foundations for the future research.

1.1 Erythrocytes

Erythrocytes, also known as red blood cells (RBC), are the most abundant of blood cells, comprising up to 45% of blood volume, maintaining the vital function of oxygen (O_2)/carbon dioxide (CO_2) transporters by carrying the respiratory gases from lungs to tissues and vice-versa (Gwaltney-Brant 2014).

1.1.1 Erythropoiesis

The average life-span of an erythrocyte in humans is 120 days. To maintain the normal count of these rapidly maturing cells, a large number of RBCs needs to be synthesized every day (around 1% of the RBCs, meaning *cca.* one billion of new cells). Erythropoiesis, a complex, dynamic and well-regulated process, allows their replenishment (Hattangadi et al. 2011).

In the fetus, erythropoiesis begins in the liver during the early first trimester, which was preceded by extraembryonic erythropoiesis of the yolk sack. RBC production occurs in the liver through the rest of gestation, although the production starts to diminish during the second trimester as bone marrow erythropoiesis increases (the first erythroblasts occur in the bone marrow at 8 to 9 weeks of gestation and the first area of bone marrow appearance is near the clavicle). By the end of the third trimester, almost all erythropoiesis is localized in the bone marrow, although other sites may be involved (Richard A. Polin 2017).

After birth, erythropoiesis is restricted primarily to the bone marrow, although occasionally it may be found in the spleen or liver as well. In adults, extramedullary hematopoiesis (EMH) may occur during hematological disorders when bone marrow production is insufficient or ineffective. Typical sites include liver, spleen, lymph nodes, paravertebral areas with the intra-spinal canal, pre-sacral region, nasopharynx and paranasal sinuses as less common locations for EMH (Gwaltney-Brant 2014).

Bone marrow is protected by bone casing and composed of hematopoietic cells, adipose tissue and various supportive cells and tissues. Its structural and blood flow configurations, including bone – bone marrow portal capillary systems, provide the appropriate environment for the proliferation, differentiation and maturation of cellular components of the blood. Stromal stem cells are progenitors of adipocytes,

skeletal tissue components (the hematopoiesis-supporting stroma, osteoblasts, chondroblasts, etc.) and reticular cells that provide a structural support for the marrow and secrete mediators essential for the maintenance, differentiation, and growth of hematopoietic stem cells (Gwaltney-Brant 2014).

Hematopoietic cells are formed continuously from a small population of pluripotent stem cells that are capable of self-renewal. A regular hematopoietic stem cell develops into a common lymphoid progenitor, which lineage results in T, B, and natural killer cells, or follows a myeloid lineage and becomes a common myeloid progenitor (CMP), giving rise to granulocytes, macrophages, megakaryocytes and erythrocytes. Differentiation of cells is a series of lineage restriction steps that results in the progressive loss of differentiation potential to other cell lineages (Kondo 2010).

The pathway leading to mature erythrocytes (Figure 1) begins with hematopoietic stem cell which later develops in CMP, megakaryocyte-erythroid progenitor, burst-forming unit – erythroid (BFU-E) and colony-forming unit – erythroid (CFU-E). CFU- E undergoes many substantial changes, including a decrease in cell size, chromatin condensation and hemoglobinization, leading up to its enucleation and expulsion of other organelles, resulting in formation of the reticulocyte and finally, after additional cellular remodeling and loss of organelles, the mature erythrocyte (Bresnick et al. 2018, Dzierzak & Philipsen 2013, Kondo 2010).

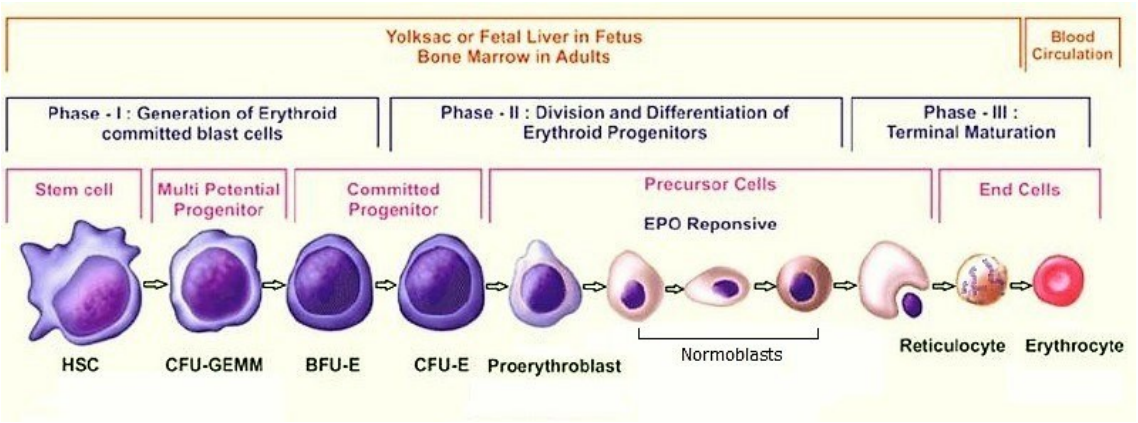

Figure 1: The pathway leading to mature erythrocytes. Hematopoietic stem cell (HSC) can either develop to lymphoid progenitor (resulting in lymphocytes and natural killers cells) or colony forming unit – granulocyte erythrocyte megakaryocyte macrophage (CFU-GEMM). The CFU-GEMM can take a pathway leading to platelets and white cells or to erythrocytes. The latter continues as follows: burst-forming unit – erythroid (BFU-E) and colony-forming unit – erythroid (CFU-E), proerythroblast, various stages of normoblasts and finaly reticulocytes which mature into RBC. Adapted from EpoMedicine, 2016 (Anonymous c2016).

The process of erythropoiesis is regulated by various growth factors. An exquisite short-term control of the process is accomplished by the kidney-derived glycoprotein hormone erythropoietin, which production is induced by hypoxic conditions and stimulates BFU-E cells. Other growth factors include granulocyte-macrophage colony-stimulating factor, stem cell factors or interleukins (IL): IL-3, IL-6, IL- 9 and IL- 12 (Hattangadi et al. 2011).

1.1.2 Structure

Red blood cells first appear in the circulation as reticulocytes, that are enucleated cells containing residual RNA and some mitochondria capable of oxidative respiration. In a healthy adult they make up 1 - 2% of the circulating RBCs, while in neonates the number is higher, up to 2.5% - 6.5% but drops to normal values within two weeks after birth. Once 24h in circulation, as the reticulocytes pass through the splenic vessels and lose their organelles and RNA, they mature into normal RBCs (Bukhari & Zafar 2013, Gordon-Smith 2013).

Healthy mature erythrocytes are biconcave cells of approximately 7.5 to 8.7 μm in diameter and 1.7 to 2.2 μm in thickness. These structural proprieties allow the RBC to accomplish its vital function: 1) the flexible membrane, composed of proteins and lipids, enables the erythrocyte to travel through the vascular system and deform reversibly to squeeze into the smallest capillaries during microcirculation (2–3 μm in diameter) and, 2) the discocyte shape with a central depression on each side provides a high surface-to-volume ratio that facilitates a rapid diffusion of the respiratory gases to and from the cell (Diez-Silva et al. 2010).

As already mentioned, mature erythrocytes lack nucleus and most of the organelles so that the maximum space in cytosol can be occupied by hemoglobin, an iron-containing biomolecule essential for gas transport.

Hemoglobin

Hemoglobin (Hb) is the main protein component of the RBCs, making up to 95 % of their dry content. Hb is a tetramer of two α and two non-α globin chains, each chain enclosing one of the four heme groups. HbA, the most common type of Hb in healthy adults, is composed of two α chains and two β chains, thus the tetramer can be represented as $\alpha_2\beta_2$,

whereas fetal hemoglobin, HbF, is known for its structure of $\alpha_2\gamma_2$, containing a γ chain that binds to the O_2 molecule with greater affinity. HbA_2, $\alpha_2\delta_2$, is found in small amounts in all adults (1 - 2 % of all Hb molecules) but other variants of Hb exist and can be seen mainly in various pathologies (Marengo-Rowe 2006).

Hb is synthesized during the RBC maturation process: mitochondria produce heme, which then combines with the globin chains synthetized on the polyribosomes. Heme iron constantly undergoes a recycling loop after the destruction of the erythrocyte so that it could be used again for new RBCs (Chung et al. 2012).

Heme, an iron-containing tetrapyrrole ring, known as proto-porphyrin, is an essential prosthetic group of many biomolecules, including Hb. Four nitrogen atoms within the ring coordinate with the central ion and there are methene bridges among the four pyrrole rings. Iron in heme is bound to a histidine (His) residue of the globin chain and to O_2 molecule that binds at other coordinated position of the ion; its ferrous state (Fe^{2+}) allows a reversible binding to O_2, while ferric state (Fe^{3+}) in methemoglobin (metHb) is unable to carry O_2 (Capece et al. 2006, Tsiftsoglou et al. 2006).

RBC membrane

The flexibility of the RBCs is primarily attributed to the cell membrane, as there are no organelles and filaments inside the cell. The RBC membrane is approximately 19.5% water (H_2O), 39.5% protein, 35.1% lipids and 5.8% carbohydrates. It consists of a cytoskeleton and an asymmetrical lipid bilayer tethered together via “immobile” macromolecular complexes centered on band-3 proteins at the spectrin-ankyrin binding sites and via glycophorin at the actin junctional complexes. The protein composition determines the shape and flexibility of the RBC. Transmembrane proteins usually have both cytoplasmic and plasma domains. The outer side includes binding sites for immune complexes and the external parts of transmembrane channels and signaling proteins. The lipid bilayer includes various types of phospholipids, sphingolipids, cholesterol, and integral membrane proteins, such as band-3 or glycophorin. (Oliveira & Saldanha 2009).

A significant feature of the lipid bilayer organization is that various phospholipids are distributed asymmetrically following their different tasks and functions. Thus, when a reorganization occurs, it has an important signaling effect. For example, phosphatidylserine (PS), which is normally localized in the inner layer of the bilayer, can

set off RBC destruction by macrophages; once the organization of the bilayer is disrupted, PS gets exposed at the cell surface and marks the RBC for phagocytosis (An & Mohandas 2008).

The cytoskeleton is composed principally of spectrin, actin and its associated proteins (tropomyosin, tropomodulin, adducin and dematin), protein 4.1R and ankyrin (Li & Lykotrafitis 2014, Lux 2016, Pandey & Rizvi 2011).

1.1.3 Function

The primary purpose of the RBCs is to carry Hb and thus allow gas exchange of O_2/CO_2 among lungs and tissues. Besides that, erythrocytes help to maintain the systemic acid/base and osmotic equilibria, participate in control of nitric oxide metabolism, redox regulation, blood rheology and viscosity (Kuhn et al. 2017). Lastly, RBCs are equipped with an effective AOX system that, together with their natural ability to circulate through the whole body, makes them invaluable free radical (FR) scavengers: they provide AOX protection not only to themselves but also to other tissues and organs in the body (Pandey & Rizvi 2011).

Hemoglobin plays a crucial role as it carries O_2 from lungs to tissues and takes carbon dioxide back to lungs. There, after O_2 binds to the first heme group in Hb, its configuration changes and the O_2 affinity of the remaining hemes increases, allowing the tetramer to easily bind and carry four O_2 molecules, thus enabling each RBC to carry over 1 billion O_2 molecules. Once the RBC reaches the tissue and the O_2 is unloaded, globin chains rearrange and diminish the O_2 affinity with the aid of 2,3- diphosphoglycerate (2,3-DPG) (Kanias & Acker 2010).

Concerning carbon dioxide transport, CO_2 diffuses from the intracellular space into blood's two components, plasma and erythrocytes. Within the organism, it is transported in three different forms: dissolved in solution; buffered with H_2O as carbonic acid or bound to proteins, particularly Hb. After CO_2 diffuses into the RBC, it combines with H_2O to form carbonic acid, during a reaction accelerated by carbonic anhydrase. The newly-emerged hydrogen ion of carbonic acid is accepted by the deoxyhemoglobin (deoxyHb) and the remaining bicarbonate ion diffuses into the plasma via the chloride shift, mediated by protein band 3. Carbonic acid can be converted back to CO_2 allowing the gas to be exhaled. Minor ways of CO_2 disposal include its direct binding

to the deoxyHb, forming carbamino-hemoglobin (20% of CO_2 removal) and lastly, carbon dioxide can be carried to the lungs dissolved in plasma (5% of total CO_2) (Arthurs & Sudhakar 2005, Geers & Gros 2000).

1.1.4 Metabolism

Mature RBCs have a limited metabolic capacity since they lack nucleus and other organelles, particularly mitochondria. The major source of energy is glucose that enters the erythrocyte by facilitated diffusion, independently of insulin. It is then metabolized by the anaerobic glycolytic Embden-Meyerhof pathway to lactate. For each glucose molecule that enters the pathway, two molecules of adenosine triphosphate (ATP) are generated. Besides that, reduced nicotinamide adenine dinucleotide phosphate (NADPH) is generated, which is essential for metHb reductase to keep the Hb in a reduced state. The obtained ATP molecules provide energy for maintenance of red cell volume, shape, the membrane flexibility and for regulation of sodium-potassium pump. The Luebering-Rapoport bypass, a side arm of the main pathway, provides 2,3-DPG, a molecule necessary for regulating the Hb O_2 affinity. Only about 90% of glucose entering the RBC is used to generate ATP through glycolysis and the remaining 10% is processed by the hexose monophosphate shunt. This alternative pathway generates NADPH molecules that are later used for glutathione (GSH) reduction, an important player in AOX protection of the cell (Brown 1996, Hoffbrand & Moss 2016, Pandey & Rizvi 2011).

1.1.5 Antioxidant capacity

RBCs are extremely susceptible to oxidative damage for two main reasons: 1) due to their primary function as gas carriers they are constantly exposed to high levels of O_2 and, 2) since mature erythrocytes are enucleated, they are unable to synthesize new proteins and enzymes for themselves. Erythrocytes are not defenseless though. They are equipped with powerful AOX defense system that provides the needed protection both for them and other cells and tissues as well. This is the reason why RBCs by fulfilling their purpose of gas transporters and by traveling through the organism serve as perfect scavengers of free radicals (Cimen 2008).

There are enzymatic and non-enzymatic mechanisms in the RBC´s AOX defense system.

Enzymatic

AOX enzymes detoxify reactive ROS by catalytically reducing or oxidizing the oxygen intermediates to less harmful products. Generally, cytosolic superoxide dismutase (SOD) provides the first line protection as it converts superoxide radical into less reactive hydrogen peroxide (H_2O_2). The H_2O_2 can be then decomposed to O_2 and H_2O by either catalase (CAT), glutathione peroxidase (GPx) or peroxiredoxin 2 (Prx2) (Culotta 2000).

Superoxide dismutase

SODs compose a family of oxidoreductase enzymes, the role of which is to protect the organism from ROS. There are three known isoforms of SOD in mammals depending on the protein fold and the metal cofactor: SOD1 (CuZnSOD), SOD2 (MnSOD) and SOD3 (extracellular SOD) (Fukai & Ushio-Fukai 2011).

In erythrocytes, cytosolic CuZnSOD plays an important role on the first line of defense against ROS by catalyzing the dismutation of superoxide radical into harmless O_2 or H_2O_2, which is further decomposed to O_2 and H_2O (Fukai & Ushio-Fukai 2011).

Eukaryotic SOD1 is described as a stable homodimer with the active sites oriented on the opposite sides of its subunits. The subunits are held together mainly by hydrophobic and electrostatic interactions. Each SOD1 monomer contains two metal ions, copper and zinc, bound together by His side chain and by a secondary linkage of hydrogen bond. Both of the metal ions are believed to play important catalytic and structural roles: while copper is essential for the catalysis of the superoxide dismutation, zinc mostly plays a structural role and acts as a positive charge sink (Rakhit & Chakrabartty 2006, Tainer et al. 1983).

Superoxide anion is one of the main ROS, hence SOD1 key role in the cell´s AOX defense. The enzyme, formerly known as erythrocuprein, catalyzes a reaction where two superoxide molecules are turned into O_2 and H_2O by the “ping-pong” mechanism: first superoxide is oxidized and the second is reduced in the disproportionation reaction (Rakhit & Chakrabartty 2006).

Catalase

Human CAT belongs to a large group of monofunctional heme-containing CATs which are found in almost all aerobic organisms. It is mostly an intracellular enzyme with highest concentrations in RBCs and liver. Under normal conditions, RBCs contain

1.31– 2.71 μg CAT /mg Hb. CAT is a tetrameric protein composed of four subunits, each containing a Fe^{3+} heme group and a bound NADPH molecule that protects the enzyme against oxidation and inactivation by its substrates. Supposedly, it does not take part in the enzymatic activity of H_2O_2 dissociation (Aksoy et al. 2004, Kodydkova et al. 2014). When CAT is exposed to H_2O_2 for a long time, the bound NADPH gets oxidized and the activity of CAT fells to about one third of its initial activity (Kirkman et al. 1987).

The enzyme has two different activity modes: 1) catalytic activity, the major one, of decomposition of H_2O_2, generated by dismutation of superoxide (catalyzed by SOD), into H_2O and O_2 (α-phase) and, 2) peroxidatic activity (β-phase), that involves H_2O_2 elimination by oxidizing alcohols, formate or nitrate. The catalytic reaction predominates in higher H_2O_2 concentration ($>10^{-4}$ M), while below this concentration the peroxidatic reaction dominates if there is an acceptable hydrogen donor (Mate et al. 1999).

The main function of CAT is H_2O_2 removal by catalytically decomposing it to H_2O and O_2; this enzyme is believed to take action mainly in removing higher intracellular H_2O_2 concentrations, while other AOX enzymes work under different conditions: GPx and Prx act at low H_2O_2 concentrations (Kodydkova et al. 2014). It has also been described that CAT is the major RBC defense enzyme against exogenous H_2O_2 and, unlike other AOX enzymes, is highly specific for H_2O_2 and, thus, does not participate in detoxifying organic peroxides (Johnson et al. 2010).

Glutathione peroxidase

Out of eight GPx recognized in mammals, only GPx1 is present in the RBCs as an intracellular cytosolic AOX enzyme. GPx catalyzes the reduction of both H_2O_2 and organic hydroperoxides and peroxynitrites using GSH as a reducing agent (Brigelius-Flohe & Maiorino 2013).

GPx1 is a tetramer of four subunits of 21kDa each containing a selenocysteine (Sec). It was described that GPx1 binds 10–15% of circulating Se (Zachara 2015). The catalytic center is a tetrad formed by cysteine (Cys), glutamine, tryptophan and aspargine and the reactive Sec is surrounded by four arginines and a lysine of an adjacent subunit. Studies suggest that these residues are essential for binding the GSH. The proposed mechanism of the reaction is as follows (Figure 2): peroxide reduction involves formation of selenic acid (Se-OH) at the selenol (Se-H) active site. First molecule of GSH is used for the reduction of the Se-OH, forming glutathiolated selenol (Se-SG),

the selenadisulfide bond of which is afterwards reduced by the second GSH molecule. Active site of the enzyme is then restored and the newly emerged oxidized GSH (GSSG) is regenerated to GSH by the action of glutathione reductase (GR) (Brigelius-Flohe & Maiorino 2013, Lubos et al. 2011).

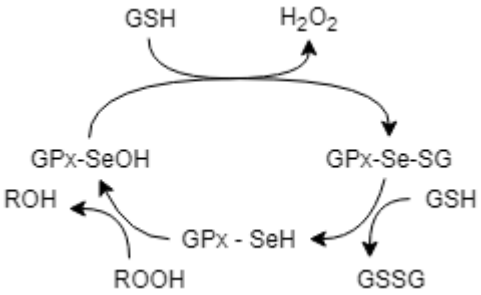

Figure 2. Reaction mechanism of GPx1. The active site of the enzyme (GPx-SeH) is oxidized (GPx- SeOH) after its reaction with hydroperoxides (ROOH). Regeneration of the enzyme involves two molecules of GSH. The first molecule reduces the selenic acid in GPx-SeOH forming a glutathiolated selenol (GPx- Se -SG). The disulfide bond is then reduced by the second GSH molecule yielding regenerated enzyme (GPx-SeH) and oxidized glutathione (GSSG). Adapted from Lubos *et al*., 2011 (Lubos et al. 2011).

For some time, GPx and CAT used to be considered as the major players of the RBC´s AOX defense system. Nowadays, as more knowledge has been gained, attention is paid to Prx2 as well, and the function of each of the enzymes has been studied in detail, although the interplay among these three enzymes still requires more research. While CAT is believed to work mainly at higher concentrations, GPx1 seems to be effective at low H_2O_2 concentrations, mostly of endogenous origin (Hb auto-oxidation). This fact might be attributed to CAT having higher Km for H_2O_2 but according to *Flohé* (Flohé 1982), since neither of the enzymes saturates with H_2O_2, the rate limiting step for GPx1 is the recycling of GSH at high H_2O_2 concentrations (Low et al. 2008, Rocha et al. 2015).

Glutathione reductase

GR is an important component of the erythrocyte´s AOX defense system as it protects RBC´s Hb, enzymes and cell membranes against oxidative damage. Unlike previously described enzymes, its effect is not pointed directly at ROS, but it is an essential catalyst of the reaction that recycles GSH (Chang et al. 1978).

GR belongs to the family of flavoenzymes which contain either flavin adenine dinucleotide (FAD) or flavin mononucleotide. GR has a homodimeric structure with two

active sites and two FAD molecules incorporated in the subunits (Kamerbeek et al. 2007). The main function of the enzyme is to keep GSH in its reduced state.

The two subunits are bond by a disulfide bridge. There are two flexible NH_2 residues arms with unknown role and the remaining residues are organized into four domains in each subunit. The first two of them bind FAD and NADPH molecules, respectively. The catalytic center lies between the subunits and is shared by five domains (four of one subunit a one of the other) which is quite unusual (Pai & Schulz 1983).

The reaction is described to happen in two steps (equation 1). First, the enzyme (E) is reduced to its stable EH_2 (reduced enzyme) form, and NADPH is oxidized to NADP. Then, EH_2 forms a complex with GSSG later yielding in regenerated GSH molecules and the enzyme (Pai & Schulz 1983, Staal & Veeger 1969).

$$H^+ + NADPH + E \Leftrightarrow NADP^+ + EH_2$$

$$H_2 + GSSG \Leftrightarrow 2\,GSH \qquad \text{(equation 1)}$$

Because of its close cooperation with NADP/NADPH, GR also regulates the proper balance of NADP and NADPH in the hexose monophosphate shunt of glycolysis (Pai & Schulz 1983).

Peroxiredoxin 2

Prx family, a group of H_2O_2, organic hydroperoxide and peroxynitrite reducing enzymes, is vastly distributed among various living organisms, from anaerobes to humans. Six isoforms have been identified in mammals, and human RBCs possess Prx1, Prx2 and Prx6. The levels of Prx2 (5.5mg/ml of packed RBC) by far exceed the other isoforms; it was even described that Prx2 is the third most abundant RBC´s cytosolic protein (following Hb and carbonic anhydrase) (Low et al. 2008).

Prx2, formerly known as torin, calpromotin, NKEF-B, HRPRP or band 8, is an intracellular cytosolic enzyme the importance of which was only described and grounded several years ago, much later than that of previously presented enzymes (CAT, SOD, GPx…) (Harris et al. 2001, Manta et al. 2009). To point out the crucial AOX role of Prx2 in RBC: the work by Lee *et al.* showed that the RBCs from Prx2 *knockout* mice proved to display a significantly shorter life span compared to wild type mice RBCs,

as well as, showing increased levels of oxidized proteins and Heinz bodies (Lee et al. 2003).

All Prxs contain in their active site (Cys), the peroxidatic Cys (CysP), highly specific for peroxides. Subfamilies of the enzymes differ in the architecture of the active site with Prx2 belonging to the Prx1 (typical 2-Cys Prx) subfamily (Peskin et al. 2016). In its functional state, Prx2 appears as a homodimer with its subunits non-covalently associated in a head to tail manner (Low et al. 2008).

The peroxidase cycle of Prx2 starts with the reaction of CysP and the H_2O_2 generating a sulfonic acid (–SOH) which can either react with the resolving Cys on the opposing chain of the dimer and form a disulfide bond (major pathway) or, it can be further oxidized by H_2O_2 to the sulfinic acid. Hyperoxidation is not common in RBCs though, as sulfiredoxin manages to counteract the reaction (Cho et al. 2010). The cycle is completed by Prx2 regeneration by thioredoxin (Trx) and Trx reductase, using NADPH as a donor of reducing equivalents. This has been accepted as a major mechanism but redox reactions with other thiols have been described, as well as, in vitro recycling of Prx2 by dithiothreitol, as alternative pathways in the Prx2 cycle (Low et al. 2008, Peskin et al. 2016).

Quaternary structure of Prx2 has been studied and it has been shown that the enzyme exists in various forms, depending on the ionic strength, protein concentration, pH, phosphorylation and, most importantly, the redox state: its minimal functional unit, monomer, oxidizes into a disulfide-linked dimer upon reaction with H_2O_2 (Ogasawara et al. 2012). During catalysis, the homodimer is shifted into a doughnut-shaped decamer, a pentamer of dimers, which immediately falls apart upon disulfide formation. The role of the transition is still unclear (Hall et al. 2009). Prx2 is even believed to form 12-decamer dodecahedron cage under certain conditions (Low et al. 2008).

Prx2 has shown a remarkable sensitivity to extremely low concentrations of H_2O_2, proving to be oxidized even when CAT and GPx1 were active. It has been suggested that Prx2 detoxifies the RBC of the low endogenous levels of H_2O_2, similarly to GPx1. These two enzymes also share the ability to reduce hydroperoxides and peroxynitrites (Low et al. 2008, Rocha et al. 2015). Another important role of Prx2 has been described

by Han *et al.*: Prxs containing 2-Cys residues act as molecular chaperons under increased OS by binding to Hb and thus stabilizing it against oxidative damage (Han et al. 2012).

Association with the membrane of RBCs has been described both under normal conditions (cca 0.05% of Prx2), in hematological disorders and under OS (Rocha et al. 2015).

Non-enzymatic

Apart from enzymatic mechanisms, RBCs AOX defense system comprises of numerous endogenous AOX molecules. The major ones are GSH, ascorbic acid and α- tocopherol (Kuhn et al. 2017, Pandey & Rizvi 2011). Besides these, carotenoids, ubiquinone, melatonin, uric acid ceruloplasmin, transferrin or haptoglobulin are described to play an important role as well (Cimen 2008).

Glutathione (GSH/GSSG)

Structurally, glutathione (L-γ-glutamyl-L-cysteinyl-glycine) is a thiol-containing tripeptide of L-glutamate, L-cysteine and L-glycine, irreplaceable in RBC´s defense against ROS (Monostori et al. 2009).

De novo synthesis of GSH is localized in the cytosol of all mammalian cells including RBCs, regardless of their limited biosynthetic capacity (Lu 2009). The biosynthesis of GSH is described to happen in two steps: 1) γ-glutamylcysteine is formed from L-glutamate and cysteine, in a reaction catalyzed by glutamate cysteine ligase and, 2) the rate limiting step, catalyzed by GSH synthetase, adding glycine to the C-terminal of γ-glutamylcysteine (Shan et al. 2015).

The crucial role of GSH as a central redox agent lies in its nucleophilic character. The reduced form serves as a proton donor in several oxidation-reduction processes while oxidizing itself into the oxidized form GSSG, which is two GSH molecules bound together by a S-S disulfide bond (Lu 2009, Zitka et al. 2012). To maintain the redox state of the cell and close the GSH cycle, GSSG is regenerated back to GSH by GR, using NADPH as a proton donor (Shan et al. 2015).

The process of GSH regeneration consists of four steps: 1) glucose (Glc) is transported through RBC membrane, 2) Glc molecule is phosphorylated to glucose-6-phosphate (G-6-P) by hexokinase, 3) nicotinamide adenine dinucleotide phosphate (NADP) is reduced to NADPH by G-6-P dehydrogenase and 6-phosphogluconate

dehydrogenase and, 4) GSSG is reduced by GR utilizing NADPH, resulting in two GSH molecules (Kurata et al. 2000).

Besides its well-known AOX role as a cofactor of GPx (Lu 2009), GSH also serves as a direct scavenger of hydroxyl radicals and peroxinitrites (Griffith 1999, Wu et al. 2004), takes part in lipid peroxides detoxification (Raftos et al. 2010) and helps to keep the thiol groups of important RBC´s structures in reduced state (Hb, enzymes, membranes…) (Lu 2009). GSH is also able to detoxify the RBC from xenobiotics by forming a water-soluble conjugates that can be exported from the RBC and excreted, and it also serves as a Cys storage, since extracellularly this amino-acid undergoes a rapid auto-oxidation into cystine (Lu 2009). Not to forget its cooperation with other AOXs molecules, enabling their regeneration (e.g. ascorbate, α-tocopherol) (Monostori et al. 2009).

At all moments, GSH is present in two forms: as a reduced molecule (GSH) and an oxidized form (GSSG), the amount of which is kept at low concentration (1:100 to 1:1000) by the action of NADPH-dependent GR. The GSSG/GSH ratio is a valuable indicator of cell functionality and the level of OS (Monostori et al. 2009). To maintain constant concentrations of GSH within the erythrocyte at ~2.3 mM, the rate of synthesis must equal the rate of its export in the form of GSH conjugates and/or GSSG (Raftos et al. 2010). It was suggested that GSSG could leave the RBC in two ways – directly through the cell membrane, which can be damaged under OS conditions, or by an active MgATP-dependent transport, both resulting in decreased GSH levels in OS associated conditions (Pandey & Rizvi 2011, Raftos et al. 2010), as has been described in Parkinson's disease, liver disease, cystic fibrosis, sickle cell anemia, AIDS, cancer, heart attack, stroke or diabetes (Pandey & Rizvi 2011).

Ascorbate/dehydroascorbate

Ascorbic acid, also known as vitamin C, is an essential hydrophilic vitamin, which, after its intake from food, is mostly localized in plasma where its serves as one of the most important AOX (it directly protects against peroxidation of plasma low-density lipoprotein (LDL)). Ascorbate´s AOX activity is based on its ability to reversibly donate one or two hydrogens and electrons to a variety of ROS, thanks to its carbon-carbon double bond and high reducing potential. Newly emerged dehydroascorbate is taken up by RBC´s glucose transporter (GLUT1) and recycled. The mechanism of the reduction is

GSH- and NADPH-dependent, catalyzed by various enzymes (dehydroascorbate reductase, glutaredoxin, thioredoxin etc.). Once recycled, ascorbate is trapped inside the RBC and only very slowly diffuses back, thus maintaining its plasma concentrations. It was suggested that ascorbate is more sensitive to oxidation by exogenous H_2O_2 than GSH and α-tocopherol. Although ascorbate does not directly protect the RBC membranes from peroxidation, it performs its function via the reduction of tocopheroxyl FR at the aqueous-lipid interface of the membrane bilayer (May 1998).

α-tocopherol

In RBCs, lipid-soluble α-tocopherol, the most widely distributed AOX in nature, acts as a potent scavenger of peroxyl radicals to protect polyunsaturated fatty acids (PUFA) of membranes from lipid peroxidation (LPO) (Pandey & Rizvi 2011). α-tocopherol intervenes in the propagation step during auto-oxidation of lipids. It donates its phenolic hydrogen atom to a peroxyl radical and, thus, converts it to less dangerous hydroperoxide. The newly formed tocopheroxyl radical is adequately stable to stop the reaction and can leave the cycle by reacting with another peroxyl radical forming an inactive non-radical product (Yamauc 1997). Ascorbate reduces the tocopheroxyl radical to regenerate vitamin E (Niki 1987).

1.2 Oxidative stress

There are various definitions of the term „oxidative stress“; *Sies* refers to it as „an imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage” (Sies 1997).

1.2.1 Oxidants

Oxidants, continuously attacking all cells living under aerobic conditions, may be of either endogenous or exogenous origin, all together known as ROS. There are two types of ROS, free radicals (molecules with unpaired electrons) and nonradicals (free radicals that share their unpaired electrons). The ROS and their important equations are depicted in Table 1 with the three major ROS being superoxide anion (O_2^-), hydroxyl radical ($^{\cdot}OH$) and H_2O_2.

Table 1. Reactions of selected reactive oxygen species.

Oxidant	Formula	Reaction equation
Superoxide anion	O_2^-	$NADPH + 2O_2 \leftrightarrow NADP^+ + 2\mathbf{O_2^-} + H^+$
Hydrogen peroxide	H_2O_2	$2O_2^- + H^+ \rightarrow O_2 + \mathbf{H_2O_2}$
		$HPX + H_2O + O_2 \leftrightarrow XAN + \mathbf{H_2O_2}$
		$XAN + H_2O + O_2 \leftrightarrow UA + \mathbf{H_2O_2}$
Hydroxyl radical	$^{\cdot}OH$	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \mathbf{^{\cdot}OH}$
Peroxyl radical	$ROO^{\cdot}$	$R + O_2 \rightarrow \mathbf{ROO^{\cdot}}$

NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; $NADP^+$, nicotinamide adenine dinucleotide phosphate, oxidized form; HPX, hypoxanthine; XAN, xanthine; UA, uric acid.
Adapted from *Birben et al.* (Birben et al. 2012).

Endogenous sources of ROS are the normal products of aerobic metabolism but under pathological conditions their levels may rise. Generally, they are produced in mitochondrial respiratory chain, as byproducts of oxidative phosphorylation, but in other organelles as well: in endoplasmic reticulum, in peroxisomes (xanthine oxidase) and during auto-oxidation process of small molecules (including Hb auto-oxidation), as well as, in immune reactions such as the respiratory burst of phagocytes.

Exogenous sources include cigarette smoke, ionizing radiation, heavy metals ions, ozone exposure, pollutants, drugs and toxins including oxidizing disinfectants or hyperoxia. (Birben et al. 2012, Pandey & Rizvi 2010, Sarniak et al. 2016)

1.2.2 Antioxidants

To compensate for the actions of oxidants, organisms have integrated protective systems of enzymatic and non-enzymatic AOXs to block the harmful effects of ROS. AOXs can be synthesized *in vivo* (e.g. GSH and SOD) or taken in from the diet.

According to *Gutteridge and Halliwell*, AOX is "any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" (Halliwell 2007).

Generally, there are two groups of AOX, enzymatic and non-enzymatic ones. The three major classes of AOX enzymes are SODs, CATs and GPxs (Sies 1997).

Ascorbic acid (vitamin C), α-tocopherol (vitamin E), GSH and carotenoids represent non-enzymatic AOXs (Cimen 2008).

1.2.3 Mechanisms and consequences of oxidative damage

Due to the unpaired electrons, ROS are highly reactive molecules and tend to attack and alter the function of cell's macromolecules - nucleic acids, lipids, and proteins (Birben et al. 2012).

PUFA residues of phospholipids are extremely susceptible to oxidation. LPO leads to formation of hydroperoxides that, if not reduced by AOX enzymes, break down into reactive aldehyde products (e.g. 4-hydroxy-2-nonenal, 4-hydroxy-2-hexenal, malondialdehyde (MDA), or acrolein) which have been proven to be mutagenic. Besides that, LPO in general leads to loss of membrane fluidity and elasticity, impaired cellular functioning and even cell lysis (Catala 2009, Valko et al. 2007).

Proteins can be damaged either by direct oxidation of their amino-acid residues and cofactors or by secondary attack via LPO end-products. The side chains of all amino-acid residues of proteins are prone to oxidation by the action of ROS, especially cysteine. Its oxidation may lead to the reversible formation of disulphide bridges between protein thiol groups (–SH) and low molecular weight thiols, in particular GSH (S- glutathiolation). Such conformation changes may lead to decrease in enzyme activity if the targeted amino-acid residues are close to active sites (Costa et al. 2007, Valko et al. 2007).

Oxidative damage has been proven to play an important role in the etiopathogenesis of many common pathological conditions, including neurological disorders, atherosclerosis, hypertension, ischemia/perfusion, diabetes, acute respiratory distress syndrome, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease or asthma (Birben et al. 2012).

Although ROS are mostly regarded as toxic by-products of oxidative metabolism and their participation in pathologic processes is indisputable, low levels of these reactive molecules are supposed to be essential for normal functioning of the organism. Namely, they play a significant role in modulation of gene expression, in cell signaling, as second messengers, or in various immune reactions (phagocytosis, inflammation) (Cimen 2008, Remacle et al. 1994).

1.2.4 Oxidative stress & erythrocytes

Erythrocytes are constantly exposed to risk of oxidative injury since incessant contact with the O_2 molecule and high content of Hb is inevitable for O_2 carriers. Even though oxyhemoglobin ($HbFe(II)O_2^-$) is rather stable molecule, its auto-oxidation (equation 2) occurs up to a rate of 3% per day. The process is characterized by a dissociation of the O_2 molecule without an electron transfer to yield O_2^- and metHb (HbFe(III)) (Kanias & Acker 2010).

$$HbFe(II)O_2 \xrightarrow{autooxidation} HbFe(III) + O_2^- \qquad \text{(equation 2)}$$

Both of these products under normal conditions are effectively detoxified: NADH-cytochrome b5–metHb reductase reduces Hb to deoxyHb and ROS are eliminated by AOX enzymes (Kanias & Acker 2010).

When the capacity of the AOX enzymes (cytosolic SOD and CAT, mostly) is exceeded, metHb can be oxidized to so called hemichromes, which are precipitates of denaturated Hb. These structures have been known to covalently bind to cell membrane, resulting in clustering of integral membrane protein band 3 and interfering with the integrity of the cytoskeleton. Therefore, membrane-bound hemoglobin (MBH) has been suggested as a reliable marker of oxygen radical-induced injury to RBCs (Sharma & Premachandra 1991, Welbourn et al. 2017).

Another widely used biomarker of RBCs OS, namely of LPO, is MDA content. This secondary oxidation product reacts with the free amino groups of proteins and phospholipids which leads to structural modification, inducing dysfunction of various cell systems. The bright side of the MDA´s reactivity is that its reaction with thiobarbituric acid (TBA), which yields a colored chromogen, offers an easy way to assess the OS of the cell. Measurement of thiobarbituric reactive substances (TBARS) has been considered as a reliable and easily reproducible biomarker of OS for many years, although a discussion about the low specificity of the test continues (Ayala et al. 2014, Pandey & Rizvi 2010, Rizvi & Maurya 2007).

To evaluate the state and potential of OS in cells, total antioxidant status (TAS)/capacity can be assessed. From a number of methods, the one developed by Benzie and Strain stands out due to its simplicity and easy and reliable performance.

The ferric reducing antioxidant power assay is measured as a reduction of ferric ion ligand complex to intensely colored ferrous complex by all available AOXs in a sample (Pandey & Rizvi 2010).

1.3 Specifics of newborns & neonatal oxidative stress

A newborn represents the culmination of various developmental steps from conception, through organogenesis to birth. The composition of fetal blood changes significantly during the second and third trimester, preparing the fetus for birth and extrauterine environment. Mean Hb increases (from 9.0 ± 2.8 g/dl at the age of 10 weeks to 16.5 ± 4.0 g/dl at 39 weeks), the percentage of nucleated cells decreases (from 12% at 18 weeks to 4% at 30 weeks) and large numbers of committed hematopoietic progenitors are found circulating in the fetal blood (Lichtman et al. 2016).

Birth introduces dramatic changes in the circulation and oxygenation, as the newborn undergoes the transition to a separate biological existence. These events are reflected in the composition of blood of the newborn, during the first hours and days of life there are rapid fluctuations in the quantities of all hematologic elements. Studies suggest that most of the measured parameters were highest on the first day of life and declined thereafter (Esan 2016, Ogundeyi M.M. et al. 2011).

Levels of cord blood Hb vary between approximately 165 and 170 g/L, depending on the timing of cord clamping. Later, at around 8 weeks, there is a progressive fall in Hb levels to 100-110g/L (or even lower in preterm infants which make them more susceptible to iron and folate deficiency), followed by a recovery to 125g/L at 6 months. Nucleated cells can be seen for first days in the blood film (longer in preterm infants). The reticulocyte count, which is initially up to 2-6%, falls to 0.5% during a week due to increased oxygenation of tissues which leads to suppression of erythropoiesis. The RBC count increases during the first day of life, then it remains at that level for about 2 weeks and slowly declines afterwards. The leukocyte count often fluctuates at all ages but most significantly in infants. Leukocytosis is typical at birth, the range of normal values is wide and the count slowly decreases until approximately 4 years of age (Esan 2016).

Considering OS, birth itself is a stressful process for both mother and fetus, and delivery is a cause of hyperoxic challenge for the newborn (Friel et al. 2004, Norishadkam et al. 2017). The neonate is exposed to an environment that is relatively hyperoxic (pO_2 of 100 mm Hg) compared to the intrauterine one (pO_2 of 20 – 25 mm Hg). The increased O_2 bioavailability leads to a greatly enhanced generation of FR and OS (Perrone et al. 2010, Tsukahara 2007).

Newborns, especially preterm, are particularly susceptible to the oxidative damage for number of reasons: structural and functional immaturity of organs, overloading of aerobic tissues metabolism with rapidly growing energy demand, the reduced ability to induce efficient homeostatic mechanisms. The same goes for neonatal RBCs: increased release of transitional metals like free iron, increased production of superoxide radicals, increased content of fatty acids, and last but not least, lack of AOX systems (low enzyme activities such as GPx and SOD; low content of vitamin E and ascorbic acid) (Claster et al. 1984, Perrone et al. 2010). It was described that at the end of pregnancy, AOX enzymes are induced and the transfer of other AOX molecules (vitamins E, C, β-carotenes, ubiquinone) across the placenta is increased during last days of gestation (Friel et al. 2004, Torres-Cuevas et al. 2017); however, the AOX systems only come to full maturity during the first year of life of the infant (Norishadkam et al. 2017).

1.3.1 Oxidative stress related diseases in newborns

Serious imbalance between the action of ROS and insufficient defensive mechanisms, which is even more likely to appear in preterm newborns, not only leads to oxidative damage but several secondary conditions are reported as well. OS related neonatal diseases include: chronic lung diseases, intraventricular hemorrhages of newborns, necrotizing enterocolitis (Norishadkam et al. 2017, Perrone et al. 2010), bronchopulmonary dysplasia, retinopathy of prematurity, patent ductus arteriosus, periventricular leukomalacia, respiratory distress syndrome, intrauterine growth retardation, congenital malformation (Ozsurekci & Aykac 2016).

There has been number of evidence that (neonatal) OS plays an important role in various diseases, yet very few OS markers have made it into routine clinical practice. This might be accounted to variety of reasons: e.g. low specificity of methods,

the properties of the oxidative modifications, such as the lability of some structures, or their low abundance, poses significant challenges to translate them into a high-throughput, cost-effective clinical diagnostic (Frijhoff et al. 2015).

Further measurements and examination of OS biomarkers are required in order for them to contribute to prediction of high-risk patients (Negi et al. 2015), proper diagnosis, and early/optimal administration of effective pharmacotherapy.

2 AIMS

Although there have been many publications concerning OS and mechanisms of AOX defense, very few or even none have dealt with the OS in erythrocytes of healthy newborns specifically. Therefore, our aims were as follows:

- to collect the physiological OS related values from healthy young individuals (early infancy)
- to observe the changes of OS levels between newborn *vs* 2 months old babies
- to contribute to the knowledge on how healthy newborns adapt to "hyperoxic challenge" caused by a sudden exposure to extrauterine environment and how their AOX mechanism copes with it in time

3 MATERIALS AND METHODS

3.1 Population

The study was performed under the approval of the Ethics Committee of Centro Materno-Infantil from Centro Hospitalar do Porto, Portugal. Blood samples were collected from newborns after obtaining mother´s informed consent to participate in the study.

Umbilical cord blood (UCB) samples were collected at birth ($n = 8$) and, after 2 months, peripheral blood samples ($n = 8$) were collected from the subjects.

There were 4 girls and 4 boys among the subjects of the study, all of which were full-term, born by vaginal delivery to healthy mothers.

Collection of samples was slightly complicated due to inevitable elimination of several participants (health reasons) and due to temporarily restricted operation with the collaborating hospital.

3.2 Sample processing

Blood samples were delivered from the hospital and processed within 2 hours after their collection, in tubes with and without anticoagulant; K_3EDTA was used as an anticoagulant.

Our intention was to isolate several different fractions of each sample – whole blood, serum, plasma, RBC membranes and total erythrocytes. Aliquots of these sample fractions were stored at -80 °C until assayed.

Serum was isolated from tubes without anticoagulant by centrifugation (1000 g, 20 min., room temperature (R.T.)) and used for biochemical parameters assessment in an automated analyzer (Cobas Mira S, Roche) using commercially available assay kits: uric acid (Uric Acid ver.2, Roche Diagnostics); albumin (Albumin Plus, Roche Diagnostics); total (Bilirubin Total Gen.3, Roche Diagnostics), direct (Bilirubin Direct; Roche Diagnostics) and indirect bilirubin; gamma glutamyl transferase (γ-Glutamyltransferase ver.2, Roche Diagnostics); aspartate transaminase (Aspartate

Aminotransferase Test ASTL, Roche Diagnostics) and alanine transaminase (Alanine Aminotransferase Test ALTL, Roche Diagnostics).

Erythrocyte, leukocyte and reticulocyte count, Hb concentration, hematocrit, hematimetric indexes – mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) – and red cell distribution width (RDW) were measured using an automatic blood cell counter (Sysmex K1000, Sysmex).

Anticoagulated whole blood was centrifuged at 2000 g, 20 min., 4 ºC and, afterwards, plasma was aliquoted and stored. Leukocytes were isolated from RBCs after centrifugation on a double density gradient (Histopaque 1077; Histopaque 1119; 700 g/30 min/R.T.).

RBCs were then washed with saline solution (NaCl 9.0 g/L; centrifuged at 1000 g/5 min/4 °C) and small portion was frozen as total erythrocyte aliquots. Another portion of these RBCs was mixed with $HClO_4$ 5 % and following a centrifugation (10000 g/15 min/ 4 °C), aliquots for GSH measurements were prepared.

The rest of the RBCs were submitted to hypotonic lysis, according to Dodge *et al.* (Dodge et al. 1963) in order to isolate membranes. Obtained membrane suspensions were carefully washed in 5mM pH8 phosphate buffer, using phenylmethylsulfonylfluoride (final concentration 0.1 mM) in the first two washes, as a protease inhibitor.

3.3 Materials

3.3.1 Reagents

From Sigma-Aldrich: Triton X-100; Tiobarbituric acid; Coomassie Brilliant Blue G-250; CAT from bovine liver; purpald; H_2O_2 30%; GPx from bovine erythrocytes; GSH (L-glutathione reduced); GSSG (L-glutathione oxidized disodium salt); GR from baker´s yeast – S. cerevisiae; β-nicotinamide adenine dinucleotide 2´-phosphate reduced tetrasodium salt hydrate (NADPH); Cumene hydroperoxide; 5,5´-dithiobis-2-nitrobenzoic acid (DTNB); 2-vinylpyridine; $KHCO_3$; Iron(II) sulfate heptahydrate; Iron(III) chloride hexahydrate; 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ).

From Merck: ethanol; methanol; $HClO_4$ 70-72%; Phosphoric acid 85%; HCl 37%; acetic acid 100%; KOH; KIO_4.

From Panreac: NaCl; KH_2PO_4; K_2HPO_4; NaH_2PO_4; Na_2HPO_4; sodium acetate.

From VWRChemicals: *n*-butanol; ethylenediamine tetraacetic acid (EDTA)

Bovine serum albumin (BSA) from Calbiochem and formaldehyde 36.5% from Chem- Lab.

3.3.2 Consumables

96 wells plates; Pauster Pipettes; Micro-pipette Tips; Eppendorf tubes; etc.

3.3.3 Instruments

Centrifuge (1580R, GYROZEN); Micro-centrifuge (Heraeus Freco 21, Thermo Scientific); Microplate reader (Power Wave XS, Bio-Tek); Water bath (FALC); Vortex (VELP Scientifica); Ultra-Low Temperature Freezer (MDF-U5386S, Panasonic); Fumehood (Industrial Laborum)

3.3.4 Software

KC junior (microplate reader software)
IBM SPSS (Statistics Package for Social Sciences), *version* 24.0

3.4 Methods

3.4.1 General

To be able to assess the changes of measured data credibly, both samples of the same subject – newborn and 2 months follow-up – were always assayed at the same time, under the same conditions.

3.4.2 Determination of total protein concentration

The modified Bradford method was used to estimate the total protein concentration of membrane and total erythrocyte samples (Bradford 1976).

The Bradford´s reagent, (100 mg of Coomassie Brilliant Blue G-250; 50 mL of ethanol 95%; 100 mL of phosphoric acid 85%) exists in two different color forms, red and blue. The Reagent´s binding to protein molecules is accompanied by its color change and, thus, by a shift in absorption maximum (from 365 to 595 nm). The absorbance at 595 nm is spectrophotometrically monitored (microplate reader) and compared with a set of protein standard solutions.

Membrane samples were diluted 1:100 and the total erythrocytes ones 1:15000. The standard curve was obtained using a set of BSA standards (concentration 0.0 to 0.05 mg/mL). An Internal Control (dilution 1:2000) was used to standardize and validate the obtained data.

3.4.3 Membrane bound hemoglobin

MBH was measured by spectrophotometry (microplate reader) in erythrocyte membrane suspensions samples (Santos-Silva et al. 1995).

Triton X-100 (5% w/v in 5mM pH8 phosphate buffer) was added to the samples for protein dissociation and the absorbance was measured at 415 nm (maximum Hb absorbance) and at 700 nm (background absorbance). The MBH percentage was estimated using the corrected absorbance value and total protein concentration of the samples to normalize its percentage (equation 3).

$$\%\ \mathrm{MBH} = \frac{\mathrm{A415} - \mathrm{A700}}{[\text{Total protein concentration}]}\ \mathrm{x}\ 100 \qquad \text{(equation 3)}$$

3.4.4 Erythrocyte membrane lipid peroxidation

The TBARS test, adapted from Mihara and Uchiyama (1977), was used to determine the erythrocyte membrane LPO in membrane and plasma samples (Mihara & Uchiyama 1978).

To assess the LPO of each sample, the quantity of MDA and its precursors was determined. MDA is one of the main LPO products and the method makes use of its reaction with TBA which results in a formation of a red colored chromogen adduct with absorption maximum at 535 nm (Ayala et al. 2014).

Membrane samples were incubated 10 min. at R.T. with Triton X-100 20% to dissociate proteins. The following procedure was the same for both membrane and plasma samples: H_3PO_4 1.0% (v/v) and TBA 0.6% (m/v) were added to the samples, the tubes were thoroughly homogenized and incubated on a boiling water bath for 45 minutes. Afterwards, samples were put on ice to cool down and n-butanol was added to each tube to extract the TBA-MDA complex. The organic layer was separated by centrifugation (1000 g; 2 min.; R.T.) and its absorbance was measured by spectrophotometry at 535 nm.

For RBC membrane samples, the LPO was determined after correcting this value with background absorbance (520 nm) and normalizing it with total protein concentration for each sample. For plasma samples, we used directly the value of absorbance at 535 nm.

3.4.5 Quantification of catalase activity

To determine the activity of CAT in membrane and total erythrocytes samples, a spectrophotometric method adapted from Johansson and Borg was performed (Johansson & Borg 1988).

The method is based upon the CAT peroxidatic activity: the reaction of the enzyme with a hydrogen donor (methanol) in the presence of optimal concentration of H_2O_2 results in a formation of formaldehyde. The latter forms a bicyclic heterocycle with a chromogen, 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald), which upon oxidation changes from colorless to a purple compound with absorption maximum at 540 nm (Figure 3). The absorbance was compared to a set of standard formaldehyde solutions and its value was used to calculate the activity of the enzyme.

$$H_2O_2 + CH_3OH + \text{Purpald} \xrightarrow{\text{CAT}} \boxed{CH_2O} + 2\,H_2O$$

Figure 3. Quantification of catalase activity. Hydrogen peroxide (H_2O_2) reaction with methanol (CH_3OH) in the presence of catalase (CAT) yields formaldehyde (CH_2O) and water (H_2O). Formaldehyde and Purpald form a compound which, upon oxidation, changes from colorless to purple. Adapted from Melo, 2017 (Melo 2017).

The membrane samples were diluted 1:25 and total erythrocyte samples 1:10000. A set of standard formaldehyde solutions (0 – 75 uM) was prepared. All samples,

standards and the control were incubated with methanol and H_2O_2 35.0 mM for 20 minutes, then KOH 10.0 M (to stop the reaction) and Purpald (chromogen) were added. After 10 minutes of incubation, KIO4 300.0 mM (color amplifier) was added to all wells and following a 5 minutes incubation, the absorbance was measured at 540 nm. A CAT activity internal control was used to validate the results.

The enzyme activity was determined by interpolation of the sample values in the standard curve and by applying a formula (including normalization of the activity by dividing by total protein concentration) as seen in equation 4:

$$Cat\ activity\ (nmol/min/mL/mGP_t) = \frac{formaldehyde\ concentration\ x\ (0.17/0.02)}{20\ (\text{min})}\ x\ D.F.x\ \frac{1}{P_t}$$

(equation 4)

3.4.6 Quantification of glutathione peroxidase activity

The activity of GPx was measured in total erythrocyte samples by an indirect assay adapted from Weydert & Cullen (Weydert & Cullen 2010).

This spectrophotometric method enables GPx activity quantifying through the actions of other enzymes and cofactors that are necessary for its function.

GPx catalyzes the reduction of various hydroperoxides (e.g., H_2O_2) to H_2O via oxidation of reduced GSH into its disulfide form GSSH. GSSG is regenerated to GSH by GR with consumption of NADPH. The decrease of NADPH can be easily monitored spectrophotometrically and is proportional to GPx activity.

Samples were diluted 1:200 and a set of standard GPx solutions (0 – 100.0 mU/mL) was prepared, as well as, a GPx internal control to validate the procedure. GSH/GR solution (with final well concentration 1.0 mM/1.0 U/mL) and NADPH 0.2 mM (final well concentration) were added followed by 10 minutes of incubation at 25 °C. After adding the substrate, cumene hydroperoxide 1.5 mM (final well concentration), a kinetic absorbance reading was performed at 340 nm for 5 minutes (30 seconds intervals). The value of GPx activity was obtained after interpolation of the samples absorbance delta per minute in the standard curve and after normalizing this value with the total protein concentration for each sample.

3.4.7 Determination of total antioxidant status

To determine the TAS in both plasma and total erythrocyte samples, the Ferric Reducing Ability of Plasma (FRAP) Assay was used according to a method adapted from I. F. F. Benzie & J. J. Strain. (Benzie & Strain 1996)

Ferric and ferrous ions can form a complex with TPTZ. The ion ferric to ferrous reduction can be monitored at low pH, as a blue colored complex of Fe^{2+}-TPTZ with absorption maximum at 593 nm is formed. By comparing the absorbance change in samples with the absorbance in a set of standards of known concentration of ferrous ion, the FRAP values were determined.

Total erythrocytes samples were diluted to 1:50, the plasma samples were applied directly to the plate. A set of standard solutions (ferrous ion concentrations 0.0 – 1.0 mM) was prepared to obtain the standard curve. A ferrous internal control ensured the validation and standardization of the procedure.

The FRAP reagent (acetate buffer 300.0 mM + ferric ion 20.0 mM + TPTZ 10.0 mM, 10:1:1) was added to all the samples and the absorption at 0 min and 4 min was measured spectrophotometrically at 37 ºC. After comparing the values with those obtained from the standard curve, total protein concentration was used to normalize the values of TAS.

3.4.8 Quantification of total glutathione and oxidized glutathione

To determine the reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio, total GSH and GSSG were quantified in the total erythrocyte samples according to a method adapted from Shaik & Mehvar (Shaik & Mehvar 2006) and from Griffith (Griffith 1980).

The principle of this spectrophotometric method is the enzymatic recycling of GSH by GR with consumption of NADPH. GSH oxidation by DTNB, is accompanied by formation of a yellow chromogen, 5-thio-2-nitrobenzoic acid (TNB), the quantity of which is monitored and thus, total GSH amount is determined as these two values are proportional (Figure 4).

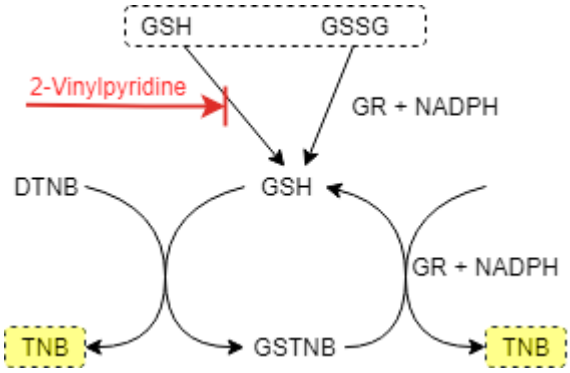

Figure 4. Quantification of total glutathione and oxidized glutathione. Glutathione (GSH) can reduce different compounds, including 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) while oxidizing itself (GSTNB). This reaction yields a yellow chromogen, 5-thio-2-nitrobenzoic acid (TNB) the amount of which can be monitored as it amount is proportional to available GSH amount. Oxidized glutathione (GSSG) is regenerated by glutathione reductase (GR) in a nicotinamide adenine dinucleotide phosphate (NADPH) consuming reaction. 2-Vinylpyridine serves as a masking agent. Adapted from Melo, 2017 (Melo 2017).

GSSG quantification employs exactly the same principle/protocol that total GSH, with only one additional step: to distinguish the GSSG in the total amount of GSH, an agent (2- Vinylpyridine) is used to mask reduced GSH (McGill & Jaeschke 2015).

Total erythrocyte samples were diluted with $HClO_4$ 5%, 1:250 and 1:50 for total GSH and GSSG quantifications, respectively. Sets of standard GSH (0 – 15.0 uM) and GSSG (0 – 8.0 uM) solutions were prepared. Internal controls of GSH and GSSG were used to validate the results.

To neutralize the samples, $KHCO_3$ 0.76 M was added to samples/internal control and standard solutions. After centrifugation (16000 g, 2 min, 4 °C), the samples were transferred to a 96 well plate and the reagent solution (240 µM NADPH, 1.3 mM DTNB final well concentration) was added to each well and the plate was incubated for 15 min at 30 °C. Afterwards, GR (final well concentration of 2 U/mL) was added and a kinetic reading was performed at 412 nm for 3 minutes (30 seconds intervals). Values of the slope were used, plotted against the standard curve and finally, normalized by the total protein concentration for each sample to get the total GSH values. The GSSG quantification required a pre-incubation of the samples with 2-vinylpyridine (10 µL) for 1 h at 4 °C.

3.5 Statistical analysis

For statistical analysis, the Statistical Package for Social Sciences (IBM SPSS, *version* 24.0, SPSS Inc) for Windows was used.

Descriptive statistic tools were employed to characterize the data, including Shapiro-Wilk normality test. As some of our data presented non-Gaussian distribution, we used the non-parametric Wilcoxon Signed-Rank Test for related samples to evaluate the differences between the studied groups (baby at birth and in 2 months of age). Spearman's rank correlation coefficient was used to assess relationships between sets of data. Data are presented as median (interquartile range). The level of significance (p) was set at 0.05.

4 RESULTS

The first task of our work was to obtain physiological data linked to OS. These can be divided into three groups: hematological data, biochemical data (both performed in the hospital) and our own measurements of RBC-specific OS related values (OS biomarkers).

Hematological and biochemistry tests were performed at birth (from UCB samples) and then in two months (64 days in average, peripheral blood samples). Experimental values were obtained in our laboratory after receiving the follow-up samples matching the newborn samples, as the importance was given to having the same assay conditions for both of the paired samples.

To assess the relationships between different parameters, Spearman's rank correlation coefficient was used.

4.1 Hematological data

RBC count, Hb, hematocrit, MCV, MCH, MCHC, RDW, reticulocytes and leukocytes count were measured.

The results (Table 2) show that there was a significant difference ($p<0.05$) between newborn and 2 months follow-up measurements in all examined parameters.

Significant decrease was observed in the following indices: RBCs, Hb, hematocrit, MCV, MCH, RDW, reticulocytes and leukocyte count.

These changes were expected and are in an accordance what was already known (Esan 2016, Ogundeyi M.M. et al. 2011, Soldin et al. 2005)

Comparison of the follow-up samples with reference values shows that apart from RDW, all data fall into the reference range (Soldin et al. 2005). Given references relate to peripheral blood samples of males, aged 61 d to <181 d, as in *Pediatric Reference Intervals* (Soldin et al. 2005).

Table 2. Hematological data of healthy newborns and of their 2 months follow-up.

	newborn	2 months old	*p*	reference value - 2 months
RBC [x10^{12}/L]	4.35 (4.21 - 4.57)	3.71 (3.54 - 3.85)	***0.012***	3.67 - 4.61
Hemoglobin [g/dL]	14.8 (14.9 - 15.9)	10.8 (10.7 - 11.7)	***0.012***	10.5 - 13.0
Hematocrit [%]	44.9 (43.4 - 46.8)	31.0 (30.5 - 35.5)	***0.012***	30.5 - 37.7
MCV [fL]	103 (101 - 106)	84.7 (83.2 - 85.7)	***0.012***	79.6 - 86.3
MCH [pg]	34.9 (33.6 - 35.2)	29.9 (29.3 - 30.5)	***0.012***	27.6 - 29.9
MCHC [g/dL]	33.8 (33.0 - 34.1)	35.4 (34.9 - 35.6)	***0.012***	33.9 - 35.4
RDW [%]	16.3 (16.3 - 17.3)	13.3 (12.8 - 13.7)	***0.012***	13.5 - 15.3
Reticulocytes [x10^{9}/L]	166 (162 - 193)	72.7 (57.6 - 110)	***0.017***	37.0 - 104
Leukocytes [x10^{9}/L]	16.0 (12.9 - 18.8)	8.4 (6.9 - 10.3)	***0.012***	7.9 - 13.4

RBC, red blood cell; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; RDW, red cell distribution width.
Data are presented as median (inter – quartile range) values. *p<0.05* between newborn *versus* 2 months old groups was considered statistically significant; Wilcoxon Signed-Rank Test was used.
Reference values were obtained from *Pediatric Reference Intervals* (Soldin et al. 2005).

Positive and statistically significant correlations were found between the gestational age and the count of leukocytes both in newborn and in 2 months samples ($r = 0.766$; $p = 0.027$; $n = 8$; $r = 0.717$; $p = 0.046$; $n = 8$, respectively).

4.2 Biochemical data

Uric acid (UA) concentration, bilirubin levels, liver enzymes (gamma-glutamyl transferase, GGT; aspartate aminotransferase, AST; alanine aminotransferase, ALT) and albumin concentration were quantified and calculated.

The data can be seen in Table 3. Uric acid, GGT, AST, ALT (and thus AST/ALT ratio) and albumin values changed significantly over two months. A decrease was found in UA, GGT and albumin levels; AST, ALT and albumin levels showed a significant increase.

The follow-up (2 months old) values were compared with reference values and all but direct bilirubin were within the reference range. Reference values relate to peripheral blood samples of infants, aged approximately 2 months (the age intervals differ), according to *The Harriet Lane Handbook* (Kahl & Hughes 2017).

Table 3. Biochemical data of healthy newborns and of their 2 months follow-up.

	newborn	2 months old	*p*	reference value 2 months
Uric acid [mg/dL]	4.85 (4.35 - 5.88)	2.60 (2.43 - 3.00)	***0.012***	1.1 - 5.6
Total bilirubin [mg/dL]	1.46 (1.23 - 1.65)	1.08 (0.45 - 1.59)	*0.208*	< 1.2
Direct bilirubin [mg/dL]	0.58 (0.46 - 0.66)	0.40 (0.20 - 0.55)	*0.262*	< 0.2
Indirect bilirubin [mg/dL]	0.90 (0.57 - 1.10)	0.68 (0.24 - 1.10)	*0.528*	< 1.0
GGT [UI/L]	148 (91 - 203)	53 (33 - 111)	***0.025***	8.0 - 90.0
AST [UI/L]	28.5 (20.0 - 33.0)	37.5 (32.5 - 101)	***0.035***	9.0 - 80.0
ALT [UI/L]	10.5 (5.3 - 12.8)	28.0 (25.3 - 72.0)	***0.012***	13.0 - 45.0
AST/ALT	2.95 (2.35 - 3.38)	1.30 (0.85 - 1.53)	***0.011***	0.7 - 1.8
Albumin [g/dL]	3.84 (3.70 - 3.92)	4.14 (3.97 - 4.41)	***0.017***	2.2 - 4.8

GGT, gamma-glutamyl transferase; AST, aspartate aminotransferase; ALT, alanine aminotransferase.
Data are presented as median (inter – quartile range) values. $p<0.05$ between newborn *versus* 2 months old groups was considered statistically significant; Wilcoxon Signed-Rank Test was used.
Reference values were obtained from *The Harriet Lane Handbook* (Kahl & Hughes 2017).

4.3 Oxidative stress biomarkers

While assessing OS biomarkers we worked with different fractions of the blood samples: RBC membranes, total erythrocyte and plasma.

All the presented OS biomarkers data are values that were normalized by protein concentration of each sample. Mentioned reference values were obtained in previous research and projects of our laboratory and belong to adult subjects, so they only serve as a rough comparison, since they are biased by age and development.

4.3.1 RBC membranes

Three tests were performed using erythrocyte membranes from our subjects. Graphic representation can be seen in Figure 5.

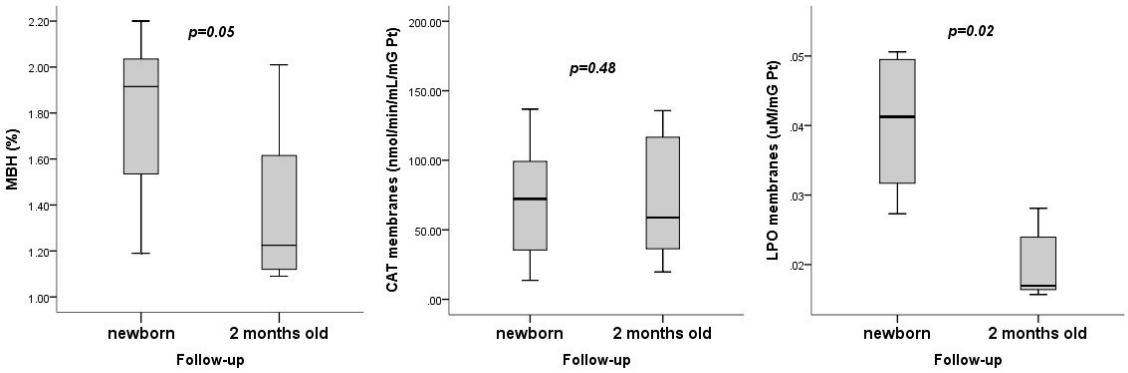

Figure 5. Oxidative stress biomarkers - RBC membranes of healthy newborns and of their 2 months follow-up: MBH, CAT, LPO. MBH, membrane bound hemoglobin; CAT, catalase activity; LPO, lipid peroxidation. Data are presented as median (inter – quartile range) values. *p<0.05* between newborn *versus* 2 months old groups was considered statistically significant; Wilcoxon Signed-Rank Test was used.

MBH percentage decreased significantly from newborn to 2 months follow-up; reference range for adults is 0.52 - 0.97 %.

Statistically significant positive correlations were found in newborns between MBH and the following indices: membrane's CAT activity ($r = 0.810$; $p = 0.015$; $n = 8$), total RBC GPx activity ($r = 0.810$; $p = 0.015$; $n = 8$), plasma LPO ($r = 0.778$; $p = 0.023$; $n = 8$). A negative statistically significant correlation was observed with GSH/GSSG ratio ($r = -0.762$; $p = 0.028$; $n = 8$).

Quantification of CAT activity in membranes contrary to our expectation has not shown a significant difference between newborns and 2 months samples; reference range for adults is 6.5 - 57.1 nmol/min/mL/mG_{Pt}.

Statistically significant correlations were discovered in these cases: membrane's CAT activity in newborns correlates positively with GPx activity in 2 months samples ($r = 0.762$; $p = 0.028$; $n = 8$) and with plasma LPO in newborns ($r = 0.717$; $p = 0.046$; $n = 8$), and negatively with GSH/GSSG ratio in newborns ($r = -0.762$; $p = 0.028$; $n = 8$).

LPO measured in RBC membranes seems to drop significantly after two months and a tendency to decrease to common adult reference range (0.012 - 0.017 μM/mG_{Pt}) is clear. Strength of this OS biomarker is also supported by statistically significant

correlations: a negative one was found between LPO 2 months and TAS 2 months (r = - 0.786; p = 0.021; n = 8); a positive link was discovered between LPO in newborns and CAT activity (measured in total erythrocytes, 2 months) and GPx activity (newborn) levels (r = 0.738; p = 0.037; n = 8 and r = 0.762; p = 0.028; n = 8, respectively) and, in the 2 months follow-up, there was an interesting (positive) connection with hematological indices, RBC count and Hb, in newborns (r = 0.714; p = 0.047; n = 8 and r = 0.759; p = 0.029; n = 8, respectively).

4.3.2 Total erythrocytes

Quantification of CAT activity, of TAS and of GPx activity was performed using total erythrocyte samples; results are presented in Figure 6.

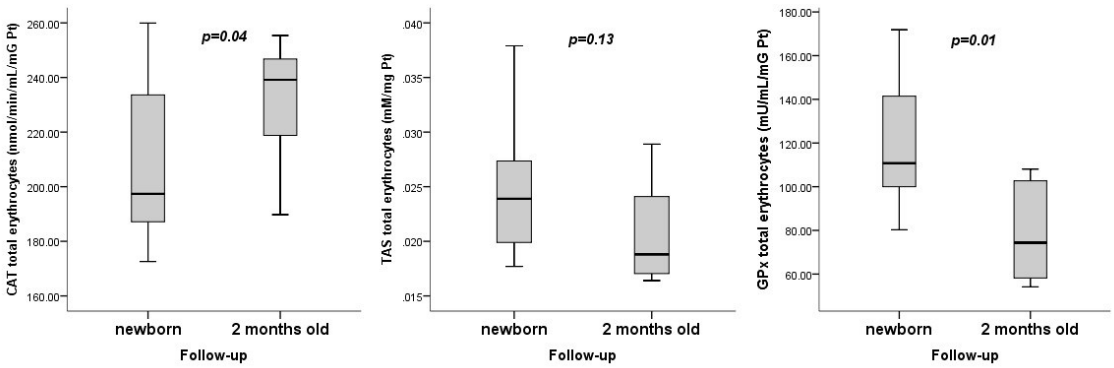

Figure 6. Oxidative stress biomarkers – total erythrocytes of healthy newborns and of their 2 months follow-up: CAT, TAS, GPx. CAT, catalase activity; TAS, total antioxidant status; GPx, glutathione peroxidase activity. Data are presented as median (inter – quartile range) values. *p<0.05* between newborn *versus* 2 months old groups was considered statistically significant; Wilcoxon Signed-Rank Test was used.

CAT activity showed an increase from newborns to 2 months follow-ups, with reference values being 181 – 275 nmol/min/mL/mG$_{Pt}$. Positive and statistically significant correlation with gestational age was discovered in newborn samples (r = 0.766; p = 0.0278; n = 8); on the other hand, in the follow-up samples, it correlates negatively and significantly with follow-up reticulocytes value (r = -0.786; p = 0.021; n = 8).

Despite our expectations, TAS levels have not proven to change with statistical significance; both values met the reference range 0.040 - 0.056 mM. However, there are some interesting correlations (statistically significant): 2 months TAS samples showed

a positive links to reticulocyte count in newborns (r = 0.738; p = 0.037; n = 8) and to GSH/GSSG ratio in 2 months samples (r = 0.881; p = 0.004; n = 8).

For GPx, we recorded a significant decrease. From values above the reference range in the newborns, the levels dropped 2 months after birth; reference: 51.5 - 88.7 mU/mL.

While still using the total erythrocytes fraction, a set of tests to measure GSH related values was carried out (Figure 7).

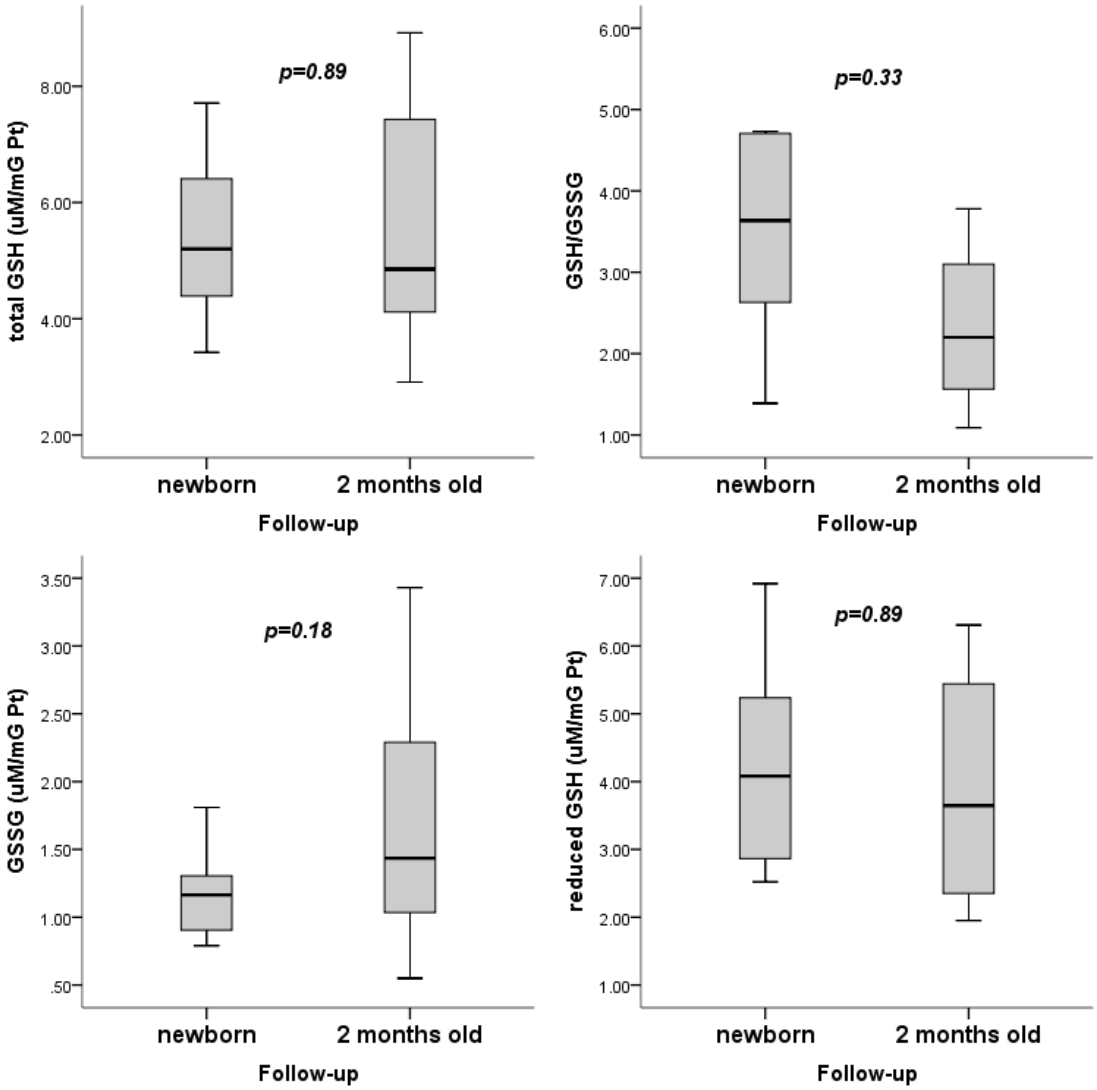

Figure 7. Oxidative stress biomarkers - total erythrocytes of healthy newborns and of their 2 months follow-up: glutathione. GSH, glutathione; GSSG, oxidized glutathione. Data are presented as median (inter – quartile range) values. $p<0.05$ between newborn *versus* 2 months old groups was considered statistically significant; Wilcoxon Signed-Rank Test was used.

None of the indices showed significant changes over the 2 months after birth. All the examined values (total glutathione, oxidized glutathione, reduced glutathione and reduced/oxidized glutathione ratio) were within the reference ranges: total GSH 9.48 – 15.3 μM, GSSG 1.19 – 1.94 μM, reduced GSH 8.29 – 13.4 μM and GSH/GSSG 6.89 – 6.97.

Several statistically significant correlations support the data: positive correlation in newborns - total GSH and both reduced GSH and GSH/GSSG ($r = 0.976$; $p < 0.001$; $n = 8$ and $r = 0.762$; $p = 0.028$; $n = 8$, respectively). In 2 months samples there is a connection between total GSH and reduced GSH ($r = 0.952$; $p < 0.001$; $n = 8$). Oxidized glutathione has a negative link to GSH/GSSG, both in newborns ($r = -0.738$; $p = 0.037$; $n = 8$). On the other hand, GSH/GSSG correlates positively with reduced GSH, in newborns as well ($r = 0.833$; $p = 0.010$; $n = 8$). All these connections are not surprising, since they only support the knowledge of GSH metabolism.

More of novelty could be these: reduced GSH in 2 months samples correlates significantly and positively with the reticulocytes count in newborns and negatively with the UA levels in newborns ($r = -0.810$; $p = 0.015$; $n = 8$ and $r = -0.735$; $p = 0.038$; $n = 8$, respectively). GSH/GSSG ratio in newborns showed a negative link to plasma LPO in newborns and a positive one to reticulocytes count in newborns ($r = -0.766$; $p = 0.027$; $n = 8$ and $r = 0.762$; $p = 0.028$; $n = 8$, respectively).

4.3.3 Plasma

LPO and TAS were determined in the plasma fraction of the samples. Graphic representation of the evolution of the values can be seen in Figure 8.

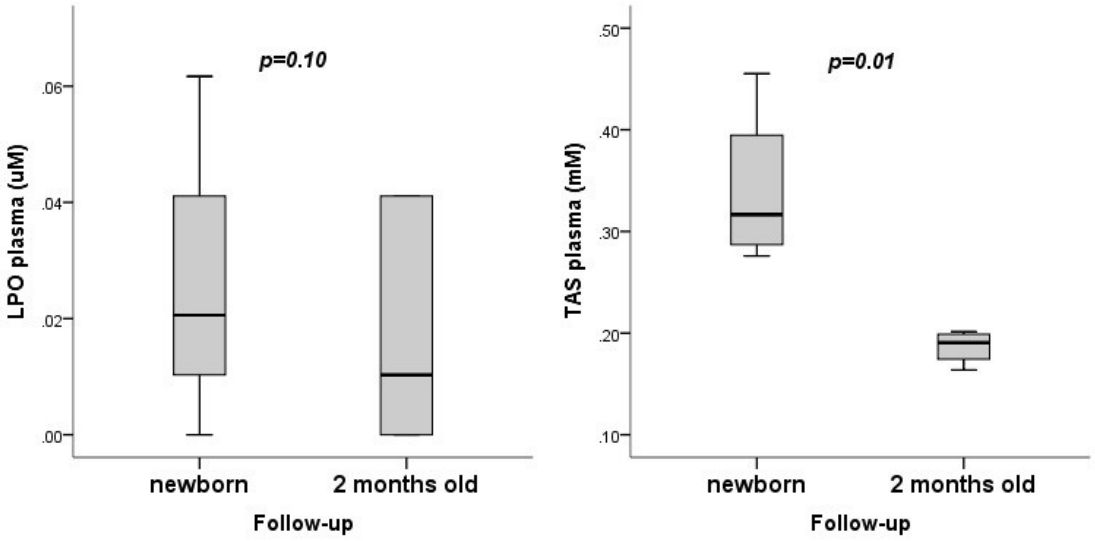

Figure 8. Oxidative stress biomarkers – plasma of healthy newborns and of their 2 months follow – up: LPO, TAS. LPO, lipid peroxidation, TAS, total antioxidant status. Data are presented as median (inter – quartile range) values. *p<0.05* between newborn *versus* 2 months old groups was considered statistically significant; Wilcoxon Signed-Rank Test was used.

Contrary to LPO measured in membranes, plasma LPO has not shown a significant difference, though a decrease was observed. Both LPO plasma values seem to be even lower than the reference ones; reference for adults: 0.022 - 0.043 μM.

TAS decreases in time; reference range being 0.28 - 0.40 mM.

5 DISCUSSION

Erythrocytes, cells responsible for O_2/CO_2 transport in the human organism, are continuously exposed to ROS, either from endogenous or exogenous origin. Though they are equipped with a powerful AOX defense system, these mechanisms can get overwhelmed on some occasions, resulting in damage to both RBCs and other cells and tissues. A state when harmful pro-oxidants outweigh the AOX capacity and, thus, disturb the balance, is called OS. The cause of OS is usually associated with various pathologies and diseases, but it has been documented in healthy newborns as well. Neonates, after being suddenly exposed to relatively hyperoxic extrauterine environment, with their immature organisms working autonomously for the first time, are extremely susceptible to FR. By evaluating reliable biomarkers of OS, we can analyze how the newborns cope with this newly acquired autonomy.

In this study we present experimental data obtained by measurements of several OS biomarkers, we follow the changes in hematology and biochemistry of neonates and with the support of correlations we estimate the connections and mechanisms among various AOX agents, focusing on the erythrocyte as a model of OS.

5.1 Hematological data

Although basic hematology assays are carried out in every hospital laboratory on a daily basis, it is not very common to have them from healthy newborns, right after birth, from the UCB. But these values, when compared with a follow-up, assayed under the same conditions, give us a precious insight on how the biochemistry of a newborn infant changes with age and the hematological values themselves are often required for monitoring and diagnosis of a possible pathology.

All the measured hematological parameters were found to be normal (Table 2), and they correspond with common knowledge about the topic (Esan 2016, Ogundeyi M.M. et al. 2011, Soldin et al. 2005). Overproduction of various blood elements and substances is an important part of the organism preparing for being literally cut off the mother. All the components necessary for gas carriage, as well as white cells responsible for the so needed immunity, exceed the counts that are usually found in older

children and adults. Attention must be paid while comparing values with reference ranges; not only the age of an infant matters but also the type of collected blood – venous *versus* UCB. In our study, newborn samples don't have reference values to compare them to as there are no official ranges for cord blood, which is an improvement given to this field of knowledge by this work. Little insight can be seen from the work of Catarino *et al*. (Catarino et al. 2009) who also evaluated UCB for several parameters as we did, plus, they worked with a Portuguese population as well, while our reference values for 2 months samples come from American authors and population (Soldin et al. 2005). A different set of reference ranges can be found in the work of Qaiser, Sandila, Ahmed, & Kazmi (Qaiser et al. 2009) who worked with Pakistani newborns, measured values are in accordance with our results. Another study was conducted by Ogundeyi M.M. *et al.* in Nigeria. The researches there worked with around 150 infants whose samples of venous blood were taken at birth, in 3 days and in 6 weeks of life. Although we cannot directly compare the values with our results, the tendency of the indices to drop in time is obvious and corresponds with our findings and general knowledge (Ogundeyi M.M. et al. 2011).

It was described that in the neonatal period the complete blood count highly correlates with birth weight, blood sampling site, crying, physical therapy, mode of delivery and gestational age (Esan 2016). In accordance, we found a positive correlation of gestational age (38-70 weeks) with the leukocytes count in our newborns.

5.2 Biochemical data

Liver, besides its metabolic and other functions, is the principal detoxifying organ in the human organism (Casas-Grajales & Muriel 2015).

UA, as powerful plasmatic AOX, providing up to 60% of AOX capacity of plasma (Fabbrini et al. 2014), acts as a FR scavenger and chelator of transitional meatal ions (Kang & Ha 2014, Pasalic et al. 2012). But the role of UA in the AOX defense system is somehow dual: while it neutralizes peroxynitrite and hydroxyl radicals to inhibit protein nitration and LPO, the reaction, in which the UA is formed, produces ROS as by-products (Kang & Ha 2014, Pasalic et al. 2012). UA is the end product of purine metabolism in humans, the reaction being catalyzed by xanthine oxidase:

hypoxanthine is oxidized to xanthine, xanthine to UA. In both reactions, molecular O_2 is reduced and superoxide radical and H_2O_2 are formed (Casas-Grajales & Muriel 2015).

In newborns, it is normal that serum UA is increased. *Raivio* stated that the mean value in cord blood is 6.0 mg/dL, increases to 7.0 mg/dL in the next 24h and decreases to 3.5mg/dL over next three days (Raivio 1976). High UA levels could be explained by many morphological changes occurring in the blood of the neonate: nuclei of the former RBCs disappear, polynuclear neutrophils predominance changes in favor of lymphocytes, total leukocyte count drops significantly, which results in a large amount of purines (Kingsbury & Sedgwick 1917).

Bilirubin, a natural AOX, is well-known as the end product of heme metabolism. Heme is released from aging RBCs and in the turnover of heme-containing enzymes. Free-heme can be toxic though, so a family of heme oxygenase enzymes degrades it: the heme ring is cleaved to form biliveridin which can be reduced to bilirubin in the NADPH consuming reaction catalyzed by biliveridin reductase (Sedlak & Snyder 2004). Bilirubin is toxic and insoluble and thus must be conjugated with glucuronic acid before excretion into the bile. Or, bilirubin can be oxidized back to biliveridin, while reducing ROS. This biliverdin – bilirubin cycle appears to affect OS–induced membrane lipid degradation. Although concentrations of bilirubin are relatively lower than the ones of GSH, the continuous recycling of biliverdin to bilirubin amplifies its effect by more than 10 000 times and therefore even small increase in bilirubin levels could account for a significant increase in its AOX potency (Marconi et al. 2018).

A significant number of term and pre-term newborns display some level of neonatal jaundice. It is accompanied by an increase in serum of (unconjugated, indirect) bilirubin levels, mostly caused by breakdown of RBCs. Extremely high levels of unconjugated bilirubin can be neurotoxic, leading up to kernicterus, but majority of the jaundice cases, a "physiologic jaundice", respond well to phototherapy, which is a gentle and effective way of reducing bilirubin levels (Mitra & Rennie 2017, Sedlak & Snyder 2004).

Our results (Table 3) are in accordance with known reference ranges and follow expected developmental tendencies in healthy, term infants. We recorded a significant decrease in UA levels, slight changes in liver enzymes levels that settle up at known physiological values in follow-ups and significant increase of albumin. Reshad *et al.*

suggested that association between UCB albumin levels and the tendency to develop significant neonatal hyperbilirubinemia in both term and preterm neonates could lead to albumin´s application as a marker in screening the newborn infants for possible development of neonatal hyperbilirubinemia (Reshad et al. 2016).

5.3 Oxidative stress biomarkers

The focus of our work was pointed towards the AOX capacity of RBCs. Even though we expected more of our biomarkers to document significant changes between newborn *versus* follow-up, the fact that several of them exhibited a significant difference despite the small number of samples ($n = 8$) suggests that those biomarkers are strong and reliable ones (MBH, LPO membranes, GPx, CAT total erythrocytes, plasma TAS) (Figures 5, 6, 7 and 8).

Since no official reference values for OS biomarkers in newborns exist, we could only compare our follow-up data with experimental values obtained in previous research of our laboratory from samples of adult individuals and there was no reference for the newborn samples (from UCB).

In RBC membranes, the decrease in the MBH and LPO (Figure 5), which are generally believed to be good OS biomarkers (Ayala et al. 2014, Pandey & Rizvi 2010, Rizvi & Maurya 2007, Sharma & Premachandra 1991, Welbourn et al. 2017), corroborates the theory of the OS being at its highest right after birth, reflecting the sudden exposure to relatively hyperoxic environment (Perrone et al. 2010). Contrary to our expectations, plasmatic TAS decreased (Figure 8). For a better explanation, this assay would probably need to be repeated to discover whether this preliminary result would change with a larger number of samples or whether it would follow the same trend (decreasing after birth). The latter option could represent a developmental stage in infant where plasmatic TAS in 2 months of age is decreased and then increases with age. Strength of the MBH and LPO as biomarkers can be supported by several correlations: not only they correlate with each other, but a negative link between LPO and total erythrocytes TAS was found in the follow-up samples thus, the lower the OS levels get, the higher the AOX capacity is. Activity of GPx, which decreased over 2 months after birth, also seems to have a significant positive correlation with the two biomarkers while the other AOX enzyme, CAT, significantly increases (Figure 6). This observation could

be explained by the different targets of these enzymes. Although both of them detoxify the cell from H_2O_2, CAT is described to work at higher H_2O_2 concentrations, of mostly exogenous origin, while GPx, on the other hand, catalyzes the decomposition of low levels of endogenous H_2O_2, often generated by auto-oxidation of Hb (Rocha et al. 2015). The decrease of GPx activity could be explained by the newborn´s adaptation to the outer environment accompanied by the documented reduction of birth-related OS. However, the newly acquired autonomy of the older infant brings along an increasing exposure to various oxidants of exogenous origin. Here, supposedly, CAT steps in, to protect the cells, and thus its activity is increased.

Although OS is usually related to pathological conditions, many studies (Norishadkam et al. 2017, Ozsurekci & Aykac 2016, Perrone et al. 2010), including our work, show that it also accompanies a physiological event such as birth of a healthy, full- term baby. Broader collection of physiological reference values for OS biomarkers and proper knowledge of the mechanism behind them could help physicians in the evaluation of development of newborns or early prediction of potential future disorders.

Nevertheless, we cannot omit the role of these oxidative damage parameters in various OS related diseases; several of them were described in the literature: chronic lung diseases, intraventricular hemorrhages of newborns, necrotizing enterocolitis (Norishadkam et al. 2017, Perrone et al. 2010), bronchopulmonary dysplasia, retinopathy of prematurity, patent ductus arteriosus, periventricular leukomalacia, respiratory distress syndrome, intrauterine growth retardation, congenital malformation (Ozsurekci & Aykac 2016). Reference values of OS biomarkers could be a valuable source of information for the diagnosis, effective treatment and disease monitoring.

Tsukahara also suggests that restoration of redox balance either by decreasing exposure to ROS or by augmenting AOX capacity represent a promising way of therapeutic intervention in OS related diseases. Classic AOXs include tocopherols, carotenoids and ascorbate but it has been suggested that their effect is only symptomatic, since they scavenge already existing ROS and, thus do not serve as a causal treatment (Tsukahara 2007).

Several AOX strategies were involved in clinical trials in the pediatric field, including administration of corticosteroids for bronchial asthma and bacterial meningitis,

melatonin for neonatal asphyxia and epilepsy, tocopherols and ascorbates for endothelial dysfunction in hyperlipidemia, L-arginine for endothelial dysfunction in cardiac transplantation, folinic acid, betaine and methylcobalamin for autism (Tsukahara 2007), enteral glutamine and arginine for prevention of necrotizing enterocolitis, epicatechin (for blockage of peroxynitrite-mediated tyrosine nitration and peroxynitrite formation) in ischemic proliferative diseases of the retina (Ozsurekci & Aykac 2016). Some favorable results of these strategies were described but still, further studies with broader population are required, and, as *Tsukahara* mentions, other techniques for measurement of OS status because the establishment of reference values for OS biomarkers is paramount for these studies.

Generally, we believe that OS and its mechanisms need to be further explored, new studies conducted, and data collected. Our contribution, despite the limited number of subjects, provides insight in the issue as we bring a number of reference values for OS biomarkers in early infancy Portuguese individuals which, to our knowledge, have not been described before.

6 CONCLUSION

In the present work we focused on the study of OS in newborns with the aid of appropriate biomarkers of OS, focusing mainly on the erythrocyte.

We obtained physiological OS-related data, including complete blood count, general biochemical data and experimental values, from a small population of Portuguese healthy, full-term newborns. The sample collections occurred twice: at birth and in 2 months of age. By observing how the values change, newborn *versus* follow-up, we gained a good insight about the development of the infant.

Both hematological and biochemical data follow the reference values, as anticipated. Our experimental values support the theory of so called hyperoxic challenge which induces high levels of OS in healthy neonates: several of our biomarkers recorded a significant change in OS levels. MBH and LPO in membranes decreased as well as GPx activity, while CAT activity in total erythrocytes increased. The first three biomarkers document neonate´s adaptation to the environment and CAT/TAS values suggest autonomy development of the newborn, who starts to encounter various exogenous oxidants.

As there are no official reference values for OS biomarkers in newborns, our results are a novelty and provide an invaluable progression in this field of knowledge, though our number of samples is small and further studies are warranted.

OS has been linked with a number of pathological conditions both in newborns and older individuals. Proper knowledge of the mechanisms behind OS and oxidative damage and large quantity of reliable data documenting the OS-related reference values could be a precious tool in the assessment of human health and disease.

7 LIST OF ABBREVIATIONS

˙OH	hydroxyl radical
2,3-DPG	2,3-diphosphoglycerate
AIDS	acquired immune deficiency syndrome
ALT	alanine aminotransferase
AOX	antioxidant
AST	aspartate aminotransferase
ATP	adenosine triphosphate
BFU-E	burst-forming unit - erythroid
BSA	bovine serum albumin
CAT	catalase
CFU-E	colony-forming unit - erythroid
CMP	common myeloid progenitor
CO_2	carbon dioxide
Cys	cysteine
CysP	peroxidatic cysteine
deoxyHb	deoxyhemoglobin
D.F.	dilution factor
DTNB	5,5´-dithiobis-2-nitrobenzoic acid
EHM	extramedullary hematopoiesis
FAD	flavin adenine dinucleotide
FR	free radicals
FRAP	ferric reducing ability of plasma
G-6-P	glucose-6-phosphate
GGT	gamma-glutamyl transferase
Glc	glucose
GLUT1	glucose transporter
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	oxidized glutathione
H_2O	water
H_2O_2	hydrogen peroxide
Hb	hemoglobin
His	histidine

HPX	hypoxanthine
IL	interleukine
K_3EDTA	tripotassium ethylenediaminetetraacetic acid
Km	Michaelis constant
LDL	low-density lipoprotein
LPO	lipid peroxidation
MBH	membrane-bound hemoglobin
MCV	mean cell volume
MCH	mean cell hemoglobin
MCHC	mean cell hemoglobin concentration
MDA	malondialdehyde
metHb	methemoglobin
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
O_2	oxygen
O_2^-	superoxide anion
OS	oxidative stress
Prx	peroxiredoxin
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
Purpald	4-amino-3-hydrazino-5-mercapto-1,2,4-triazole
R.T.	room temperature
RBC	red blood cells
RDW	red cell distribution width
RNA	ribonucleic acid
ROS	reactive oxygen species
Sec	selenocysteine
SOD	superoxide dismutase
TBA	thiobarbituric acid
TBARS	thiobarbituric reactive substances
TNB	5-thio-2-nitrobenzoic acid
TPTZ	2,4,6-Tris(2-pyridyl)-s-triazine
Trx	thioredoxin
UA	uric acid
UCB	umbilical cord blood
XAN	xanthine

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