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DISSERTATION

“Effect of L-methionine on RBCs metabolism during storage”

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Abstract

Packed Red blood cells (RBCs) remain the most widely transfused blood component in the clinical setting. For clinical purposes, erythrocyte concentrate can be stored at 4 ± 2° C, for up to 42 days. However, their storage under blood banking conditions is associated with deleterious morphological and metabolic alterations, so called “storage lesions”, which have a negative effect on the quality of stored RBCs.

Application of omics technology in transfusion medicine significantly improved the study of RBC storage lesions. Metabolomics analysis showed a dramatic decrease in methionine concentration in stored RBCs, while homocysteine level increased [1]. Methionine is an essential methyl donor for several metabolic reactions, such as regulation of glycolytic enzymes, synthesis of glutathione and conversion of phosphatidylethanolamine (PE) to phosphatidylcholine (PC). Methionine can be generated either by homocysteine and betaine, or by homocysteine and 5’-methyltetrahydrofolate (5’-MTHF). The latter reaction uses Cobalamin (vitamin B12) as a cofactor.

Based on this finding, we attempted to restore the methyl-group pool in stored RBCs by supplementation of L-methionine, or to convert the accumulated homocysteine into methionine by supplementation of 5’-MTHF and vitamin B12.

Study Design and Methods: A pool-and-spilt design of leukodepleted RBCs stored in PAGGS-M additive solution was carried out to obtain three identical groups for PAGGS-M (control), PAGGS-MM (supplemented with L- methionine) and PAGGS-MB (supplemented with 5’-MTHF and vitamin B12). RBC units were stored at 4° C for up to 42 days and assessed for complete blood count, blood gas analysis, ATP, 2, 3 DPG, NAD/NADH, NADP/NADPH and total GSH and GSSG levels, hemolysis rate and extracellular metabolomics on a weekly basis.

Results: Supplementation of L-methionine significantly reduced the hemolysis rate and activity of PPP in packed RBCs throughout the storage period. However, the hemolysis rate was lower at the beginning of storage, rather than the end of storage period. RBCs stored in PAGGS-MM additive solution demonstrated the highest MCV, which was not related to cation leakage. The metabolic fate of supplemented methionine was substantially shifted

towards transamination pathway. Unlike L methionine, supplementation of 5'-MTHF + B12 had neither a beneficial, nor a harmful effect on stored RBCs

Conclusion: Reduced hemolysis rate and activity of PPP in RBCs stored PAGGS-MM, can be the consequences of decreased oxidative stress. Higher MCV of RBCs caused by L-methionine supplementation may be related to lower vesiculation of RBC membrane. Transamination of L-Methionine may prevent RBCs from accumulation of homocysteine. *In vivo* study is needed to estimate the effect of L-methionine on stored RBCs.

Abstract in German

Erythrozytenkonzentrate (EKs) sind immer noch die am häufigsten transfundierten Komponenten im klinischen Alltag. Klinisch zu nutzende EKs können bei 4 ± 2° C für bis zu 42 Tage gelagert werden. Eine solche Lagerung, selbst unter stringenten Blutbank-Bedingungen, führt jedoch bisher immer zu morphologischen und metabolischen Veränderungen, den sogenannten Lagerungsschäden, die einen negativen Effekt auf die Qualität der EKs haben.

Der Einsatz der omics-Technologie in der Transfusionsmedizin konnte die Untersuchungen zu Lagerungsschäden signifikant verbessern. Die metabolische Analyse zeigte einen dramatischen Abfall der Methionin Konzentration in den gelagerten Erythrozyten, während das Homozystein-Niveau anstieg [1]. Methionin, als ein wesentlicher Methyldonor, ist für eine Vielzahl von metabolischen Reaktionen wichtig, wie z.B. der Regulierung von Glykolyse-Enzymen, der Synthese von Gluthathion und der Umwandlung von Phosphatidylethanolamin (PE) zu Phosphatidylcholin (PC). Methionin kann entweder durch Homozystein und Betain, oder durch Homozystein und 5'-Methyltetrahydrofolat (5'-MTHF) generiert werden. Die letztgenannte Reaktion nutzt Cobalamin (Vitamin B12) als Kofaktor.

Ausgehend von diesen Ergebnissen wollten wir versuchen, den Methylgruppenpool in gelagerten Erythrozyten durch die Ergänzung mit L-Methionin aufzufüllen, oder durch Zugabe von 5'-MTHF und Vitamin B12 das akkumulierte Homozystein in Methionin zu konvertieren.

Studiendesign und Methoden: Ein pool-and-split-Design von leukozytendepletierten Erythrozyten in PAGGS-M Lösung wurde genutzt, um drei identische Gruppen für PAGGS-M (Kontrolle), PAGGS-MM (Zugabe von L-Methionin) und PAGGS-MB (Zugabe von 5'-MTHF und Vitamin B12) zu erhalten. Die Erythrozyten wurden anschließend bei 4°C für bis zu 42 Tage gelagert und in dieser Zeit wöchentlich auf ihre Gesamtzellzahl, ATP-, 2,3-DPG-, NAD/NADH-, NADP/NADPH- Konzentrationen, die Menge an Gesamt- und oxidiertem Gluthation und ihre Hämolyserate untersucht. Des Weiteren wurden Blutgasanalysen und extrazelluläre Metabolomics-Untersuchungen durchgeführt.

Ergebnisse: Die Zugabe von L-Methionin reduzierte signifikant die Aktivität des PPP und die Hämolyserate in Erythrozytenkonzentraten, auch wenn die Rate im Vergleich zum Ausgangswert während der Lagerung anstieg. Erythrozyten, die in PAGGS-MM gelagert wurden, zeigten die höchsten MCV-Werte. Dies war nicht assoziiert mit dem Verlust von Kationen. Das metabolische Schicksal des zugegebenen Methionins wurde in erheblichem Maße in Richtung des Transaminase-Weges verschoben. Im Gegensatz zum L-Methionin, hatte die Zugabe von 5'-MTHF + B12 weder einen positiven noch negativen Effekt.

Zusammenfassung: Die Reduktion der Hämolyserate und der Aktivität des PPP in Erythrozyten, die in PAGGS-MM gelagert wurden, kann eine Folge von vermindertem oxidativen Stress sein. Der signifikant höhere MCV der Erythrozyten unter L-Methionin-Zugabe könnte mit der geringeren Vesikulierung der Membran zusammenhängen. Die Transaminierung von L-Methionin könnte die Erythrozyten vor einer Akkumulierung von Homocystein schützen. *In vivo*- Studien sind unabdingbar, um den Effekt von L-Methionin auf gelagerte Erythrozyten einschätzen zu können.

1. Introduction

1.1. Human red blood cell (RBC)

RBCs are cellular component of human blood. They are produced through a process called erythropoiesis, which is divided into prenatal and postnatal period. In the prenatal period, blood is produced in several organs, such as liver, spleen, bone marrow. After birth, bone marrow is mostly responsible for the maturation of RBCs from committed stem cells to mature erythrocytes that takes 7 days. Mature erythrocytes leave the bone marrow and enter

peripheral blood circulation, where they live about 100 to 120 days. Unlike other cells, RBCs do not possess a nucleus, mitochondria, a Golgi apparatus, ribosomes, or an endoplasmic reticulum, but they are rich in hemoglobin. RBCs lose their nucleus at an early stage of development. The enucleated cells are called reticulocytes. They lose other cellular organelles on reaching full maturity. The absence of these organelles plays an important role in RBCs function and limits the metabolic capacity of the cells. Hemoglobin is a complex of proteins with heme group. It is red in color and contain iron atoms. This group temporarily binds to oxygen molecules (O_2) in the lungs and release them throughout the body. Oxygen (O_2) transport to the tissues depends on hemoglobin's ability to bind oxygen in the alveoli of the lungs. Oxygen rich RBCs circulate thousands of miles through blood vessels of different size and provide all tissues with oxygen, which can easily diffuse through the membrane of red cells [2]. On the other hand, a high content of oxygen in RBCs contributes to an accumulation of reactive oxygen species (ROS) and results in oxidative stress. This process has a negative effect on erythrocyte physiology, disrupts their membrane and accelerates RBC aging. The survival of RBCs in the circulation depends on the flexibility and mechanical stability of erythrocyte membrane [3]. Besides the gas transport, RBCs actively participate in the regulation of the regional vascular tone, vascular antioxidant system and physiologic response of regional and systemic hypoxia [2]

1.1.1. Red blood cell membrane

RBC membrane consists of lipid bilayer and cytoskeleton [4]. The predominant lipids in the lipid bilayer are phospholipids and cholesterol. They are almost equally distributed in the two leaflets of the lipid bilayer, but four of the phospholipids: phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), sphingomyelin(SM) are asymmetrically distributed. The internal surface of the plasma membrane is rich in amino-containing phospholipids (PS, PE), but the external surface is rich in choline-containing phospholipids (PC, SM) [5, 6]. This asymmetry is caused by the lipid flip-flop mechanism. This is a very slow process, which takes hours and even days. The adenosine triphosphate (ATP) dependent amino phospholipid flippase and floppase are responsible for the transport of phospholipids on both sides of the membrane. Flippase is an amino phospholipid translocase. It transports phosphatidylserine and phosphatidylethanolamine from the outer to the inner monolayer [7, 8]. The P-glycoprotein-floppase is the energy-dependent translocator. It transports phospholipids and amphiphilic drugs from the inner to the outer leaflet of the membrane with a low selectivity [9-11]. The asymmetrically located lipids: PC, PE, PS and

phosphatidylinositol (PI) are synthesized on the cytoplasmic side of endoplasmic reticulum [12]. However, lipid translocators distribute newly synthesized lipids across the plasma membrane and prevent lipid accumulation on the one side of the membrane. These translocators play a key role in the maintenance of membrane asymmetry. In addition, RBC membrane contain the energy-independent, but calcium-dependent flippases called scramblases. They non-selectively transport lipids on both sides of the membrane. In physiological conditions, flippases move PS from the outer to the inner monolayer of plasma membrane. The internalization of PS is necessary for the maintenance of normal RBC function. When the cytoplasmic level of calcium ions increases, the scramblase facilitates the transport of some amino phospholipids, especially PS from the inner to the outer monolayer of the membrane. In the presence of high intracellular calcium concentration, scramblase behaves as ‘’an open door’’ for lipids, which diffuse along the concentration gradient [13]. The translocation of PS on the outer monolayer activates macrophages and results in phagocytosis of erythrocytes. Macrophages have PS receptors, which readily bind to PS on the surface of RBCs. Thus, PS externalization on the surface of the RBC membrane is considered a senescence marker and such cells are eliminated by the reticuloendothelial system (RES) [14].

The cytoskeleton of RBCs is located under the lipid bilayer and consists of several proteins (Figure 1). These proteins are connected to each other and form a special network, composed of spectrin ankyrin, actin, protein 4.1, adducin, dematin, tropomyosin and tropomodulin [15-17]. Cytoskeletal proteins interact with the lipid bilayer and maintain membrane integrity. The attachment between cytoskeleton and membrane proteins determines the cell shape and flexibility. Spectrin is the most dominant protein of the cytoskeleton. It has two subunits, namely alpha- and beta-spectrin. These subunits are twisted together and form a single tetramer. Actin connects to the tails of spectrin tetramers forming several polygons. Actin-spectrin connection is facilitated by the protein 4.1 and adducin. Actin filaments interact with tropomyosin and tropomodulin and together form microfilaments. Dematin (protein 4.9) binds actin and makes a bundle of actin filamentous. Finally, interaction between the proteins mentioned above, forms an important meshwork, which is fixed to the lipid bilayer by ankyrin. Ankyrin binds transmembrane protein band 3. The connection between ankyrin and band 3 is strengthened by protein 4.2. In addition, spectrin meshwork connects to another transmembrane protein glycophorin C. The protein 4.1 stabilizes Spectrin-glycophorin C connection and the attachment of cytoskeleton meshwork to lipid bilayer. This meshwork is

anchored at several sites of the membrane that contributes to the stability of the RBC membrane [4, 18]. RBC cytoskeleton plays an important role in erythrocyte physiology, as several inherited disorders of RBC are often caused by inherited deficiencies of cytoskeleton proteins [4, 19].

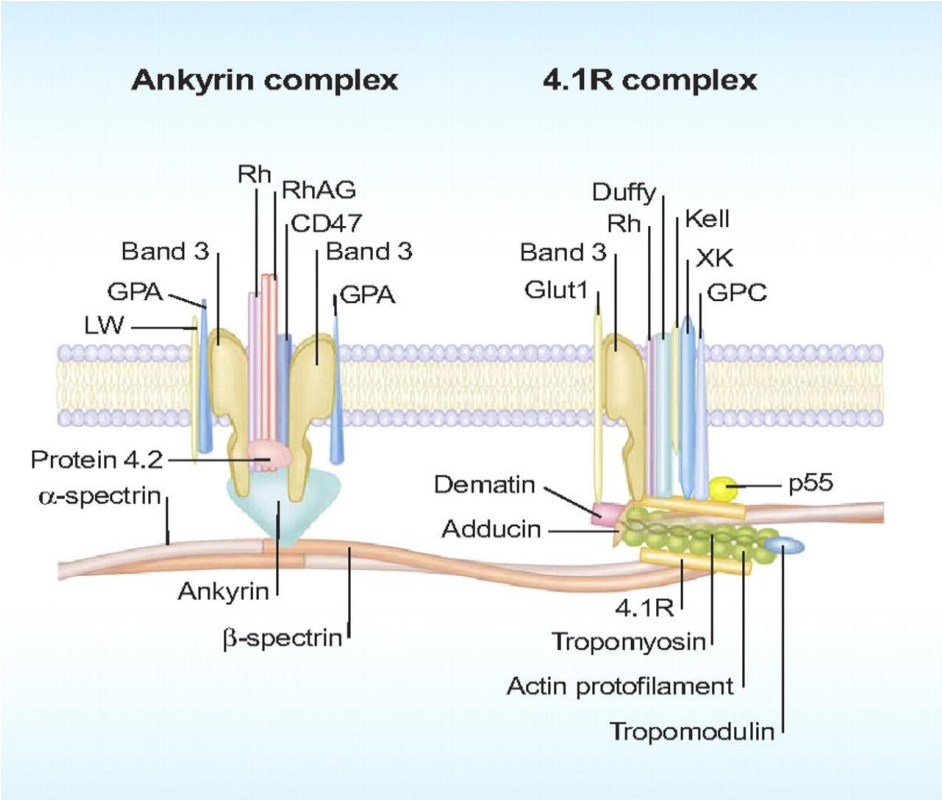


Figure 1: Protein Architecture of the erythrocyte membrane [20].

There are three major integral proteins in human RBC; band 3, glycophorin, Aquaphorin [21]. Band 3 is the most important transmembrane protein. It mediates anion transport and is termed anion exchange protein. Band 3 consists of N-terminal cytoplasmic and C-terminal membrane domains. Membrane domain is responsible for the exchange of bicarbonate and chloride across the RBC membrane. This transmembrane protein is actively involved in maintaining the integrity of the membrane and preventing RBC membrane surface loss [22].

Human genes encode the following glycophorin proteins A, B. C, E. They are rich in siliac acid. The acid gives RBC hydrophilic-charged coats and inhibits erythrocyte adhesion to another erythrocyte, or to the endothelium of blood vessel [23].

Aquaporin belong to a large family of major intrinsic proteins (MIP). They form selective pores in red cell membrane for water transport. These pores are completely impermeable to charged species such as protons and contribute to the maintenance of osmotic equilibrium of RBCs [21].

1.1.2. Hemoglobin

Hemoglobin is iron-containing, cytoplasmic organelle of erythrocyte and plays a major role in gaseous transport. It delivers oxygen from the lungs to the tissues and binds carbon dioxide in periphery. CO_2 is a metabolically produced gas, which is eliminated during passage through the lungs. Hemoglobin synthesis is a complex process. It begins within an immature erythrocyte in the bone marrow and requires three major components: Globin chains, protoporphyrin, and iron. Protoporphyrin synthesis comprises seven reactions. On the final step, one ferrous iron ($Fe2^{+}$) is added to protoporphyrin ring to form heme [24].The majority of adult hemoglobin is hemoglobin A (Hb A). This tetramer is made up of two alfa (α) and two beta (β) chains. Each chain contains one heme group, which binds oxygen. Normal oxygen transport requires a soluble state of hemoglobin. Oxidative stress, alterations in pH and physiological ion concentration affect the solubility of hemoglobin and cause its polymerization and precipitation that disrupts hemoglobin oxygen transport. Hemoglobin-oxygen affinity and correspondingly oxygen delivery to the tissue is regulated by 2, 3-diphosphoglycerate (2,3-DPG), synthetized via glycolysis in RBCs [19].

1.2. RBC metabolism

The RBC is a unique cell, with its simple but active metabolic pathway. It possesses no organelles, where the energy is produced. Glycolysis is the major energy producing pathway. It occurs in the cytoplasm of RBC. All parameters of cell such as: cell membrane stability, intracellular hemoglobin solubility, cell function and survival depend on the level of glucose inside of an erythrocyte and on the rate of glycolysis. There are four glucose-supported metabolic pathways in human erythrocyte: Embden-Meyerhoff, Luebering-Rapoport pathway, methemoglobin reductase pathway and pentose phosphate pathway (PPP) [25].

1.2.1. Embden-Meyerhoff pathway (Glycolysis)

Embden-Meyerhoff pathway is another name for glycolysis in RBC. This is a non-oxidative pathway, which is responsible for glucose catabolism and ATP production (Figure 2). Glycolysis begins with the catabolism of one molecule glucose, where six-carbon glucose is converted into two molecules of the three-carbon pyruvic acid. This reaction generates two molecules of ATP. As RBC does not possess mitochondria, it is not able to produce high energy phosphate via Krebs cycle. Thus, nearly 100% of ATP is produced through the activation of the Embden-Meyerhoff pathway. ATP is necessary for the normal functioning of the membrane to maintain cell shape, flexibility and viability. Lack of energy caused by deficiencies of glycolytic enzymes is associated with decreased RBC life span[26].

Glucose easily permeates RBC membrane [27]. The first step of glycolysis begins with phosphorylation of glucose by the enzyme hexokinase and forms glucose-6-phosphate [28, 29]. The reaction consumes 1 molecule of ATP. Hexokinase is a rate-limited enzyme and has a decreased in *vitro* function. [30]. High concentration of glucose-6-phosphate can cause the inhibition of hexokinase [31]. Moreover, the oxidized form of glutathione (GSSG) also inhibits the activity of hexokinase [32]. On the second step of glycolysis, glucose-6-phosphate undergoes the isomerization into fructose-6-phosphate that is catalyzed by glucose 6-phosphate isomerase. This enzyme has a higher activity in human reticulocyte compared to mature RBC[28]. On the third step, the rate-limited enzyme phosphofructokinase phosphorylates fructose-6-phosphate to yield fructose 1, 6-bisphosphate. This reaction also consumes 1 molecule of ATP. Subsequently, fructose 1, 6 bisphosphate is reversibly converted into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate by aldolase. From each molecule of fructose 1, 6 bisphosphate are yielded two molecules of glyceraldehyde-3-phosphate [29]. On the next step of glycolysis, each molecule of glyceraldehyde-3-phosphate undergoes first oxidation by glyceraldehyde 3-phosphate dehydrogenase, then phosphorylation to yield two molecules of 1, 3 bisphosphogycerate and 2 molecules of NADH, a reduced from of oxidized NAD^+ [29]. Glyceraldehyde 3-phosphate dehydrogenase deficiency in RBC is associated with hemolytic anemias [33]. The enzyme phosphoglycerate kinase in human RBC transfers the high energy phosphoryl group from the carboxyl group of 1, 3-bisphosphoglycerate to adenosine diphosphate (ADP) and produces two molecules of ATP and two molecules of 3-phosphoglycerate. On the next step, the enzyme monophosphoglycerate mutase converts 3-phosphoglycerate into 2-phosphoglycerate, which is dehydrated by enolase to yield phosphoenolpyruvate. Subsequently, the phosphoryl group of phosphoenolpyruvate is transferred to ADP by pyruvate kinase that results in the

formation of two molecules of ATP and pyruvate [29]. The end step of glycolysis is the production of lactate from pyruvate by lactate dehydrogenase [32]. Magnesium is required as a cofactor for most of the reactions of glycolysis cycle.

Glycolysis is regulated by the interaction of glycolytic enzymes with transmembrane protein band 3. Glycolytic enzymes: phosphofructokinase, aldolase, and glyceraldehyde-3-phosphate dehydrogenase directly bind N-terminus of transmembrane protein Band 3 and results in inactivation of enzymes [34]. Moreover, the interaction between band 3 and glycolytic enzymes depends on intracellular oxygen concentration. Deoxyhemoglobin has higher affinity to glycolytic enzyme binding site on band 3. It binds Band 3 stronger than glycolytic enzymes, whereas, Band 3 has much lower affinity for oxyhemoglobin [34, 35]. Thus, glycolysis rate in RBC is higher during hypoxia [36].

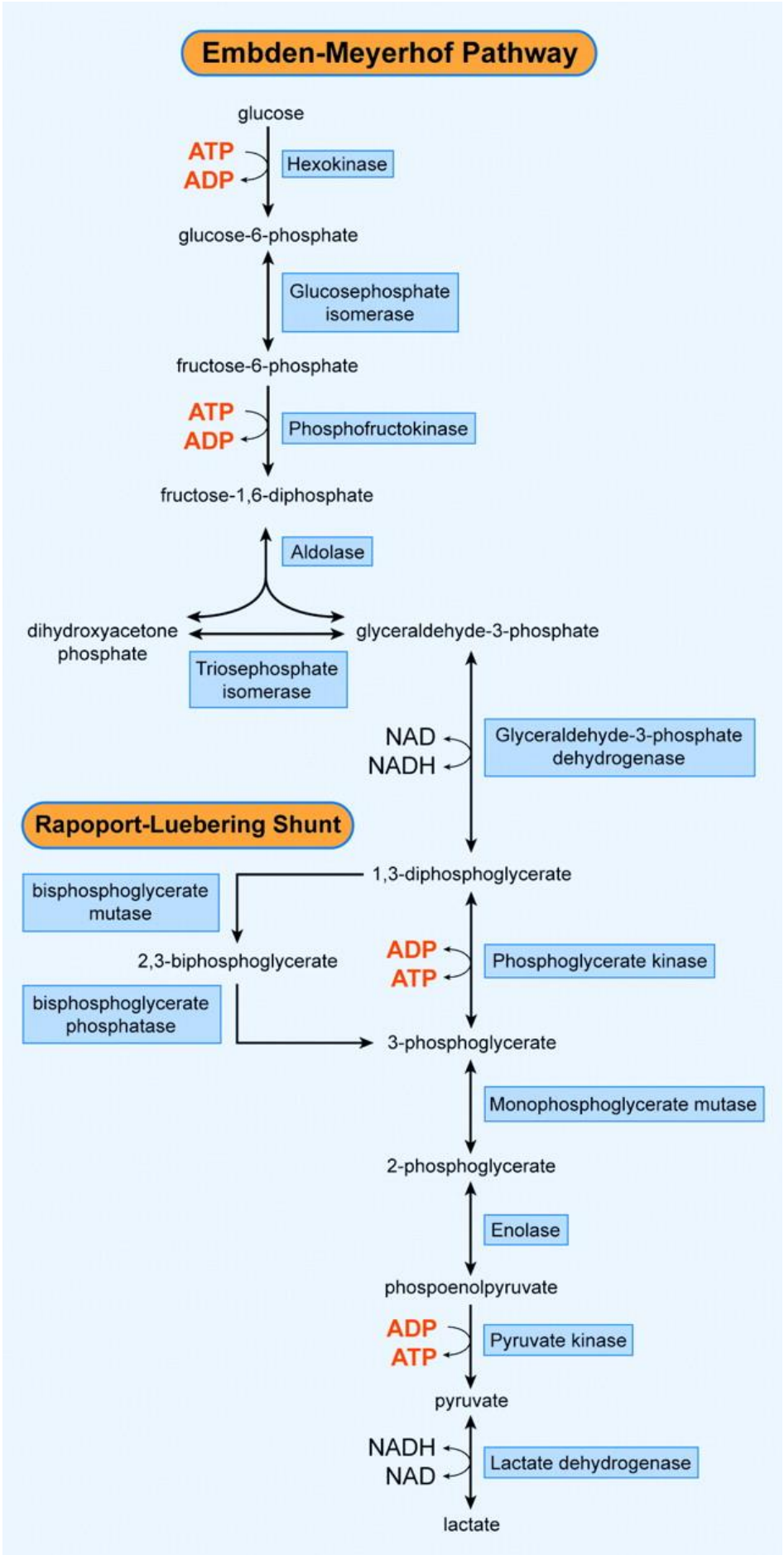

Embden-Meyerhof Pathway
glucose
ATP
ADP
Hexokinase
glucose-6-phosphate
Glucosephosphate isomerase
fructose-6-phosphate
ATP
ADP
Phosphofructokinase
fructose-1,6-diphosphate
Aldolase
dihydroxyacetone phosphate
glyceraldehyde-3-phosphate
Triosephosphate isomerase
NAD
NADH
Glyceraldehyde-3-phosphate dehydrogenase
Rapoport-Luebering Shunt
1,3-diphosphoglycerate
bisphosphoglycerate mutase
2,3-biphosphoglycerate
ADP
ATP
Phosphoglycerate kinase
bisphosphoglycerate phosphatase
3-phosphoglycerate
Monophosphoglycerate mutase
2-phosphoglycerate
Enolase
phospoenolpyruvate
ADP
ATP
Pyruvate kinase
pyruvate
NADH
NAD
Lactate dehydrogenase
lactate

Figure 2: Interaction between Embden-Meyerhoff and Luebering-Rapoport pathways [37].

1.2.2. Luebering-Rapoport pathway

2,3-DPG in mature RBC is generated by the Luebering-Rapoport pathway. It is a branch of anaerobic glycolysis and depends on the activity of the pH-sensitive enzyme phosphofructokinase. 2,3-DPG molecule regulates the oxygen release from hemoglobin and delivery to tissues [25]. 1,3-biphosphoglycerate is produced through glycolysis and enters to Luebering-Rapoport shunt (Figure 2). It is converted into 2,3-DPG by the enzyme bisphosphoglycerate mutase. Finally, 2,3-DPG is hydrolyzed by bisphosphoglycerate phosphatase. The product of this reaction is 3-phosphsglycerate, which returns to glycolysis cycle. Bisphosphoglycerate mutase is pH dependent enzyme and is easily inhibited in low pH [38].

1.2.2.1. 2, 3 Diphosphoglycerate

The 2,3-DPG molecule is a major regulator for oxygen transport to tissues, as oxygen binding capacity of hemoglobin depends on its intracellular concentration. At neutral pH and at the physiological concentrations of salts, 2,3-DPG binds to beta subunits in a molar ration1:1 in deoxyhemoglobin. One molecule of 2,3-DPG inserts into the cavity between hemoglobin beta subunits and results in low oxygen affinity of hemoglobin. During oxygenation, the distance between alfa subunits of hemoglobin increases and results in conformational changes of hemoglobin. The cavity between the beta subunits narrows that makes 2,3-DPG unable to insert into the cavity [39]. 2,3-DPG also facilitates the release of oxygen from hemoglobin. Released oxygen is delivered to tissues and consumed for cellular metabolism. RBCs are not always able to maintain the 2,3-DPG level within the normal range. Lower concentration of 2,3-DPG than the physiological range significantly reduces oxygen delivery to the tissues. The amount of 2,3-DPG depends on several factors, including the amount of its precursor 1,3 diphosphoglycerate, intracellular pH and the rate of its hydrolysis. The 2,3-DPG level is increased at high pH due to the activation of major enzyme bisphosphoglycerate mutase, while low pH inhibits this enzyme and results in reduction of 2, 3-DPG concentration. [38, 40] Furthermore, the enhancement of ADP and 3 phosphoglycerate synthesis is associated with a low level of 2, 3-DPG [40]. This unique molecule weakly binds to fetal hemoglobin, which has higher affinity for oxygen compared to adult hemoglobin. During RBC storage 2,

3DPG and ATP concentrations dramatically decrease, resulting in high affinity of hemoglobin for oxygen. Supplementation of inosine can restore ATP and 2,3-DPG levels and reduce hemoglobin affinity for oxygen [41, 42].

1.2.3. Methemoglobin reductase pathway

This pathway utilizes NADH generated from glycolysis and prevents Fe ions in heme group from oxidation. NADH is a major cofactor for methemoglobin reductase, which reduces ferric irons into ferrous form [43]. A decreased level of methemoglobin in circulation is maintained by methemoglobin reductase. Methemoglobin is unable to bind oxygen reversibly for a long time that affects oxygen transport and supply to the tissues. Methemoglobin concentration also increases during acute anemia. This may stimulate adaptive systems to hypoxia and augment nitric oxide production [44]. Under pathological conditions, up to 40% of hemoglobin is oxidized within the RBCs, while 60% is maintained in reduced form due to the other nonspecific reductants, which can prevent hemoglobin oxidation. There are two different forms of methemoglobin reductase deficiency; inherited and acquired [45]. Methemoglobin reductase strongly binds to the inner surface of plasma membrane and creates a bond, which is digested by cathepsin D or other detergents [46].

1.2.4. Pentose phosphate pathway

PPP is an aerobic pathway. It utilizes only 5-10% of glucose [40]. This pathway combines oxidative metabolism of glucose and reduction of the oxidized form of nicotinamide adenine dinucleotide phosphate ($NADP^+$). The first substrate for PPP is Glucose-6 phosphate. It is converted into 6- phosphogluconolactone by glucose 6-phosphate dehydrogenase. One molecule of $NADP^+$ is reduced through this reaction. On the next step, gluconolactonase hydrolyses 6- phosphogluconolactone and forms 6-phophogluconate, which is first oxidized, then loses carboxyl group by 6-phosphogluconate dehydrogenase. This reaction generates a second molecule of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and ribulose 5-phosphate, a final product of the oxidative phase of PPP. Ribulose 5-phosphate can be converted either into its aldose isomer ribose 5-phosphate, or xylulose 5–phosphate by phosphopentose isomerase or ribulose 5-phosphate epimerase, respectively [29]. NADPH, is used as a cofactor for reduction of GSSG [40]. GSH counteracts oxidative damage and neutralizes ROS that prevents hemoglobin from oxidation and globin chains from denaturation [47]

Glucose-6-phosphate dehydrogenase (G6PD) plays an important role in the activation of PPP and in NADPH synthesis [48]. This enzyme deficiency is presented in a large part of the population and affects nearly 400 million people [19]. G6PD deficiency is the most common enzyme abnormality in RBC and occurs in different forms [38]. The enzyme deficiency in neonates is mostly associated with jaundice, and acute hemolytic anemia [49], whereas the adults with an atypical course have mild hemolytic anemia.

1.2.5. Glutathione

RBCs are constantly exposed to highly reactive radicals due to the cellular gas exchange. They react with several oxidants such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) and are readily oxidized. Glutathione is believed to play a major protective role against oxidation. It neutralizes oxidants and prevents RBC damage. When the exposure exceeds the scavenging capacity of RBC antioxidant defense mechanisms, the oxidants accumulate within RBC causing progressive damage and senescence of cells [50].

Glutathione synthesis comprises 2 steps. Glutamate and cysteine are required to form γ-glutamylcysteine, which further reacts with glycine and forms glutathione. The first step is catalyzes by γ-glutamylcysteine synthetase and the second one by glutathione synthetase, respectively. Both of the reactions are ATP-dependent [51].

Glutathione peroxidase is the major enzyme involved in antioxidant reactions. GSH is converted into GSSG after the reduction of hydrogen peroxide and lipid peroxide. Glutathione reductase uses NADPH as a cofactor for reducing of GSSG [52]. The ratio of oxidized and reduced glutathione affects glutathione redox capacity. The activities of major enzymes such as: glutathione reductase and glutathione peroxidase and the amount of total glutathione decrease in RBCs of old human [53].

1.3. RBC senescence

The maximum life-span of a circulating human erythrocyte is 120±4 days [54]. The mature RBCs are unable to synthetize enzymes because of the lack of nucleus. During RBC aging the catalytic capacity of major glycolytic enzymes such as hexokinase, aldolase, and pyruvate kinase is exhausted and results in impairment of glycolysis. Moreover, their enzymatic

response to exogenous metabolic stimuli such as the supplementation of phosphate, or methylene blue is lower in senescent RBC compared with younger one [55]. Besides, impaired glycolysis, redox hemostasis is dysregulated causing increased oxidative stress. It affects oxygen delivery to the tissue due to the reduced cellular deformability and contributes to RBCs' senescence [56].

RBCs' senescence includes structural and biochemical changes within the cell and combines multiple signaling pathways. It is associated with low activity of major antioxidant enzymes, as well as decreased production of reduced glutathione [53]. The decreased amount of total glutathione in RBC leads to oxidation of transmembrane protein Band 3 [57], which strongly connects to cytoskeleton of RBC membrane [18]. Oxidation alters the conformation of Band 3. It binds to denatured hemoglobin and promotes the formation of senescent cell antigen (SCA) [57] [58].

Under physiological conditions, circulating RBCs lose 20% of their hemoglobin content during their life span [59]. This process is termed hemoglobin vesiculation. It is accelerated in the second half of an RBC's life [60]. Hemoglobin vesiculation and disruption of lipid asymmetry contribute to apoptosis like death (eryptosis). This is a suicidal death of mature RBCs, It is stimulated by the complex cellular machinery such as calcium influx, caspases, complement system, calpain, energy depletion and oxidative stress. The removal of extracellular Cl^- contributes to Ca^{2+} leakage into the erythrocyte and results in the externalization of PS on the outer leaflet of the membrane [61]. Dysregulation of calcium homeostasis is a main signal for eryptosis, caused by activation of calcium channels [62, 63]. It enhances calcium uptake from plasma and activates caspases, Ca-dependent cysteine proteases-calpains and scramblase. All this promotes externalization of phosphatidylserine and leads to phagocytosis of RBCs by RES [64]. This process is involved in many erythrocyte disorders with hemoglobin or membrane abnormalities [62].

1.4. The red blood cell storage lesion

The transfused RBCs have a short half-life with most common storage period of 42 day [65]. During storage, RBCs undergo numerous biochemical and structural alterations, which is

termed storage lesions [66]. They have a negative influence on RBC quality. Almost 25% of transfused RBC are removed in recipient circulation within 24 hours. Moreover, the percentage of removed RBCs correlates with storage time [67]. This means that one unit of packed RBC is eliminated by recipient RES after four units of RBCs transfusion. The aging process within packed RBC depends on storage conditions, which are not physiological. High oxidative stress within the packed RBCs exhausts cellular glutathione and accelerates the aging of RBCs. Another factor, that can influence the quality of RBC during storage, is the percentage of already aged RBCs in donor circulation at the time of blood donation. These senescent RBCs only contribute to the formation of storage lesions. Furthermore, RBCs senescence *in vivo*, is also associated with formation of cell lesions, which are rapidly eliminated by RES [68]. Such a system does not exist in packed RBC and results in accumulation of storage lesions.

RBCs are mostly stored in acid citrate dextrose (ACD), or citrate-phosphate-dextrose (CPD) anticoagulant solutions, where glucose as dextrose is utilized for the RBC metabolism. Dextrose catabolism generates lactate. Its accumulation within packed RBCs causes the reduction in intracellular pH [69]. This process inhibits major glycolytic enzyme phosphofructokinase and causes impairment of glycolysis pathway [70]. Besides the glycolytic enzymes, 2,3-DPG is also a pH-dependent parameter. It declines rapidly at the end of first week due to the inhibition of bisphosphoglycerate mutase at low pH [38]. Moreover, intracellular acidification contributes to the activation of 2,3 bisphosphoglycerate phosphatase. This enzyme dephosphorylates 2,3-DPG. However, the reduction in 2,3-DPG level is a reversible event and it is restored within 24 hours after transfusion [71]. During the storage, RBCs gradually lose ATP, which correlates with RBC *in vivo* survival [72]. Furthermore, morphological changes in packed RBC is associated with ATP depletion [73]. Shape changes of stored RBC from discoid to reversibly-deformed echinocyte or irreversibly-deformed spheroechinocyte in response to reduction at the ATP level are already well-known (Figure 3). Adenosine supplementation to stored RBCs restored not only the ATP level, but also the biconcave shape of RBCs [74]. ATP production also depends on the activity of the major transmembrane protein band 3. It strongly binds deoxyhemoglobin. This contributes to the release and activation of glycolytic enzymes [36]. During storage, RBC progressively lose the oxygen-depended metabolic modulation, due to changes in membrane proteins, in particular Band 3 [75]. Band 3 undergoes progressive oxidation and aggregation. This affects the connection between Band 3 and hemoglobin and results the inhibition of glycolytic

enzymes as well. Hemoglobin, bound to membrane protein, undergoes autoxidation. The auto-oxidized hemoglobin is inaccessible for the cytosolic RBC antioxidant system. This process becomes more profound under hypoxic conditions, when hemoglobin is partially oxygenated [34, 35]. Auto-oxidized hemoglobin readily undergoes denaturation and loses its normal function. Under the storage conditions, hemoglobin - Band 3 connection contributes to the oxidation of other membrane proteins and results in acceleration of RBC senescence [76]. Besides Band 3, other cytoskeleton proteins undergo oxidative modification such as protein 4.1, 4.2 and spectrin [77, 78].Their degradation distorts the membrane structure.

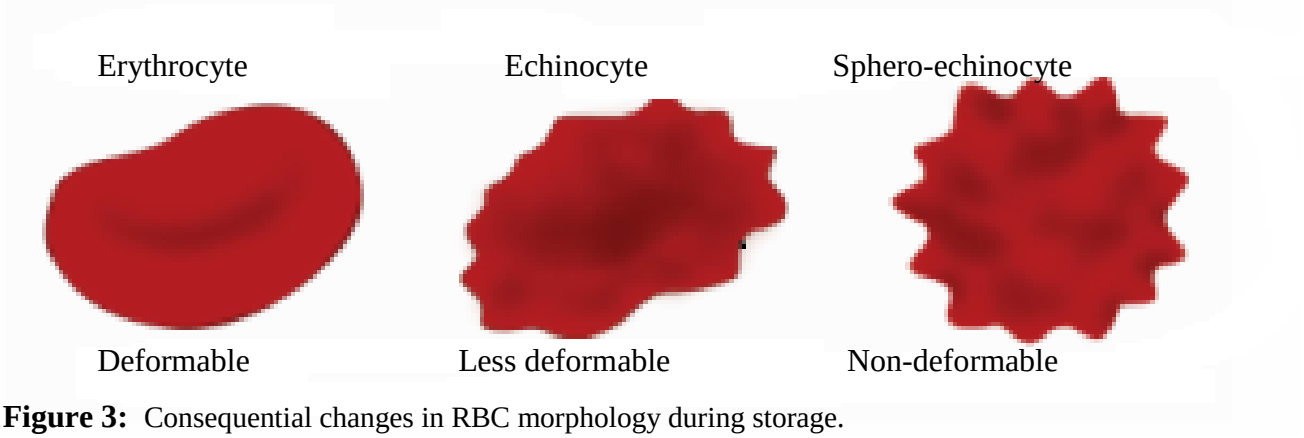


Figure 3: Consequential changes in RBC morphology during storage.

RBC irreversibly lose membrane due to microvesiculation (hemoglobin-, lipid- and protein-containing vesicles) and results in morphological changes of stored RBC [79]. Loss of RBC membrane contributes to reduction in cell deformability and an increase in osmotic fragility [80, 81]. The micro-vesicles contain only Band 3 from transmembrane proteins[82]. Membrane vesiculation also occurs during RBC senescence *in vivo* [60]. However, these vesicles are composed of nearly 65% denatured hemoglobin and differ considerably from vesicles in packed RBC [82], where the vesicles are composed of about 50% of extracellular hemoglobin [83]. In stored RBCs, hemoglobin auto-oxidation generates superoxide. It is converted into hydrogen peroxide via dismutation. Hydrogen peroxide is not completely neutralized in RBCs. It reacts with ferrous and ferric hemoglobin and oxidizes them to ferrylHb and oxyferrylHb respectively. Both forms of oxidized hemoglobin are involved in oxidative chain reaction and produce an additional source for oxidative stress such as: free iron, free radicals and degradation products of heme. They further react with hydrogen peroxide and aggravate oxidative stress. Heme and oxiferrylHb activates inflammatory responses *in vivo* through major transcription factor NF-κB signaling [84]. Nitric oxide (NO) homeostasis is also dysregulated during RBC storage. NO is the endothelium-derived relaxing

factor (EDRF), released from endothelial cells. NO plays an important role in controlling blood flow by basal vasodilation of the blood vessels [85-87]. Human RBCs synthetize NO in plasma membrane and cytoplasm. NO controls membrane deformability of RBC and inhibits platelet activation [88]. Oxygenated hemoglobin reacts with NO and forms methemoglobin (deoxygenated hemoglobin) and nitrate [89]. Interestingly, consumption of NO by extracellular hemoglobin is dramatically greater compared to hemoglobin in RBCs [90]. During RBC storage, extracellular hemoglobin gradually increases due to hemolysis and microvesicles. These microvesicles remain in packed RBCs during the storage period and scavenge NO as free hemoglobin does. Transfusion of RBC unit contains a large amount of extracellular hemoglobin (free and vesicle), causing a reduction in the nitric oxide level in recipient. After transfusion of such an RBC unit, extracellular free hemoglobin is removed by haptoglobin, but hemoglobin-containing vesicles still remain and reduce the NO level [91]. This process contributes to transfusion-related adverse reactions.

Normally, phospholipids are asymmetrically distributed in RBC membrane. This asymmetry is maintained by amino phospholipid translocases [7, 9, 92]. During RBCs storage, the activity of translocases significantly decreases in parallel with ATP depletion and is partially restored by ATP regeneration [93]. Lipid redistribution across the membrane of stored RBC is reduced in low pH [94]. Moreover, stress-induced phosphatidylserine exposure increases during RBC storage and results in RBC senescence [95].

RBC storage also has a negative effect on the transport of sodium and potassium ions. Their transport are regulated by the transmembrane protein Na+/K+ ATPase. It pumps sodium ions out of the cell and potassium ions into the cell against their concentration gradients. During RBCs storage at 4°C, Na+/K+ ATPase is inhibited resulting in an elevation of intracellular sodium, while the potassium level decreases [96]. However, this is a reversible event and is restored after transfusion. It takes 24 hours for sodium and about 4 days for potassium ions to be restored [97]. In addition, storage-related elevation of intracellular sodium contributes to water influx to cytosol, that increases the mean corpuscular value of RBCs [98].

RBC storage lesions affects calcium homeostasis. The permeability of calcium ions increases from outside to inside of RBCs inducing their senescence [99]. High intracellular calcium level activates calcium-dependent potassium channels and results in loss of potassium and chlorine ions. This process occurs simultaneously with flux of intracellular water and

causes RBC shrinkage. High intracellular calcium level activates calcium-dependent cysteine protease-calpain and contributes to eryptosis [100].

Concentration of plasma fibrinogen of packed RBC stored in additive solution decreases, while the aggregability of RBCs increases with the storage period. However, this phenomena does not occur if the additive solution is replaced with autologous plasma [101].

RBC storage lesions are complex changes, which contribute to RBC senescence and limit storage time. Oxidative stress plays a dominant role in all alterations mentioned above. Impairment of glutathione homeostasis during RBC storage results in accumulation of ROS and aggravation of oxidative stress. Storage-associated increase in the malondialdehyde (MDA) level is a good indicator for activation of oxidative stress, which induces oxidative injury of RBC and causes their senescence [102]. The pathological alterations within the packed RBCs begins after 4^{th} days of storage as some parameters such as: ATP, 2,3-DPG, reduced glutathione are gradually consumed. Permanent elevation in oxidized glutathione begins on the 14^{th} day of RBC storage. This indicates dysregulation of PPP and reduced production of NADPH caused by high oxidative stress [103]. During RBCs storage, extracellular glucose, mannitol and adenine levels are gradually decreased. They enter RBC through the membrane and are utilized during the storage period [104]. Accumulation of glycolysis products such as pyruvate inhibits glycolytic enzymes and reduces glycolysis rate as well. Metabolomics application clearly showed that the accumulation of oxidized lipids in packed RBCs is caused by dysregulation of the redox system [1]. Furthermore, RBC storage is associated with elevation of intracellular homocysteine [105].

1.5. A short history of transfusion medicine

The history of blood transfusion goes back several decades. The first documented transfusion of human blood was performed by James Blundell on 22^{nd} December 1818. He transfused 14 ounces of blood to the husband of his niece, a 35-year-old patient suffering from gastric carcinoma. This case was described as‘’scirrhrosity of the phylorus’’ by the author. The symptoms, associated with the disease, were subsided after blood transfusion, but the patient died 56 hours later. James Blundell was a physician, psychologist and an outstanding obstetrician in his days. He was a pioneer who realized the importance of blood in the treatment of various diseases and transfused human blood to another human. Between the years 1818 and 1829 ten transfusion procedures of human blood were performed by him and

his colleagues, but only four procedures were beneficial to the patients. The first successful transfusion was received a woman suffering from postpartum hemorrhage. The blood donor was the husband of the patient. Four ounces of blood was drawn from his arm and transfused to his wife, whose health state was significantly improved after the blood transfusion. This case was published in the journal ‘’Lancet’’ in 1829. The career of James Blundell is associated not only with the development of transfusion therapy, but also with the invention of various instruments for transfusion medicine [106]. Despite some success in blood transfusion, it remained a hazardous procedure due to a big risk of mortality caused by blood clotting. Platelets, ‘’spherules’’ smaller than erythrocytes were firstly described by the German anatomist Max Schultze, while Giulio Bizzozero studied their aggregation ability in circulation. He observed adhesion and aggregation of platelets in injured areas of the blood vessel that formed blood clots [106]. Besides blood clotting, the blood volume, transfused from donor to recipient was also a disputable issue. Dr. F. W. Pavy first advised to use phosphate of soda as an anticoagulant for transfusion purposes. In 1884 “phosphate blood” was for the first time transfused to several patients [107]. That was in the ‘pre-Landsteiner’ period when the blood group system was still unknown.

A considerable success in the history of blood transfusion was the discovery of blood types, when Karl Landsteiner an assistant at a pathological-anatomical institute in Vienna found individual differences between men’s blood. He noticed clumping of the blood cells after adding sera in an experiment and examining this phenomenon in detail. In the experiment, he identified three types of blood and grouped them according the first letters of alphabet. A, B, C [108]. The rarest group of blood type AB was discovered by Decastello and Sturini [109]. Independently from Landsteiner, four types of blood were found by other researchers, but they were termed I, II, III and IV [110]. Interestingly, Rhesus factor was discovered accidentally by H. R. Peters and A.S Wiener [109]. They transfused the blood of their own group to patients, but they developed severe hemolytic reactions and died. Investigators noticed the clumping of donor RBCs after addition patients’ sera. It contained the agglutinins, which had not been described before. The newly discovered blood factor was named Rhesus (Rh) factor. Donors’ Rhesus factor reacted with anti-Rh antibodies of the patients after the blood transfusion. Blood donors were Rh positive and all recipients were Rh negative.

Next successful step in blood transfusion history was the foundation of the first blood donor service by Percy Oliver, a secretary of British Red Cross in 1921[111]. At that time, the blood

was donated by the relatives or friends of the patients. The first world blood bank was established by a physician Oswald Hope Robertson. He rejected the arterial-vein anastomosis and decided to store the donated blood on ice in a glass bottle for up to 26 days. The stored blood was transfused to a solder after storage successfully. It was the first clinical case [111].

During the two world wars, blood transfusion became the most essential and common tool to treat wounded soldiers in battle, that significantly increased the demand on blood. However, the transport of the large quantities of whole blood from the donor center to battle area was quite difficult. This problem was solved by isolation of blood components. First, plasma was isolated from whole blood by American professor Edwin Cohn. He isolated and collected four major plasma protein fractions (fraction I- fibrinogen, fractions II and III –globulins, fraction IV- albumins) [110].

Each plasma fraction was successfully used in treating patients with different diseases. Later, isolation of blood components was actively used not only in the military, but also for clinical purposes. However, this procedure required more time and personnel in clinics. The first blood cell separator was invented by the professor Edwin Cohn that promoted the establishment of blood component therapy in transfusion medicine. [110].

Storage of blood components required an anticoagulant. Scientists started looking for a suitable blood anticoagulant as phosphate of soda did not have a good anticoagulant properties. The anticoagulant had to be safe for humans. For this purpose, several substances were tested, including sodium bicarbonate, ammonium oxalate, arsphenamine, sodium iodide, sodium sulfate, and hirudin [112]. Sodium bicarbonate as an anticoagulant was recommended by Neudorfer (1860). Tzanck, one of the founders of the International Society of Blood Transfusion, described the similar anticoagulant properties of sulfarsenol and sodium citrate (1922). Brines successfully used ammonium oxalate and arsphenamine as anticoagulants (1926). Sodium sulfate was recommended by MacCraken and Abelman (1926) [112]. Surprisingly, 1% concentration of sodium citrate had been already used as an anticoagulant for laboratory investigations of blood and its *in vivo* toxicity was a well-known fact [110]. Later, Richard Lewinsohn discovered that sodium citrate in a low concentration effectively maintained its anticoagulant properties and was not harmful to human health. Following long-term experiments, he was the first to describe the anticoagulant properties of 0. 2% sodium

citrate, which was not toxic either [110]. Anyway, 0. 2% sodium citrate was accepted in blood transfusion 10 years later after the publication of his findings.

Investigations showed that citrate was not sufficient for successful blood conservation. In the following years, it was suggested to add dextrose to the anticoagulant solution. Storage of blood cells was improved in citrate-dextrose anticoagulant. Dextrose decreased fragility of RBC *in vitro* and prolonged their shelf life by 2 weeks in rabbit model [113]. Citrate-dextrose anticoagulant solution became very popular and was tested by several authors. Later, phosphate was added to citrate-dextrose solution to still further improve blood conservation. A clinical study, conducted at Harvard Medical School and Peter Bent Brigham Hospital, showed that blood storage in CPD solution extended the storage time by up to 28 days [114]. Afterwards, CPD solution was accepted as an anticoagulant of choice.

Despite successful development of transfusion medicine, blood transfusion remained a risky procedure due to the febrile reactions caused by presence of leucocytes in the blood. In immunocompromised patients, granulocyte transfusion was associated with graft-versus-host disease, which was an unexpected complication. In 1962, T.J Greenwalt and his group invented a nylon fiber-containing filter and established a new method to prevent transfused blood from leucocyte contamination [115]. This method was used in a clinical study, where all blood units were filtered thereby preventing febrile reactions. The blood banks and transfusion medicine have been developing for more than 50 years. Within this period, many new and important events occurred. In this brief review, the major events are mentioned, which have brought about a fundamental change in transfusion medicine.

1.6. Human blood donation, collection and processing

Blood donation is routinely performed in blood banks. Whole blood donation is the most common type. However, blood components such as plasma, platelet, or red cells can be donated separately using apheresis. According the American or European guidelines 450-500 ± 10% ml of whole blood is collected from one donor [116-118]. The whole blood is collected in one plastic bag with an anticoagulant solution. The most common anticoagulant solution contains CPD to prevent blood clothing and maintain cellular function. In general, whole blood is usually separated into blood components such as: RBCs, platelets and plasma, as whole blood transfusion is very seldom indicated. First, the whole blood is centrifuged into primary collection packs. During centrifugation, RBCs are deposited at the bottom of the

plastic bag and the plasma remains on the top. Leukocytes and platelets are suspended in plasma over the RBCs. Afterwards, blood components are extracted into satellite bags, except RBCs. They remain in primary bag. The initial storage of whole blood depends on the type of blood component, which is produced from whole blood. For the production of RBCs, whole blood can be stored at 4°C for 48–72 hours before blood component separation. But, plasma should be produced from whole blood stored at 22°C for up to 24 hours [119]. The blood component can be collected from donors through direct apheresis. This method of separation is better as the rest of the blood is returned back to donor circulation without any loss.

All allogenic blood and blood products are leukoreduced to prevent post-transfusion complications caused by leukocytes [119]. This procedure is performed within 48 hours of donation with special leukocyte-depleting filter [119]. Filtration can remove nearly all leukocyte from packed RBC or fresh-frozen plasma (FFP), but this reduces 10-15% of the volume of whole blood.

Leukocyte depletion system is widely used almost in every country. According the standards, each platelet or RBC unit should contain less than 5×10^6 leukocytes after leukodepletion [120]. Leukocyte depletion prevents recipient from transfusion-related adverse reactions such as febrile non-hemolytic transfusion reaction, transmission of cytomegalovirus especially in patients, who have received stem cell or kidney transplantation and in neonates and children under one year of age [121]. Collected RBCs are commonly suspended with 100 ml of additive solution to achieve a hematocrit level of 50-70% and to maintain erythrocyte viability after storage. Processed red cells are termed packed RBCs. They are stored at 4± 2°C for a maximum of 35–49 days. Packed RBCs can be stored in their own plasma, but only for 28-35 days [119]. The combination of anticoagulant and additive solutions, type of plastic bag materials and any further processing step during RBC collection, significantly determine the shelf life of packed RBCs. Each RBC concentrate contains a small amount of plasma, ranging between 5 and 30 ml. The shelf life of packed RBCs depends on the hematocrit value of the donor and on centrifugal force during processing of whole blood [119].

1.7. Steady state of RBCs storage

Successful investigations have fundamentally changed medical treatment guidelines. However, blood transfusion remains an irreplaceable therapeutic intervention in medicine.

The RBC transfusion in clinical practice is indicated for the maintenance of adequate tissue oxygen delivery in patients with severe anemia of different origins [122, 123]. According the most widely used protocol, RBC concentrates are stored for up to 42 days at 4 ± 2 °C, in anticoagulant solutions [65]. However, the administration of ‘’older’’ RBC units is associated with the life-threatening complications such as: Allergic reactions, transfusion-related acute lung injury, non-immune hemolytic reactions, hyperkaliemia, or citrate toxicity [124]. Spinella et al. reported that stored RBC concentrate older than 14 days increased the risk of death of critically ill patients with trauma injuries [125]. A small retrospective study by Purdy and Tweeddale showed a rise of mortality in septic intensive care patients transfused with RBCs older than 16 days [126]. However, this finding was not proven by randomized prospective studies [127]. Nevertheless, adverse outcome of old blood transfusion remains controversial [128].

Storage of packed RBCs is associated with the formation of “storage lesions’’[66]. RBC storage lesions have been studied for decades, but storage-related pathways have not been completely identified. The quality, efficacy and safety of packed RBCs can be improved through a better understanding of the storage-related specific changes and the establishment of a better storage system.

The new era in blood transfusion has begun with the application of omics technology. It has opened up new possibilities in transfusion medicine. Two omics disciplines such as metabolomics and proteomics are widely used in investigations focused on RBC storage lesions [103]. In recent years, a large number of metabolites have been identified and quantified in packed RBCs [1, 104, 105]. Metabolomics data analysis has shown that some metabolites are depleted during the storage of RBCs, while others dramatically accumulate. On the basis of these findings, new additive solutions have been developed. They improved storage conditions of RBCs compared to well-known Saline-Adenine-Glucose-Mannitol (SAGM) additive solution [43, 129]. The improvement of additive manufacturing is one of important issues of transfusion medicine. New additives can improve storage of packed RBCs and reduce transfusion-related adverse reactions.

1.8. Past, present and future of RBC additive and anticoagulant solutions

1.8.1. An anticoagulant solution of choice: history of development of anticoagulants for RBC storage

Removal of plasma had a negative effect on RBCs' storage, but plasma was replaced with an additive solution. Development of additive manufacturing has significantly improved RBCs metabolism during storage and extend storage period. The influence of additive solutions on the length of storage period was reported by many authors [130, 131]. Moreover, the nutritional components and concentrations of each additive solution has determined the storage period of RBCs. The experiment conducted by Hess et al. was a good example of this. They have reported a successful storage of RBCs in 300 ml of EAS-76 version 6 experimental additive solution for up to 12 weeks with 78 ± 4 % of post-transfusion recovery (PTR) [130].

It was already mentioned in the short history of transfusion that glucose (dextrose) was the first additive nutrient in combination with ACD used at the beginning of the 20th century. The addition of dextrose protected RBCs against damage and reduced the cells' mechanical fragility. However, the pH of additive solution should be maintained in the range of 5.2 to 6.2 [132]. Dextrose-based solution requires acidification, to prevent caramelization of dextrose during the heat sterilization process [133]. Later, phosphate was identified to have beneficial effect on packed RBC and was added to the ACD solution. CPD was first tested in a clinical study by Gibson et al. [134]. A slow diffusional passage of phosphate across the gradient from inside to outside of stored RBCs caused intracellular phosphate depletion and a decrease in ATP production. The breakdown products of 2,3-DPG during the first two weeks was the main source of phosphate, which supported the synthesis of the new ATP molecules. From the second week, both 2,3-DPG and ATP concentration sharply dropped in parallel with the intracellular phosphate level. Supplementation of phosphate to RBC concentrates maintained its intracellular concentration and prevented early ATP depletion during RBC storage. Afterwards, packed RBC were stored in ACD or CPD anticoagulant solution for up to 21 days, with 75% and 79% of PTR [134].

In 1962, ACD solution was supplemented with adenine and inosine, which regenerated the ATP concentration and restored shape and viability of packed RBCs [73]. In the present study, a close relationship between ATP depletion and loss of RBC viability was found. Addition of adenine to anticoagulant solution prevented RBCs from early depletion of purine nucleotides. Concentrations of both adenine and adenosine concentrations were decreased in stored RBC due to deamination reaction. Furthermore, the supplementation of other purine

nucleosides regenerated the organic phosphates, which is a source for ATP synthesis and extended RBCs storage [135-137]. In 1968, CPD anticoagulant solution was modified by adding adenine (CPD-A1) [138]. The whole-blood samples were collected from 32 volunteers and stored with CPD-A1 anticoagulant solution for up to 35 days with 80.53 ± 6.44% of PTR at the end of storage. However, 19 units of RBCs (Hct about 80%), stored similarly, had 71.38±10.3% of PTR after 5 weeks of storage. CPD-A1 was widely accepted in U.S. and recommended for 35 day-storage of whole-blood or packed RBCs. The anticoagulant solutions (CPD, CPD-A1) preserved not only whole blood, but also its components without any remarkable pathological changes.

1.8.2. From the first experimental additive solution to PAGGS-M

Development of an appropriate anticoagulant solution could not solve all problems of successful RBCs storage. After plasma removal, RBC concentrates had higher viscosity and cellular energy resources were not sufficient for long-term storage. Plasma removal required the replacement with another solution in order to decrease hematocrit and improve storage conditions. The first attempts for developing an additive solution was reported in 1972. The blood was separately stored in modified CPD-ad-NaA (citrate-phosphate-dextrose-adenine-sodium-ascorbate) and BAGPM (bicarbonate-adenine-glucose-phosphate-mannitol) storage mediums. Addition of bicarbonate to additive solution inhibited the reduction of pH and prevented RBCs from metabolic deterioration caused by intracellular acidosis. Bicarbonate buffer could neutralized metabolically generated acid and increased 2,3-DPG production. The PTR of RBCs was greater than 70 % in BAGPM additive solution after 42 days and in CPD-ad-NaA after 28 days of storage [139, 140]. However, BAGPM additive solution was not accepted for routine storage of RBCs as each volume of RBCs needed a large volume of the additive solution and specific bag systems [133].

The first commercial additive solution SAG (Saline-adenine-Glucose) for packed RBCs was introduced in Europe by Högman and his research group in 1978 [141]. They used sodium chloride and developed an isotonic additive solution in order to maintain equal movement of water inside and outside of cell membrane and to prevent erythrocyte from shrinkage, or swelling. SAG was a protein-free medium and its viscosity was similar to that of whole blood. Packed RBCs were stored in 40 - 100 ml of SAG for up to 35 days. This additive solution improved storage conditions and extended the shelf life of packed RBCs with 83 ± 6.8% of

PTR. Each RBC pack contained 100 ml of SAG additive solution with 17 mg adenine to maintain a sufficient ATP level, 900 mg glucose for long-term regulation of cellular metabolism, and 880 mg of sodium chloride to prevent potential swelling or shrinkage of RBCs. SAG was recommended for five-week storage with CPD anticoagulant solution [142]. Four years later, mannitol was added to SAG additive solution that improved in vitro quality of RBCs [142]. Mannitol protected RBC membrane from mechanical damage during blood processing that prevented their spontaneous hemolysis. Moreover, it reduced RBCs lysis with 50% and extended their storage by 6 weeks [143]. SAGM (Saline-Adenine-Glucose-Mannitol) with pH 5.7 was a modification of SAG. This additive solution has been widely used not only in Europe but in the UK, Australia, New Zealand and Canada [144]. It had been used as an effective additive in Europe for several decades. However, SAGM has not been approved by the Food and Drug Administration (FDA) and was not used in the U.S.

During the development of additive manufacturing, SAGM additive solution was modified with supplementing it with guanosine. Guanosine was an alternative source of ribose-1-phosphate, which is required for production of adenosine monophosphate (AMP) from adenine. AMP was an essential molecule for ADP and ATP synthesis. Supplementation of guanosine increased not only the adenylate pool but, also guanylate one. The modified SAGM formulation was named phosphate–adenine–guanosine–glucose–saline–mannitol (PAGGS-M) storage solution. It was licensed for 42 days of storage for erythrocyte concentrate with pH 5.7. PAGGS-M was superior to SAGM due to the lower hemolysis rate after 49 days of storage [145].

1.8.3. History of additives manufacturing in U.S.

The first RBC additive solution ADSOL, later AS-1 (acronym of Additive solution) was developed in the U.S. in 1978 [142]. ADSOL and SAGM have the same nutritional composition, which differed only with regard to its concentrations of salt, sugar, adenine and mannitol. AS-1 contained 2.2 g dextrose (glucose), 27 mg adenine, 750 mg mannitol, and 900 mg of sodium chloride (134). In 1983, Heaton and colleagues reported on the preservation of erythrocyte concentrates in AS-1, with PTR greater than 75% after 49 days of storage [146]. This result was in compliance with FDA requirements (at least 70 % of PTR within 24 hours after RBCs transfusion). Concentrated additive solution AS-1 was superior to SAG and was approved by the FDA for 49 days of RBC storage in 1983. Surprisingly, the data published by

other authors did not confirm the results of Heaton and his research groups. FDA convened a meeting and resolved conflicts among the investigators. At that meeting, the researchers presented their own results of RBC storage in AS-1 for various period of time. Finally, the Committee revised different results presented at the meeting and recommended to reduce storage period of AS-1 from 49 to 42 days [142].

In 1983, second additive solution AS-2 (Nutricel) was developed in the U.S. It was accepted for 35 days of RBCs storage. Unlike SAMG and AS-1, AS-2 had a higher concentration of supplemented nutrients and 42 mg of citric acid, instead of mannitol. Citrate is readily metabolized after transfusion, however, its metabolism could cause citrate toxicity in recipients after the transfusion of RBCs stored in AS-2. This additive solution contained high amount of dextrose (396 mg), Adenine (17 mg), phosphate (285 mg) and was used in combination with CP2D anticoagulant solution, which had double the amount of dextrose compared with CPD. Similar to mannitol, citrate protected RBCs from mechanical damage. Moreover, the RBCs, stored in AS-2 maintained a high ATP concentration, as well as cell viability [142]. Citrate was impermeable to slightly permeable ions that contributed to the maintenance of osmotic balance in cryohydrocitosis red cells. These cells have a membrane abnormally permeable to Na and K ions, which tends to spontaneous lysis [147]. Later, the second formula of additive solution (AS-2, Nutricel) was modified with supplementation of large amount of dextrose (100 mg), adenine (30mg), and a low amount of sodium chloride in order to maintain isotonic conditions in packed RBCs. The new version of AS-2 was accepted as a third generation of additives in the U.S. and called AS-3. This process was carried out so quickly, that the researchers directly began investigations on the new version of Nutricel. AS-3 with pH 5.8 was licensed in the U.S. for up to 42 days of RBC storage in combination with CP2D anticoagulant solution. High concentrations of dextrose and adenine in 100 ml of AS-3 were considered to support the production and maintenance of energy-rich phosphates that increases the viability of stored RBCs. However, AS-3 did not show any significant effect on the energy production capacity compared with other additives. RBCs stored in AS-3 had 81.8% of PTR after 6 weeks of storage. The next generation of additive solutions was AS-5 with pH 5. 5. It contained saline, adenine, glucose, mannitol and sodium chloride in double concentration and no sodium phosphate compared to AS-3. AS-5 was similar to SAGM and was used for 42 days of storage with CPD anticoagulant solution [142].

1.8.4. Concept of “chloride shift” in additive manufacturing

All conventional additive solutions, mentioned above had acidic pH and limiting buffer capacity. A rapid decrease in 2,3-DPG concentration and ATP level during storage was no guarantee for long-term preservation of RBCs with favorable PTR (>75%). During refrigerated storage, intracellular pH in packed RBC rapidly decreases below 7 and results in degradation of diphospho-glycerate mutase. This enzyme in RBCs is responsible for the catalytic synthesis of 2,3-DPG, which is not detectable after 14 days of RBCs storage [65]. Low pH within erythrocytes or storage media reduces glycolytic enzyme activities as well as the rate of glycolysis and inhibits the ATP synthesis in packed RBCs. The maintenance of intracellular pH within a physiological range during RBCs storage has a beneficial effect on 2,3-DPG concentration. Meryman and Hornblower first promulgated the concept of so-termed ‘’chloride shift’’. It is based on Gibbs–Donnan equilibrium [133]. This is a bicarbonate and chloride ions exchange process, which contributes to the maintenance of a high 2,3-DPG level. Carbon dioxide is generated as a waste product of normal cellular metabolism. It passively diffuses through the capillaries in circulation, enters RBC and balances intra- and extracellular CO_2 partial pressures. Inside of erythrocyte, CO_2 undergoes hydrolysis and forms carbonic acid (H_2CO_3) by the enzyme carbonic anhydrase. H_2CO_3 spontaneously dissociates into bicarbonate and hydrogen ion. Hydrolysis speed is very fast inside of erythrocytes due to the presence of carbonic anhydrase. RBC membrane is impermeable to hydrogen ions, but the exchange of cellular bicarbonate (HCO_3^-) and chloride (Cl^-) in plasma occurs across the membrane due to Gibbs–Donnan equilibrium. The activity of major integral membrane protein band 3 plays an important role for ion exchange process. [133, 148-150]. This process leads to an acidification of intracellular pH from light to the strong acidic state and is termed “Chloride shift”. It results in an increase in chloride concentration in stored RBCs. Acidic pH induces the breakdown of oxygen from oxyhemoglobin(HbO_2) which produces CO_2 for further neutralization of metabolic acidosis [149].

RBCs, as well as other cells, have selectively permeable membrane [151], which separates extracellular and intracellular fluid. Besides small ions, extracellular and intracellular fluids contain charged macromolecules like proteins, which are not able to pass through the cell membrane via the pores. Instead, small diffusible ions move readily across the membrane and balance anion and cation concentrations on both sides of the membrane. At physiological pH,

proteins have negative charge and attracts cations. Thus, the concentration of anions on one side of the membrane is equal to the concentration of cations on another side of membrane. The distribution of diffusible ions creates electrical potential between the inner and outer sides of the cell membrane and develops different osmotic pressure on both sides of the membrane that causes passage of some ions. This phenomena is known as Gibbs–Donnan equilibrium and exists between two solutions separated by a semipermeable membrane [133, 152, 153]

Based on the Gibbs–Donnan equilibrium, Meryman and Hornblower developed an experimental alkaline additive solution, where sodium chloride was replaced with citrate in order to prevent "chloride shift" in the stored RBCs from. RBC membrane was impermeable to citrate, therefore intracellular chloride anion left RBCs due to Donan equilibrium and hydroxyl anion entered the cells to maintain charge balance. Transport of hydroxyl anions from outside of the membrane to inside of RBCs caused elevation in intracellular pH, while extracellular pH was reduced. The RBCs, stored in this additive solution, maintained high 2,3-DPG levels for six weeks of storage. Besides the "chloride shift", the buffer system in RBCs was achieved through the buffer property of the additive suspension, which consisted of adenine, glucose, phosphate and citrate. The concept of chloride shift was very popular by that time and widely used in development of RBC additives. Alkaline additive solution hindered chloride diffusion in RBCs and prevented ''chloride shift'' in packed RBCs during storage. The disadvantage of alkaline additives was glucose caramelization during heat sterilization [154, 155].

1.8.5. Development of alkaline additive solutions

Maximal duration of storage of packed RBCs with conventional additive solutions was 35-42 days, which was ample time to balance the supply and transfusion of packed RBCs in hospitals with minimal outdating. The prolonged storage of RBCs had a practical importance for military blood transfusion services. The research group of Greenwalt and Hess used chloride shift principle and developed new additive solutions, which extended RBCs storage by 9, 10, 11 and 12 weeks [130, 131, 156, 157]. The investigators split additive solution in two different packs in order to avoid chloride shift and to prevent glucose from caramelization. The first acid solution contained glucose, while the second alkaline solution consisted of other nutritional components. After heat sterilization of acid solution, these two additives were mixed and added to erythrocyte concentrate. Later, these experimental additive

solutions were named EAS 61, EAS 64, EAS 67 and EAS 76v6. Packed RBCs, stored in these additives, were analyzed for hemolysis rate, extracellular potassium concentration and PTR. RBCs, stored for 9 weeks in EAS 61 had a mean PTR of 77 ± 7 %, in EAS 64 for 10 weeks 84 ± 8 %, in EAS 67 for 11 weeks 79 ± 5% and in EAS 76v6 for 12 weeks 78 ± 4% [130, 131, 156, 157]. On the basis of these studies, next additive solution (AS-7) was designed with pH 8.5. AS-7 contained sodium phosphate and sodium carbonate in order to maintain buffer capacity during storage. It was licensed by the FDA with CPD anticoagulant solution. AS-7 was also investigated by Cancelas et al. who reported a successful storage of RBCs with 88 ± 5% and 82 ± 3% of autologous PTR after 42 days and 56 days of storage, respectively [158]. Interestingly, the whole blood, stored overnight at room temperature in AS-7, demonstrated an outcome similar to that of RBCs stored for 42 days in respect of PTR (>75%). However, packed RBCs in AS-7 had a lower PTR after 56 days of storage, compared to RBCs stored for 42 days [159]. Elevated intracellular pH within the stored RBCs contributed to the production and maintenance of 2,3-DPG, which resulted in better oxygen delivery to the tissue. Later, another alkaline additive solution was developed on the basis of "chloride shift". The RBCs were collected in modified CPD anticoagulant solution with half-strength citrate (0.5CPD), processed and stored in additive solution containing citrate, adenine, mannitol, phosphate and glucose. This additive solution was termed red-cell additive solution 2 (RAS2). The investigators used CPD-SAGM additive solution as a control and studied the effect of RAS2 on RBCs during storage. RBCs stored in 0.5CPD-RAS2 had higher 2,3-DPG concentration, which was maintained at baseline level for up to 28 days compared to CPD-SAGM, where 2,3-DPG concentration was maintained only for up to 14 days. Moreover, RBCs stored in RAS2 had a lower hemolysis rate compared with the RBCs in CPD-SAGM. The 24-hour post-transfusion survival of RBCs in RAS2 was 78.9 ± 7.1% after 49 day of storage [160]. Later, the researchers reported a better preservation of RBCs in 150 ml of Erythro-Sol 2 with pH 8.8. It was modified form of 0.5CPD-RAS2 with full-strength CPD, which kept the ATP level for 3 week at the initial level [161]. The next generation of additive solutions was Erythro-Sol 5 (E-Sol 5). It contained adenine, dextrose, mannitol, citrate and sodium phosphate with a pH of 8.4. E-Sol 5 was investigated by Radwanski et al. who reported that RBCs stored in E-Sol 5 showed better results compared to red cells in AS-1 after 42 days of storage. In comparison to AS-1, alkaline pH of E-Sol 5 contributed to maintaining the 2,3-DPG level of stored RBCs for a long time and increased the glycolysis rate [162]. This result was confirmed by Greenwalt and Hess [130, 131, 156-158]. Unlike Erythro-Sol 1 and Erythro-Sol 2, E-sol 5 contained trisodium citrate (CPD and ACD-A anticoagulant).

1.8.6. Replacement of citrate with gluconate in PAGGG-M additive

The presence of citrate in additive solutions contributed to maintain a high intracellular pH, which facilitates the production of 2,3-DPG and ATP, respectively. Citrate has also been widely used as anticoagulant for blood products. It is rapidly metabolized in the liver. However, citrate toxicity has been a well-known adverse effect during massive transfusion to humans [163, 164]. Chelation of calcium or magnesium by citrate can cause coagulopathy or myocardial depression in recipients. Neonates who have an immature liver, or patients with liver diseases are at risk of citrate toxicity after massive transfusion. Therefore, the next step in additive manufacturing was to develop a storage solution without citrate, or with a minimal content of citrate to prevent severe reactions mentioned above. Citrate-free additive solution (PAGGG-M) with a pH of 8.2 was developed due to the disadvantages associated with citrate. It was a modified form of acidic additive solution PAGGS-M, where citrate anions was replaced with gluconate anion. The RBC membrane was also impermeable to gluconate. PAGGG-M increased the production of 2,3-DPG until 35th day of RBC storage. Moreover, PAGGG-M stored RBCs were able to be kept at a constant ATP level (5-6 mmol/g Hb) due to guanosine supplementation. The hemolysis rate of RBCs stored in PAGGG-M was below 0.2 % after 6 weeks of storage [165]. Later, in other studies Burger et al. showed that independently of intracellular pH, phosphofructokinase was strongly activated during the first two weeks of RBCs storage in PAGGG-M additive solution. The activation of a rate-controlling enzyme of glycolysis resulted in sufficient energy reserve and prevented stored RBCs from depletion of 2,3-DPG and ATP. In agreement with present study, other authors also confirmed the advantage of PAGGG-M in RBCs stored for 35 days [166].

1.8.7. Improvements RBC additive solution as a consequence of omics based researches

All innovations in additive manufacturing mentioned above contributed to a better preservation of packed RBCs. However, they couldn't prevent RBCs from all lesions caused by storage. Better preservation has received more attention when fresh and old RBC units were investigated for clinical purposes. The investigators studied the post transfusion effect of fresh and old RBC units on recipients and reported that post-transfusion complications of

RBCs in critically ill patients was associated with storage time that increased the risk of death [167] However, other studies did not show any advantage of fresh RBCs over the older ones [168-170]. Nevertheless, a large clinical study is required in order to evaluate the role of storage lesions in post-transfusion complications, as well as the risk of mortality. The existence of storage lesions in old RBC packs was obvious but the pathophysiological mechanism for all lesions was poorly understood. There were two alternatives: either reducing the shelf life of packed RBC or improving their preservation to minimize the deleterious changes during storage. On the basis of omics application, the investigations of packed RBCs at different points in time showed various pathophysiological changes in RBC metabolism during storage [1, 104, 105]. Omics technology is the most useful tool for investigating storage lesions within the RBC or storage media. Storage lesions are associated with consumption, or accumulation of some metabolites, which were found via metabolomics discipline [1, 105]. These findings contributed to the development of additive manufacturing and commenced the supplementation of consumed substances to the reformulated additive solution. For example, Ardiunu et al. reported storage of RBCs in AS-3 additive solution with supplementation of 245 mg of L-carnitine. The concentration of this substance gradually decreases during RBC storage. Supplementation of L-carnitine reduced hemolysis, enhanced ATP concentration and PTR (88, 9 ± 5%) [129]. L-carnitine is a natural compound and with carnitine palmitoyl transferase it modulates turnover of membrane phospholipid fatty acids in intact human RBCs and thus improves stabilization of RBC membrane under stress conditions [171, 172].

1.8.8. Oxygen-depleted additive solutions

RBCs contain a high concentration of oxygen. Therefore, they are continuously exposed to both endogenous and exogenous oxidants, which cause many deleterious changes. [76-78]. Rajendra Chaudhary and Rahul Katharia described oxidative stress in stored RBCs. they reported that oxidative stress played a major role in disrupting membrane integrity and lysis of RBCs [102]. In view of this fact, Yoshida and his colleagues devised a method of anaerobic storage conditions and studied RBC storage in oxygen-depleted additive solutions. Anaerobic storage coupled with low pH additive solution significantly extended the storage period with less hemolysis and a PTR of over 75%. However the combination of anaerobic conditions and alkaline additive solution showed a remarkably low PTR [173, 174]. Later, D'Alessandro et al. studied the effect of anaerobic storage on red cell metabolism during storage. They confirmed in *vitro* effect, which Yoshida et al. had demonstrated, but they also

found that anaerobic conditions during RBC storage impair erythrocyte capacity to maintain glutathione homeostasis due to blocking of the pentose phosphate pathway [175]. Furthermore, when the deoxygenated erythrocytes are transfused after prolonged anaerobic storage conditions, they meet oxygen-saturated RBCs in the circulation of the recipient and will be again exposed to oxidative stress.

Development of a storage solution for packed RBCs has been a slow and incremental process, due to incomplete understanding of the storage lesions. However, additive manufacturing certainly had positive effects on RBC storage. Long-term investigations gradually improved additive manufacturing and prolonged storage of RBCs. Detailed information about the development of RBC additive solutions is presented in the table 1. This table contains all widely used additives worldwide. Despite the successful additive manufacturing, new additive solutions are still being investigated in order to diminish storage lesions.

Additives	SAGM	AS-1	AS-3	AS-5	PAGGS-M	AS-7	MAP	E-Sol5	PAGGG-M
Adenine	1,25	2	2,2	2,2	1,44	2	1,5	2	1,44
Guanosine	-	0	0	0	1,44	0	0	0	1,44
Sodium Chloride	150	154	70,1	150	72	0	85	0	0
Dextrose	45	111	55,5	45	47,5	80	40	111	47,5
Mannitol	30	41	0	45,5	55	55	80	41	55
Trisodium Citrate	-	0	20	0	0	0	-	25 [a]	0
Citric acid	-	0	12	0	0	0	1	0	0
Gluconate	-	0	0	0	0	0	-	0	40
Na2HPO4	0	0	0	0	16	12	-	20[b]	8
NaH2PO4	0	0	23	0	8	0	6	0	8
NaHCO3	-	0	0	0	0	26	-	0	0
pH	5,7	5,5	5,8	5,5	5,7	8,5	5,7	8,4	8,2
Anti-coagulant	CPD	CPD	CP2D	CPD	CPD	CPD	ACD	0 5CPD	CPD
FDA Licensed	No	Yes	Yes	Yes	No	Yes	No	No	No
Storage period	42 Days	42 Days	42 Days	42 Days	49 Days	49 Days	42 Days	42 Days	35 Days
Volume, ml	100	100	100	100	100	110	92	100	110

Table 1: Composition of widely used additives with anticoagulant solutions and FDA approvals [133, 176-179]. (Concentration in mmol/L, [a]not specified trisodium citrate between citrate acid, [b]not specified Na2HPO4 between NaH2PO4).

2. The purpose of the study

Methionine is a sulphur-containing essential amino acid with various functions in the mammalian organism. It is not synthetized de novo and is only supplied by diet. Animal products and fish are considered to be the main source of methionine for humans. It is involved in DNA synthesis and initiates the protein synthesis in the eukaryotic cells [180]. Methionine is incorporated in many proteins and forms buried and surface residues, which are quite susceptible to oxidation and serve as an endogenous antioxidants for proteins. It reacts with oxidants and forms methionine sulfoxide, which is reduced by the enzyme methionine sulfoxide reductases [181]. Diminished activity of methionine sulfoxide reductases is associated with accumulation of an oxidized form of methionine, which affects the life span of cells and triggers age-related neurodegenerative diseases [182]. Moreover, three oxidation -sensitive methionine sites (in N-terminus, in the transmembrane region and at the terminal end of the C-terminus) of transmembrane protein Band 3, are time-dependently oxidized [183].

In biological systems, methionine is transformed into its active form of S-adenylmethionine (SAM) by the enzyme methionine adenosyltransferase [184]. SAM is the major methyl donor in more than 200 metabolic reactions [185]. It is characterized by anti-inflammatory properties [186]. Methionine is incorporated in the composition of globin chains of mammalian hemoglobin, including human and have different contents among the species[187] Besides the diverse and important functions of methionine mentioned above, it can also inhibit metal oxidation [188]. Bioavailability of methyl donor is necessary for main metabolic reactions such as regulation of glycolytic enzymes, synthesis of glutathione and conversion of PE to PC [189, 190]. The glutathione redox system plays an important role in counteracting oxidative stress and neutralizing ROS. The supplementation of methionine to poultry feed activated glutathione redox system and glutathione peroxidase in poultry [191]. Glutathione is one of the important antioxidants. It is endogenously synthetized almost in all cells. Glutathione is involved in antioxidant defense, DNA and protein synthesis, detoxification of electrophilic xenobiotics, storage and transport of cysteine, regulation of cell proliferation and apoptosis [192, 193].

SAM donates the methyl group and forms S-adenosylhomocysteine (SAH), which is hydrolyzed by SAH hydrolase with production of homocysteine and adenosine. This reaction is known as transmethylation. Homocysteine can be converted into methionine by the enzyme methionine synthase. For this reaction, the enzyme methylenetetrahydrofolate reductase

requires 5'-MTHF as methyl donor and vitamin B_{12} as a cofactor to form methionine, whereas the enzyme betaine-homocysteine methyltransferase BHMT uses betaine and converts homocysteine into methionine [184]. Interestingly, the level of homocysteine increases gradually during RBC storage, while methionine is fully utilized in parallel with homocysteine accumulation [105].

The goal of our study, was to evaluate the effect of methyl donor on stored RBCs. Instead of direct methyl donor such as SAM, we used L-methionine. It is better antioxidant compared with SAM. The cytoskeleton proteins of intact RBC membrane are methylated only through the supplementation of L-methionine [194]. Moreover, the RBC membrane is impermeable to SAM [195].

In order to achieve our aim, we reprogrammed RBC metabolism during storage by supplementation of PAGGS-M additive solution with methyl donors. Two interventions were assessed quantitatively:

1 – Restoration of methyl group pool by supplementation of PAGGS-M with L-methionine, which be called later PAGGS-MM

2 – Conversion of accumulated homocysteine into methionine by supplementation of Cobalamin (vitamin B_{12}) and 5'-methyltetrahydrofolate (5'-MTHF, active form of vitamin B_9) to PAGGS-M, which be called later as PAGGS-MB.

3. Material and methods

3.1. Study sample

The whole blood was collected from 27 healthy donors according the German National Blood Center guidelines (Blood donation services-ZTB, Berlin). The donors met standard blood donor criteria and gave informed consent in accordance with the Declaration of Helsinki. All units of whole blood were processed in order to obtain 27 packed RBCs. They were stored in PAGGS-M additive solution. Eighteen of RBC units were obtained from male donors and 9 from female donors (Table 2). We used DQE 7241LC blood bags from (Maco Pharma International GmbH) which contained 70 ml CPD in whole blood bag and 110 ml PAGGS-M in RBC bag.

To minimize biological differences between donors and ensure an equal volume of RBC concentrates during study period, a "pool and split" experiment design was carried out, whereby each RBC unit was split from a pool of 3 ABO- blood groups and gender-matched RBC units to produce equivalent RBC products in each condition. 5 pools were generated from gender-matched donors with 0 RH+, while 4 pools were collected from the A Rh+ donors. Each unit produced from one pool segregated into 3 experimental groups: control, (PAGGS-M, treated with 3 ml of 0, 9% NaCl), L-methionine supplementation (PAGGS-MM), Cobalamin + 5'- MTHF (vitamin B_{9+12}) supplementation (PAGGS-MB)

Table 2: The pooling of donated blood according to blood groups, Rhesus and gender.

Pool	Donation	Sex	Blood group
A	1	male	A(Rh+)
	2	male	A(Rh+)
	3	male	A(Rh+)
B	4	male	A(Rh+)
	5	male	A(Rh+)
	6	male	A(Rh+)
C	7	female	0(Rh+)
	8	female	0(Rh+)
	9	female	0(Rh+)
D	10	male	0(Rh+)
	11	male	0(Rh+)
	12	male	0(Rh+)
E	13	male	0(Rh+)
	14	male	0(Rh+)
	15	male	0(Rh+)
F	16	male	0(Rh+)
	17	male	0(Rh+)
	18	male	0(Rh+)
G	19	female	A(Rh+)
	20	female	A(Rh+)
	21	female	A(Rh+)
H	22	female	0(Rh+)
	23	female	0(Rh+)
	24	female	0(Rh+)
I	25	male	A(Rh+)
	26	male	A(Rh+)
	27	male	A(Rh+)

3.2. Treatment protocols

The supplementation compounds: L-methionine 149.21 g/mol, vitamin B_{12} 1355.37 g/mol and 5-Methyltetrahydrofolic acid disodium salt 503.42 g/mol were purchased from Sigma-Aldrich, Germany with Catalog numbers (M8439, V2876, M0132), respectively.

In the preliminary experiments, the dose of L-methionine was determined based on the hemolysis rate during storage period. We tested 5-, 15-, 25- and 35-fold higher concentrations of L-methionine than its normal plasma level. The lowest hemolysis rate was observed in RBC packs supplemented with a 25-fold higher concentration of L-methionine. The level of hemolysis and survival rate of transfused RBCs are the main standard requirements for patenting a new additive solution in USA and in Europe [65]. Thus, 25-fold higher concentration of L-methionine relative to its normal plasma level was used in our study. The purpose of supplementing vitamin B12 and 5-Methyltetrahydrofolic acid disodium salt was to convert accumulated homocysteine into methionine. Therefore, each RBC unit was also supplemented with corresponding substances at 25-fold higher concentrations of their normal plasma levels. The end concentrations of methionine, vitamin B_{12} and B_9 in corresponding RBC units were 1.125 mmol/l, 22. 5 ng/ml and 975 nmol/l, respectively. Each substance was dissolved with sterile 0.9 % NaCl solution under sterile conditions. Following sterile filtration with 0.22 µm pore sized syringe filter. 3 ml volume of each solution was first injected into a satellite blood bag and then sealed with a pool-and-split RBC unit. Afterwards, RBC packs were sealed again and the satellite blood bags were removed. All equivalent RBC products were stored for up to 42 days under standard conditions (4 ± 2 °C), while 15 ml blood of each sample was collected aseptically in satellite bag for the analysis on a weekly basis**.** At the end of storage, all RBC units stored in all tested additive solutions were checked for bacterial contamination by microbiology. None of the RBC units were contaminated.

3.3. Complete blood count

Complete Blood Count test was performed with Sysmex blood cell counter (Ca. K4500), which measures the following parameters: white blood cell (WBC), red blood cell (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelet (PLT). The stored RBCs was collected in 1.5 ml Eppendorf tubes and analyzed using the above mentioned blood cell counter.

3.4. Blood gas analysis

The measurement of following parameters: pH, pCO_2, pO_2, Cl^-,Ca^{2+}, K^+, $Na^{+,}$ glucose (Glu), lactate (Lac), total hemoglobin (tHb), sO_2 and methemoglobin (MetHb) was performed using the Blood Gas Analyzer (radiometer ABL 700 serie). Blood was collected in the 1.5 ml of syringe and the above mentioned parameters were analyzed. The storage temperature of RBC units was kept (4 ± 2 °C) during the analysis of blood samples.

3.5. Extracellular hemoglobin (Hb) /hemolysis rate

Plasma/low Hb Analyzer (HemoCue) was used to estimate extracellular/supernatant hemoglobin level, as well as the rate of hemolysis. 10 ml of blood was collected from satellite bag and centrifuged at 1800 rpm for 10 minutes without brake. Then, 750 µl of supernatant of each sample was transferred to 1.5 ml Eppendorf tubes and centrifuged again at 13000 rpm for 5 minutes in order to avoid contamination of cell particles. Finally, 20 µl of supernatant of each sample was used to measure the hemoglobin level in the supernatant with Plasma/low Hb Analyzer (HemoCue). The rate of hemolysis was determined with the following formula: hemolysis rate [%] = (100 - hematocrit [%] x Hb supernatant [g/dl]/Hb erythrocyte [g/dl]. Rest of the volume (700 µl) of each supernatant was stored under -80 °C for further metabolomics measurement.

3.6. ATP measurement

ATP concentration is a marker of glycolysis rate in stored RBCs. Bioluminescence-based "ViaLight™ plus kit" (Cat.LT07-221, LONZA) was used for rapid and safe detection of the ATP level. The reconstituted ATP monitoring reagent was prepared according the protocol, aliquoted and stored at -20 °C. Stored RBCs were diluted with PBS buffer and a suspension with 40000-50000 of cells was obtained. Afterwards, 100 µl of cell suspension of each sample was transferred to 96-well luminometer-compatible plate and 50 µl of cell lysis reagent was added to each well for the extraction of ATP from the lysed cells. Then followed, the incubation of the samples at room temperature (25 °C) for 10 minute in order to obtain a complete extraction. Finally 100 µl of reconstituted ATP monitoring reagent was added to each well. This reagent containes luciferin, which reacted with ATP released from lysed RBCs and generated luminescence signal-light by the enzyme luciferase.

$$\text{ATP + Luciferin + } O_2 \xrightarrow[Mg^{2+}]{\text{Luciferase}} \text{Oxyluciferin + AMP + PPi + } CO_2 \text{ + LIGHT}$$

The luminescence signal of each sample was measured with the Multilabel Plate Reader Victor 3 (Cat 1420-012, PerkinElmer). The intensity of light emission was proportional to the ATP concentration.

3.7. 2,3 Diphosphoglycerate test

The measurement of 2,3-DPG was performed with 2,3-DPG kit (Cat.10148334001, Roche, Mannheim, Germany). Four different basic solutions were prepared at the beginning of the experiment according to the protocol and stored at +4°C for 3 weeks, except basic solution 2, which was kept at -20°C. 1 ml of stored RBCs from each sample was transferred to 15 ml falcon tubes and denatured with 5 ml of 0.6M ice-cooled perchloric acid volumetric solution (Cat. 4A014799, AppliChem, Darmstadt Germany), mixed well and centrifuged at 4,000 rpm for 10 minutes. Afterwards, 4 ml of supernatant of each sample was collected, neutralized with 500 µl of 2.5 M potassium carbonate and stored in an ice-bath for 40 minutes. Finally the samples were centrifuged again at 4,000 rpm for 10 minutes and 100 µl of pure supernatant was removed for measuring the 2,3-DPG concentration.

The first working solution was obtained from basic solutions 1, 2 and 3 (2 ml, 50 µl, 50 µl per sample and blank, respectively) according the instructions provided with the kit. 2.1 ml of first working solution was transferred to each measuring cuvette, 100 µl of supernatant was added and mixed gently. 5 minutes later, the first absorbance- A1 at 340 nm wavelength was determined with the spectrometer Smart Spec Plus (Serial No.273, Bio Rad). The second working solution was prepared from basic solutions 4 and 5 (20 µl and 20 µl per sample, respectively), mixed well and 40 µl was added to each tested sample. The second absorbance- A2 was also determined at 340 nm wavelength with the same device.

The test principle is based on the following reactions: The formation of 3-phosphoglycerate from 2,3-DPG by the enzyme bisphosphoglycerate mutase. Following the conversion of 3-phosphoglycerate to 1,3-DPG by Phosphoglycerate kinase. Further reactions are catalyzed by the enzymes: glyceraldehyde-3-phosphate dehydrogenase, triose phosphate isomerase, and

glycerol-3-phosphate-dehydrogenase with formation of glyceraldehyde-3-phosphate, dihydroxyacetone phosphate and glycerol-3-phosphate, respectively. The reactions catalyzed by glyceraldehyde-3-phosphate dehydrogenase and glycerol-3-phosphate-dehydrogenase require NADH as a reducing agent, which is converted into NAD^+. Thus, each molecule of 2,3-DPG leads to the formation of two molecules of NAD^+.

2,3-DPG concentration was calculated using a formula which contains the following parameters: test volume, sample volume molar mass of 2,3-DPG, layer thickness of the measuring cuvettes and an absorption coefficient of NADH at 340 nm and dilution factor. 2,3-DPG level is determined by measuring the changes in the absorption through NADH consumption in the UV-test, as NAD^+ is not detected by the photometer. On the basis of hemoglobin content in the sample, 2,3-DPG concentration is calculated as followed;

$$\textbf{[2,3-DPG (mmol/l)] = (([(A1-A2)}_{\textbf{Probe}}\textbf{-(A1-A2)}_{\textbf{Blank}}\textbf{] x 11.70) / Hematocrit \%) x 100}$$

3.8. Detection of total glutathione and oxidized glutathione (GSSG)

Total glutathione consists of its reduced GSH and oxidized GSSG forms. A luminescence based system- GSH/GSSG-Glo™ Assay (Cat. V6612 PROMEGA) was used to determine total glutathione GSH+ GSSG and GSSG levels in order to assess the antioxidant capacity of RBCs stored in three different additives. According the instruction of the kit, three main reagents were prepared:

1) Total glutathione lysis reagent (luciferin-NT, passive lysis buffer and distillated water) with 25 µl of final volume per reaction in 96-well plate

2) Oxidized Glutathione lysis buffer (luciferin-NT, NEM, 25mM, passive lysis buffer, and distillated water) with 25 µl of final volume per reaction in 96-well plate.

3) Luciferin Generation Reagent (100mMDTT, Glutathione-S-Transferase, Glutathione reaction buffer) with 50 µl of final volume per reaction in 96-well plate.

Stored RBC samples were diluted with PBS buffer to obtain suspensions with 40000-50000 cells. After mixing gently, 25 µl of each cell suspension was transferred to the 96-well

luminometer-compatible plate. 25 µl of either total glutathione, or oxidized glutathione lysis reagent was added to corresponding samples and mixed on the plate shaker for 5 minutes at room temperature. Then 50 µl of luciferin generation reagent was added to each well and incubated at room temperature (25 °C) for 30-60 minutes and briefly shaken. Finally, 50 µl of luciferin detection reagent was transferred to the same wells and incubated at room temperature for 15 minutes. Luminescence signal from each well was read by Multilabel Reader Victor3.

The principle of the test is based on the following: the luminescence signal can be obtained only from a reduced form of glutathione. The total glutathione lysis reagent releases the reduced and oxidized forms of glutathione from the cell and converts oxidized glutathione into the reduced form, while N-ethylmaleimide (NEM) in oxidized glutathione lysis reagent rapidly reacts with the reduced form of glutathione and inhibits luminescence signal. The luminescence signal is produced by glutathione-dependent conversion of luciferin-NT to luciferin. Finally, luciferin detection reagent simultaneously inhibits luciferin generation reagents and initiates a luminescence signal, which is directly proportional to the amount of reduced glutathione produced by either total or oxidized form of Glutathione (Figure 4).

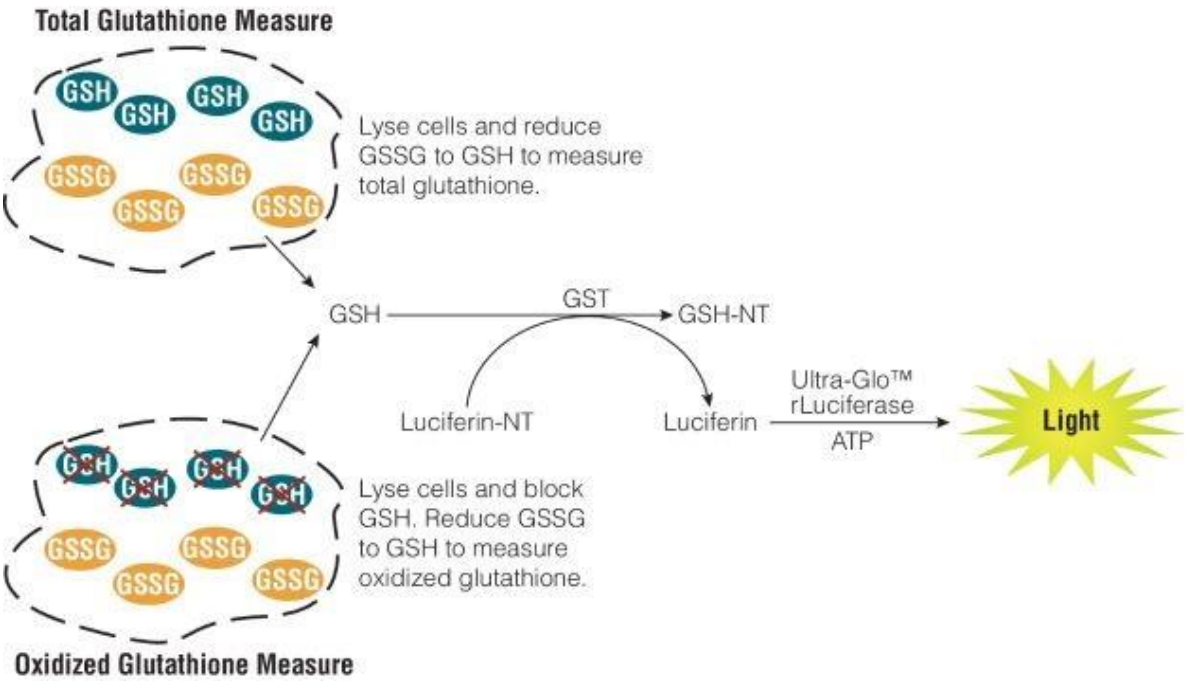


Figure 4: GSH-dependent conversion of a GSH probe, formation of luciferin from luciferin-NT by a glutathione S-transferase enzyme results in a firefly luciferase reaction.

3.9. NADP/NADPH assay

The amounts of $NADP^+$ and NADPH in stored RBCs were measured by the NADP/NADPH-Glo™ Assay (Cat. G9081 and G9082 PROMEGA). It is a bioluminescence assay, which rapidly detects the reduced and oxidized forms of nicotinamide adenine dinucleotide phosphates.

NADP/NADPH-Glo™ Detection Reagent includes:

- 55µl Reductase
- 55µl Reductase Substrate
- 1 vial NADP Cycling Enzyme (lyophilized)
- 275µl NADP Cycling Substrate
- 1 vial Luciferin Detection Reagent (lyophilized)
- 10ml Reconstitution Buffer

All substances within the kit were thawed at room temperature and briefly centrifuged. Reconstitution buffer and lyophilized luciferin detection reagent were equilibrated to room temperature. The reconstitution buffer was then transferred to the bottle of lyophilized luciferin and prepared the luciferin detection reagent, which was aliquoted and stored at - 20 °C.

$NADP^+$ and NADPH was measured individually according the protocol provided with the kit. These molecules have different stabilities in acidic and basic environments. More precisely, $NADP^+$ is easily broken down by heating in basic solution, while the stability of NADPH abruptly decreases in acidic conditions. 0.4 M HCl acid, 0.2 M NaOH base, HCl/Trizma (Merck, Darmstadt, Germany) and 0.5 M Trizma base (Cat. 4855.2, Carl Roth GmbH, Karlsruhe, Germany) solutions were prepared at the beginning of the experiment.

The stored RBCs was diluted with PBS buffer and an RBC suspension with 40000-50000 of cells was obtained. 50 µl of suspension from each samples was transferred to a 96-well luminometer-compatible plate and lysed with 50 µl of base solution, containing 1% dodecyltrimethyl ammonium bromide (DTAB) (Cat. D5047). DTAB can lyse RBCs without affecting the stability of dinucleotides. The plate was briefly shaken on the plate shaker to obtain a complete extraction of dinucleotides. Afterwards, 50 µl of lysed suspension from

each sample was transferred to empty wells. 25 µl of 0.4 M HCl was added to the wells for acid treatment. This was followed by incubation of the plate for 15 minutes at 60°C and for 10 minutes at room temperature, respectively. Acid-treated samples were neutralized with 25 µl of 0.5 M Trizma base, while 50µl of HCl/Trizma solution was added to base-treated wells. The NADP Cycling Enzyme was reconstituted with addition of 275 µl of pure water. Finally, the NADP/NADPH detection reagent was prepared with the following substrates:

- Reconstituted Luciferin Detection Reagent 1ml
- Reductase 5 µl
- Reductase Substrate 5 µl
- NADP Cycling Enzyme 5 µl
- NADP Cycling Substrate 5 µl

100µl of NADP/NADPH detection reagent was added to each acid- and base-treated sample, gently shaken and incubated for 40 minutes at room temperature. The luminescence signal was detected by the multilabel plate reader Victor 3. The luminescence signal from the acid-treated samples corresponded to the concentration of $NADP^+$ while the luminescence signal from the base-treated samples was equivalent to the amount of NADPH. The NADP Cycling Enzyme in NADP/NADPH Detection Reagent reduced $NADP^+$ to NADPH and reductase converted proluciferin reductase substrate to luciferin in the presence of NADPH. Luciferin was detected by the recombinant luciferase present in the luciferin detection reagent (Figure 5)

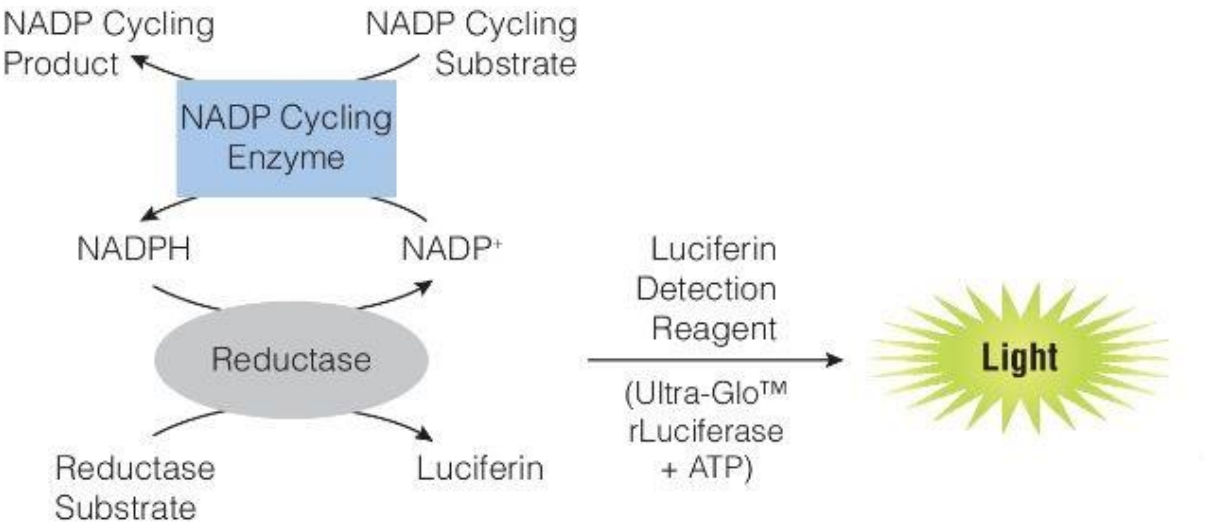


Figure 5: The principle of NADP/NADPH-Glo™ assay.

3.10. NAD/NADH assay

The NAD/NADH-Glo™ Assay (Cat. G9071 and G9072 PROMEGA) measures both forms of dinucleotides separately in the same sample. This assay is based on the same principle as NADP/NADPH-Glo™ assay and was performed in a manner similar to that described above (3.9).

3.11. Sample preparation and derivatization for metabolomics

The MMC mixture was prepared with methanol, methyl tert-butyl ether (MTBE) and chloroform (1:1:1, v/v/v) and cooled to – 20 °C. 750 µl ice-cold MMC was added to 20 µl cell-free supernatant of stored RBCs in 1.5 ml Eppendorf tubes and vortexed (10 s.). Following agitation at 1000 rpm for 30 min at 4ºC, 250 µl LC/MS grade water was added to the mixture and vortexed again. The samples were then centrifuged at 21000 g for 5 min at 4ºC for the phase separation. Finally, 100 µl of upper (polar) phase was collected into conical glass vials and evaporated at room temperature using SpeedDry vacuum concentrator. Immediately before GC/APCI-MS measurement, the evaporated extracts were derivatized on-line using a PAL RTC autosampler (CTC Analytics, Switzerland). After addition of 10 µL methoxyamine (20 mg/mL in pyridine; Sigma), GC vials were agitated for 90 min at 34 °C and 750 rpm. 90 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA; Macherey-Nagel, Germany) containing 0.2 µg/mL each of C4-C24 fatty acid methyl esters (Sigma, Germany) as retention index markers were added, followed by agitating GC vials for 30 min. Before injection, samples were allowed to rest for 2 h to complete derivatization reactions.

3.12. GC-APCI/MS acquisition

GC/APCI-MS analysis was carried out on an Agilent 7890 B gas chromatograph (Agilent, Germany) coupled to an Impact II quadrupole time-of-flight mass spectrometer via a GC-APCI II source (Bruker, Germany). 1 µL of sample was injected into a split/splitless inlet, operated at 230 °C in split mode (1:10). Chromatographic separation was carried out on a 30 m x 0.25 mm x 0.25 µm HP5-MS UltraInert capillary column (Agilent, Germany) connected to a 0.5 m x 0.25 mm RxiGuard (Restek, Germany) column as transfer capillary. The GC-APCI source was operated in positive ion mode. Full-scan line spectra were recorded in the scan range of m/z 80-1000 at an acquisition rate of 10 s-1.

3.13. Metabolomics data analysis

Automated mass spectral annotation was performed using InterpretMSSpectrum algorithm implemented as an R function / package (https://www.rproject.org/) [196]. The fully processed peak list, including all informative peaks, their potential sum formulas and quality parameter values, is returned for further manual evaluation. Metabolite levels were represented by the observed ion intensity of a selected unique ion. Ion intensity was log10 transformed and normalized using a two-way analysis of variance (ANOVA)-based model for the removal of measurement bias [197]. Graphical representations and statistical analyses (principal component analysis (PCA) and ANOVA)) of annotated metabolites (targeted metabolomics), and non-annotated peaks (untargeted metabolomics) were performed using the R software environment 3.1.1

3.14. Statistical methods

All measured quality control variables were assessed for normality by the skewness and kurtosis test. Skewed data were logarithmically transformed for statistical analysis and then back-transformed to their natural units for presentation in the tables and graphs. To analyze the influence of methyl donor supplements on quality control variables of RBC concentrate, generalized linear mixed model (GLMM) was performed on the collected data from repeated measurements. Time (0-6), treatment (PAGGS-M, PAGGS-MB, and PAGGS-MM) and interaction of time and treatment were set as fixed effects, whereas assignment of a corresponding pool was selected as a random effect. The significance level of P values was set at 5%. All calculations were performed using IBM SSPS 24 software.

4. Results

4.1. Complete blood count

The complete blood count (CBC) parameters of RBCs stored in 3 different additive solutions varied noticeably with the mean corpuscular volume (MCV), hemoglobin concentration (Hb), hematocrit (HCT) and RBC count (RBC). The MCV of packed RBCs was varied significantly among the additives throughout the storage period. It gradually increased over the storage

period (MCV: 85.0 fL (±0.94) on day 1 to 88.6 fL (±0.93) on day 42, P=0.000). Moreover, from the start of storage, the increase in MCV was much more pronounced in PAGGS-MM than in PAGGS-M and PAGGS-MB additive solution (MCV 87.3 ±0.93 fL vs. 86.8 ± 0.93 fL and 86.9 ± 0.93 fL, P=0.000, respectively) (Figure 6). Nevertheless, all packed RBCs stored in three variants of additive solutions, which were analyzed here, demonstrated acceptable MCV values as QC parameter.

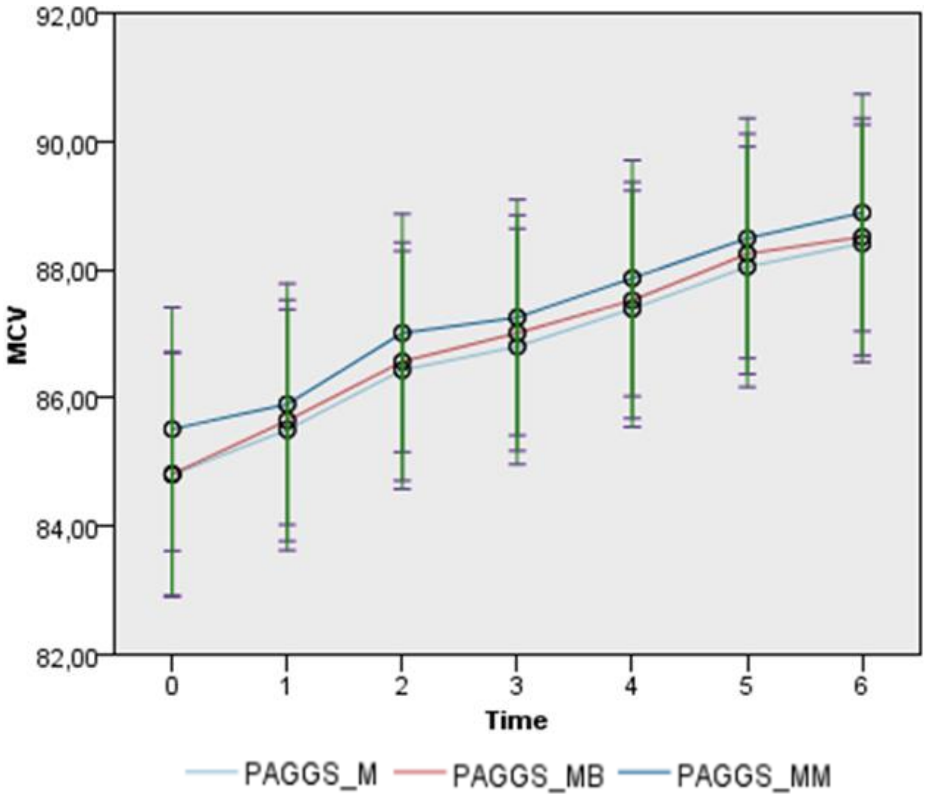


Figure 6: Increase in mean corpuscular volume (MCV, fL) of packed RBCs stored in PAGGS-M (n=9), PAGGS-MB (n=9) and PAGGS-MM (n=9) additive solutions for up to 42 days.

Interestingly, hematological indices, such as RBC count, Hb and HCT in packed RBCs which belong to the quality control parameters, demonstrated different characteristics in the additives tested throughout the storage period. The RBC count, Hb and HCT values of RBCs stored in PAGGS-MM remained relatively stable, whereas these parameters gradually increased till week 4 in RBCs stored in PAGGS-MB additive and then decreased again. These indices were lowest in PAGGS-M-stored RBCs at weeks 4 and 5. However the differences did not reach statistical significance throughout the storage period (RBC: 6.621 ± 0.096 x 10^6/µL, 6.680 ± 0.096 x 10^6/µL, 6.654 ± 0.096 x 10^6/µL, P=0.39; Hb: 19.794 ± 0.167 g/dl, 20.041 ± 0.167 g/dl, 19.975 ±0.167 g/dl, P=0.16; HCT: 57.392 ± 0.465 %, 57.998 ± 0.465%, 58.017 ± 0.465%, P=0.19; mean ± standard error; PAGGS-M, PAGGS-MB, PAGGS-MM,

respectively). In contrast, packed RBCs stored in PAGGS-MB has demonstrated a significantly higher number of RBCs, HCT and total Hb level at week 4 (RBC: 6.710 ± 0.111 x 10^6/µL, P=0.026; Hb: 20.152 ± 0.248 g/dl, P=0.031; HCT: 58.741 ± 0.699 %, P= 0.041, respectively) (Figure 7-8). This phenomenon may be explained by the different evaporation rate of packed RBC in test conditions

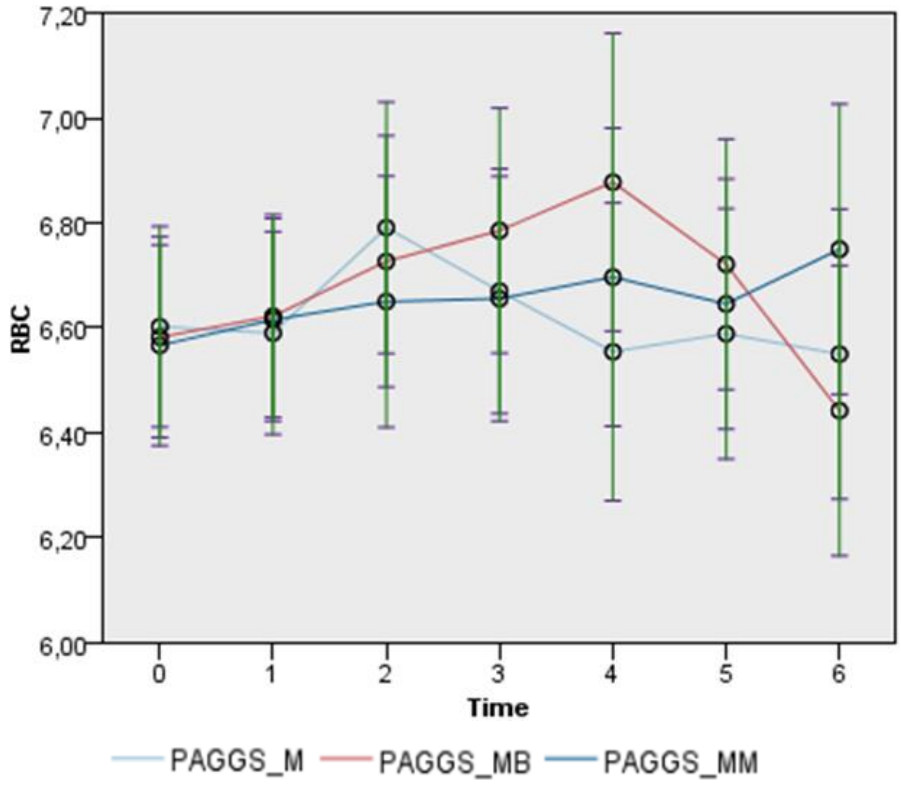


Figure 7: Changes in red blood cell count (RBC, x 10^6/µL) of packed RBCs stored in PAGGS-M (n=9), PAGGS-MB (n=9) and PAGGS-MM (n=9) additive solutions for up to 42 days.

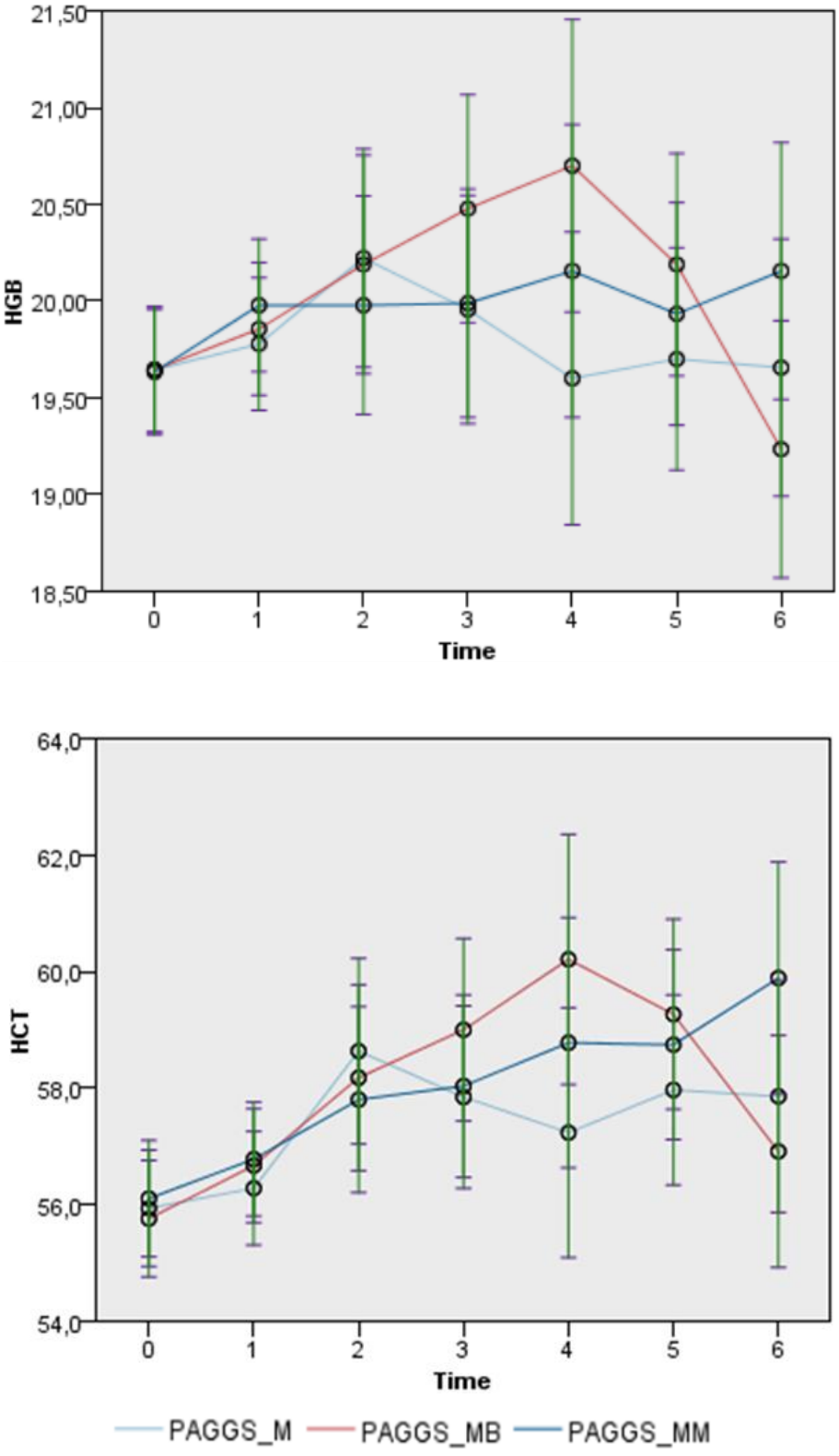


Figure 8: Changes in total hemoglobin (g/dl) and hematocrit (%) levels of packed RBCs stored in PAGGS-M (n=9), PAGGS-MB (n=9) and PAGGS-MM (n=9) additive solutions for up to 42 days.

4.2. Blood gas analysis

4.2.1. Extracellular pH and sodium-potassium balance

Extracellular pH of RBCs, stored in three different additive solutions gradually declined throughout of the storage period from 6.877 ± 0.005 on day 1 to 6.256 ±0.004 on day 42. (P= 0.000). This was consistent with values reported in the literature [198]. However, neither PAGGS-MB, nor PAGGS-MM had shown any significant differences in comparison to PAGGS-M additive solution. Moreover, extracellular pH was very similar at each weekly measurement in packed RBCs stored in three different additives (Figure 9).

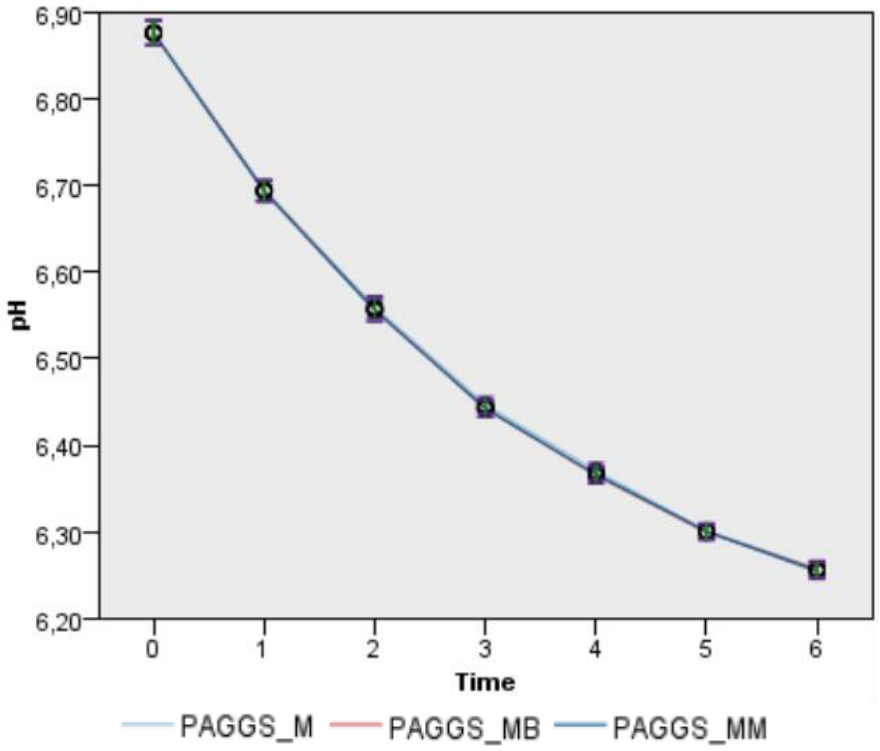


Figure 9: Gradual decline of extracellular pH in packed RBCs stored in PAGGS-M (n=9, colored), PAGGS-MB (n=9) and PAGGS-MM (n=9) additive solutions for up to 42 days.

Extracellular concentration of sodium ions in RBCs stored in PAGGS-M, in PAGGS-MB and in PAGGS-MM additives decreased over the storage period (106.148 ± 0.575mmol/L on day 1, 77.593 ± 0.544mmol/L on day 42; P=0.000, while extracellular potassium increased from 4.119 ± 0.643mmol/L on day 1 to 45.215 ± 0.563mmol/L on day 42; P= 0.000) (Figure 10). Nevertheless, no statistical differences were observed among all three types of additive solutions. This indicates that the significantly high MCV of RBCs in PAGGS-MM was not related to cation leakage.

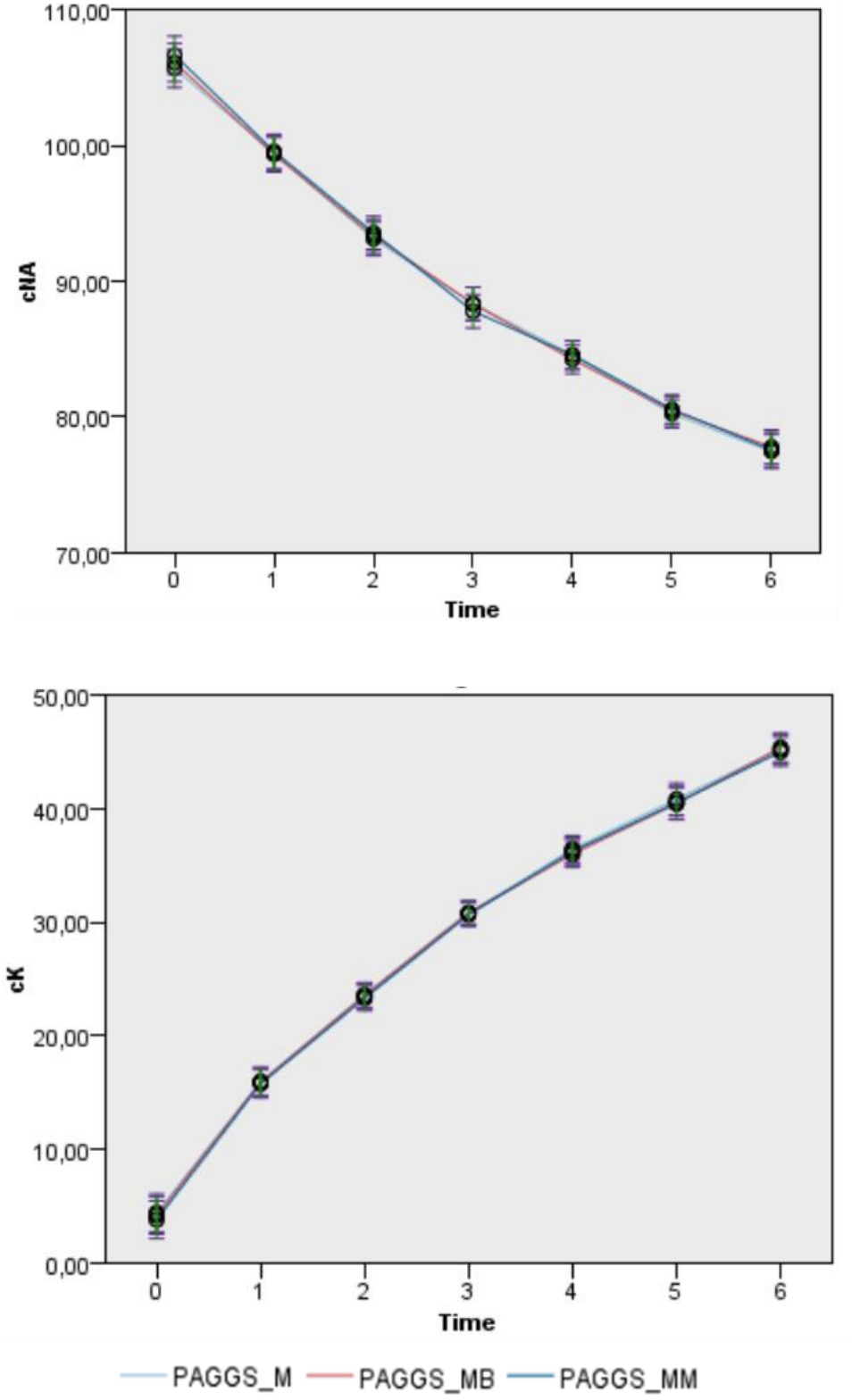
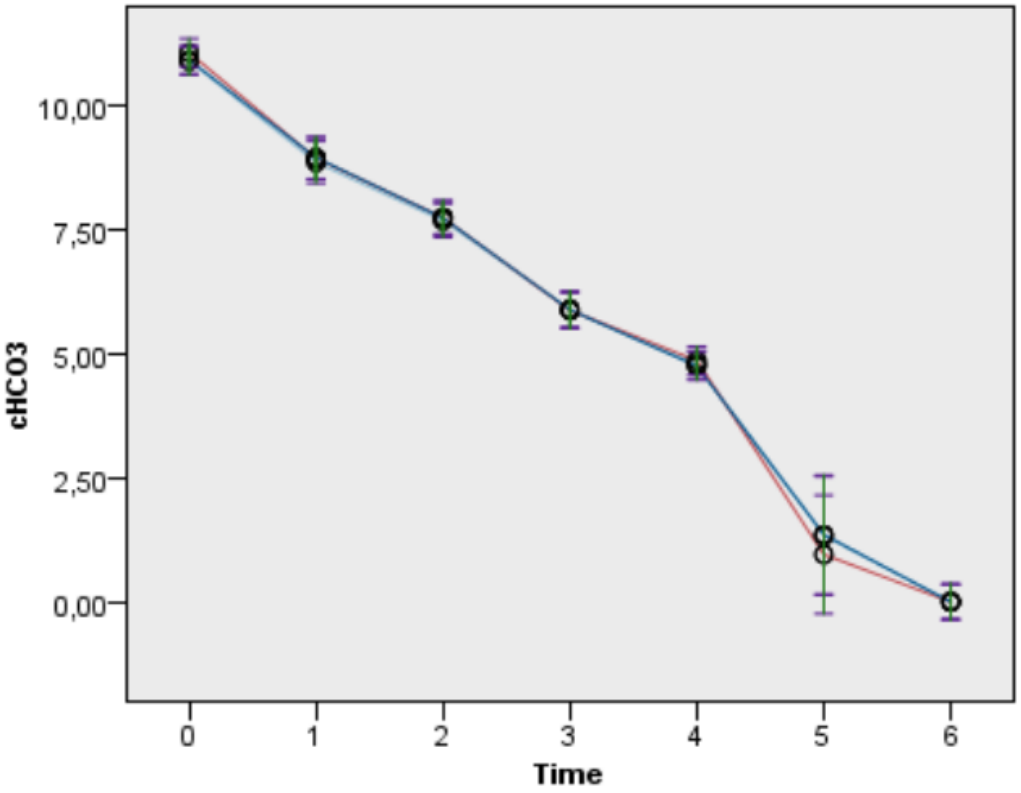


Figure 10: Changes in the extracellular sodium and potassium concentrations (mmol/L) in packed RBCs stored in PAGGS-M (n=9, colored), PAGGS-MB (n=9) and PAGGS-MM (n=9) additive solutions for up to 42 days.

4.2.2. Bicarbonate (HCO3-) chlorine (Cl-) and calcium ions

In the case of RBC storage, intracellular pH is maintained by efflux of intracellular Cl – from RBC and influx of HCO3– into RBC, which is known as the 'chloride shift' [133]. In our experiment, extracellular $HCO3^{-}$ concentration decreased progressively in all additives tested throughout the 6-week storage period (10.974 ± 0.134mmol/L on day 1, 0.000 ± 0.148mmol/L on day 42; P=0.000), while Cl^{-} concentration increased only in the first week of the storage and then remained relatively stable for the rest of the storage period (79.667 ± 0.549mmol/L on day 1, 80.667 ± 0.611mmol/L on day 42; P=0.000) (Figure 11). However, no statistical differences were found among the tested additive solutions.

Despite the chelation of calcium with citrate in packed RBCs, the extracellular calcium level gradually increased in all tested RBC concentrates during 6 weeks of storage (0.086 ± 0.002mmol/L on day 1, 0.127 ± 0.001mmol/L on day 42; P=0.000), but without statistical significance among the additive solutions. (Figure 11)



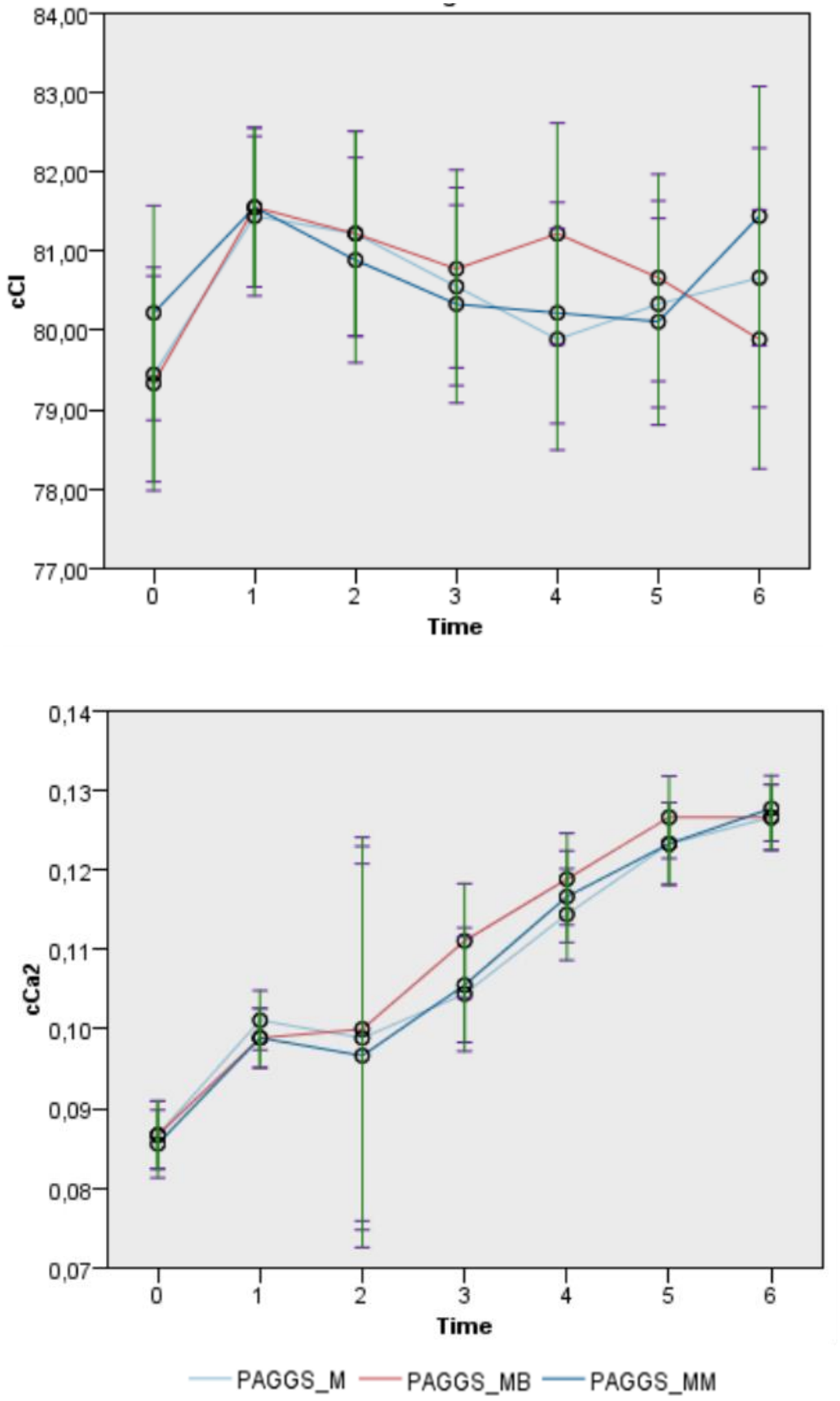
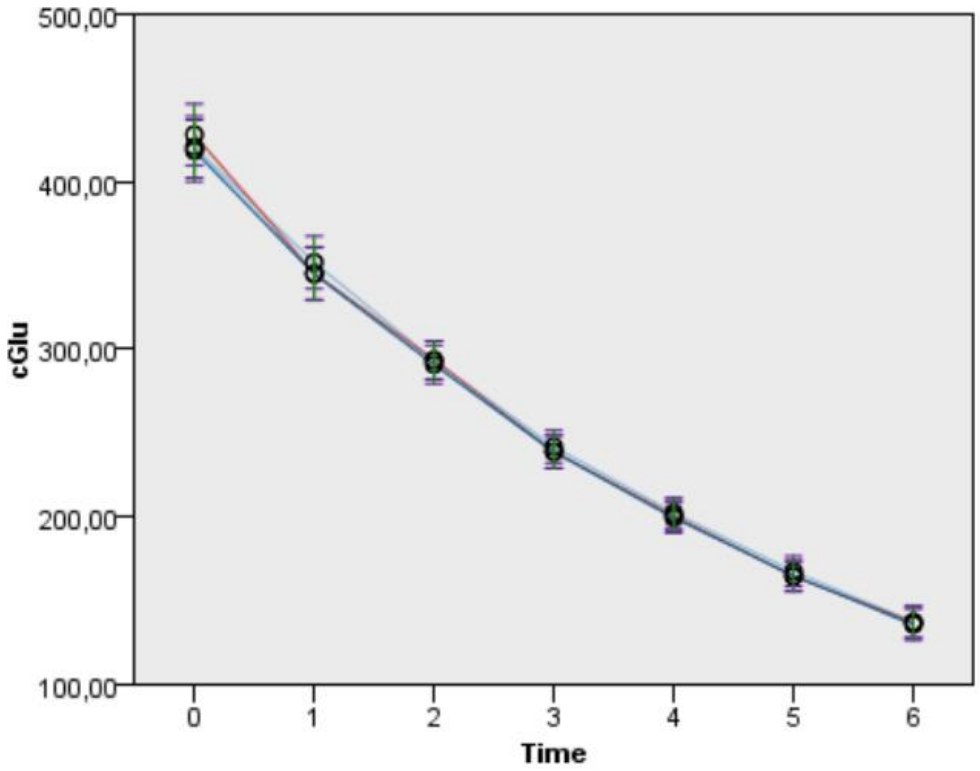


Figure 11: Changes in extracellular Cl-, HCO3– and calcium concentrations (mmol/L) in packed RBCs stored in PAGGS-M (n=9), PAGGS-MB (n=9), PAGGS-MM (n=9) additive solutions for up to 42 days.

4.2.3. Extracellular glucose and lactate concentration

Extracellular glucose concentration in all tested RBC concentrates was gradually decreased during 6-week storage (422.593 ± 6.468mg/dl on day 1, 136.556 ± 4.525mg/dl on day 42; P=0.000). As shown in Figure 12, lactate production correlated with reduction in glucose level during RBCs storage (30.926 ± 2.061mg/dl on day 1, 299.444 ± 2.815mg/dl on day42; P=0.000). However, no significant differences were observed in the two parameters among the tested additive solutions. Furthermore, extracellular glucose consumption rate was weekly reduced and it was accompanied by lactate production. On the other hand, each additive solution contained sufficient glucose concentration on day 42 for further glycolysis (137.000 ± 4.737mg/dl, 137.111 ± 4.737mg/dl, 135.556 ± 4.737mg/dl; PAGGS-M, PAGGS-MB and PAGGS-MM, respectively). However, lactate accumulation gradually reduced the glycolysis rate throughout of storage time.



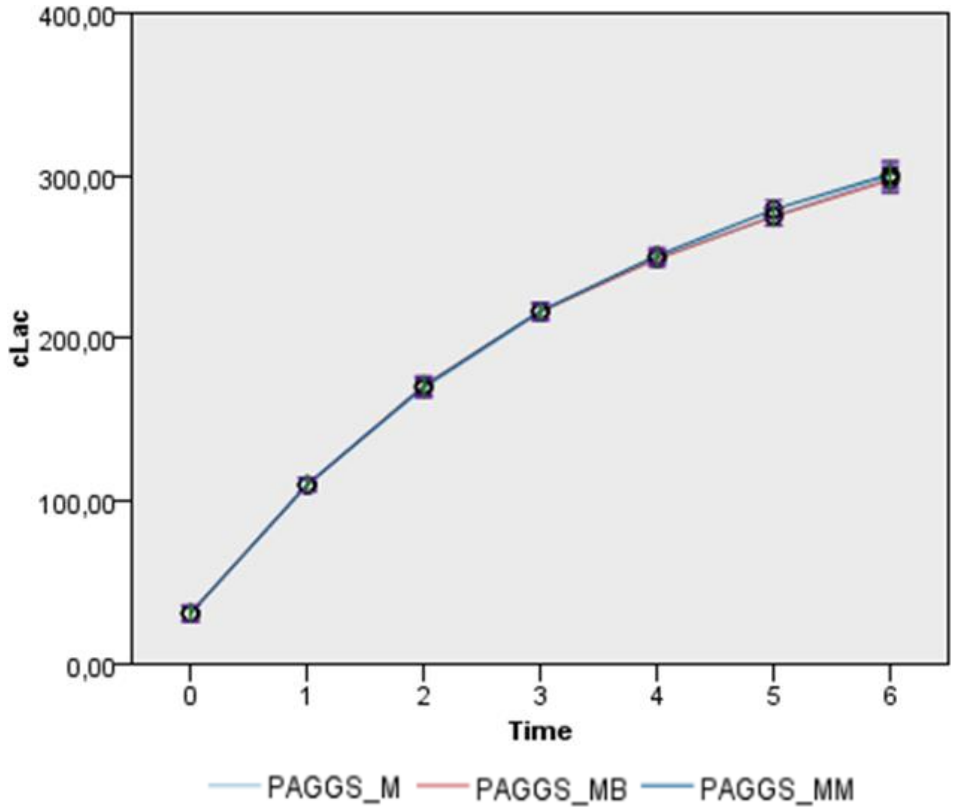


Figure 12: Decrease in extracellular glucose and increase in extracellular lactate concentrations (mg/dl) in packed RBCs stored in PAGGS-M (n=9), PAGGS-MB (n=9), PAGGS-MM (n=9) additive solutions for up to 42 days.

4.3. ATP and 2,3-DPG concentrations.

ATP concentration is one of the most important quality parameter for packed RBCs. In our experiment, the ATP level in packed RBCs stored in three different additives was reduced in the first two weeks, whereas it partially increased on the third week of storage (29047.778 ± 1707.178"relative fluorescence units" (RFU), 31067.222 ± 1707.178 RFU, 28369.444 ± 1707.178 RFU in PAGGS-M, PAGGS-MB and PAGGS-MM, respectively; P=0.204,). This may indicate a compensatory production of ATP from exogenous adenine present in additives. However, no statistical differences was observed in ATP concentrations among the RBCs stored three different additives (Figure 13).

2,3-DPG concentration dramatically decreased as reported in the literature [104] (from 4.472 ± 0.097 mmol/L on day 1 to 0.014 ± 0.010 mmol/L on day 42; P = 0.000). As shown in Figure 14, it was not measurable after 14 days of storage in RBCs stored in three different additive solutions (0.366 ± 0.091mmol/L, 0.301 ± 0.091mmol/L, 0.328 ± 0.091mmol/L; in PAGGS-M, PAGGS-MB and PAGGS-MM, respectively; P=0.881). Significant differences in

2,3 DPG level among packed RBCs stored in three different additives were not found during 6 week of storage.

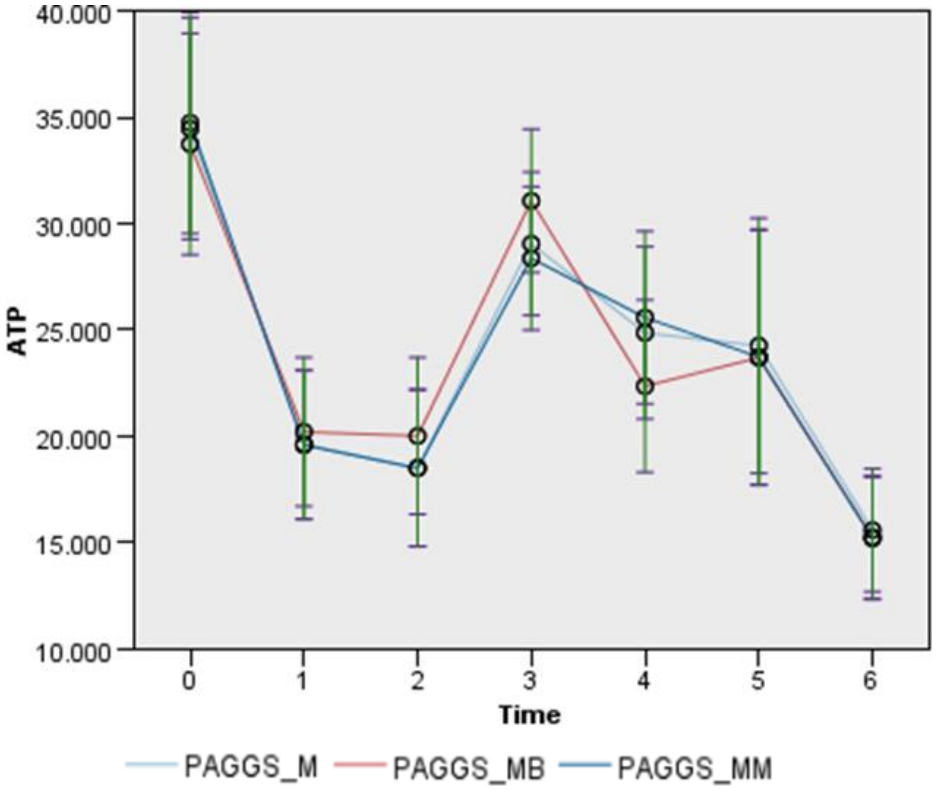


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Figure 13: Dynamic changes in ATP level (RFU) in packed RBCs stored in PAGGS-M (n=9), PAGGS-MB (n=9), PAGGS-MM (n=9) additive solutions for up to 42 days.

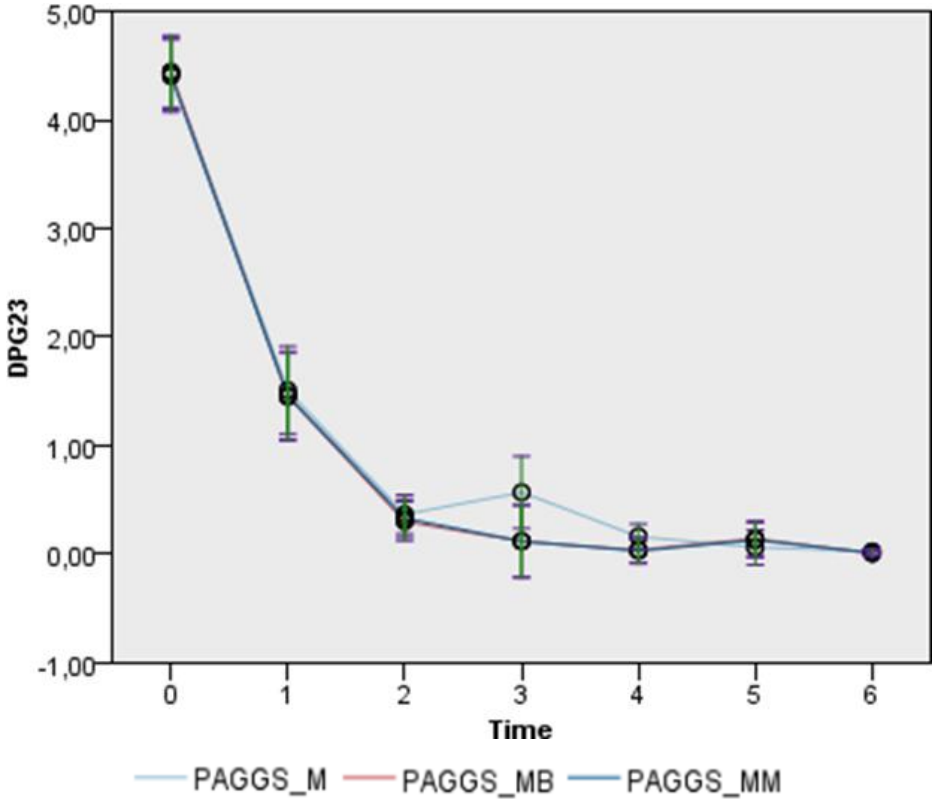
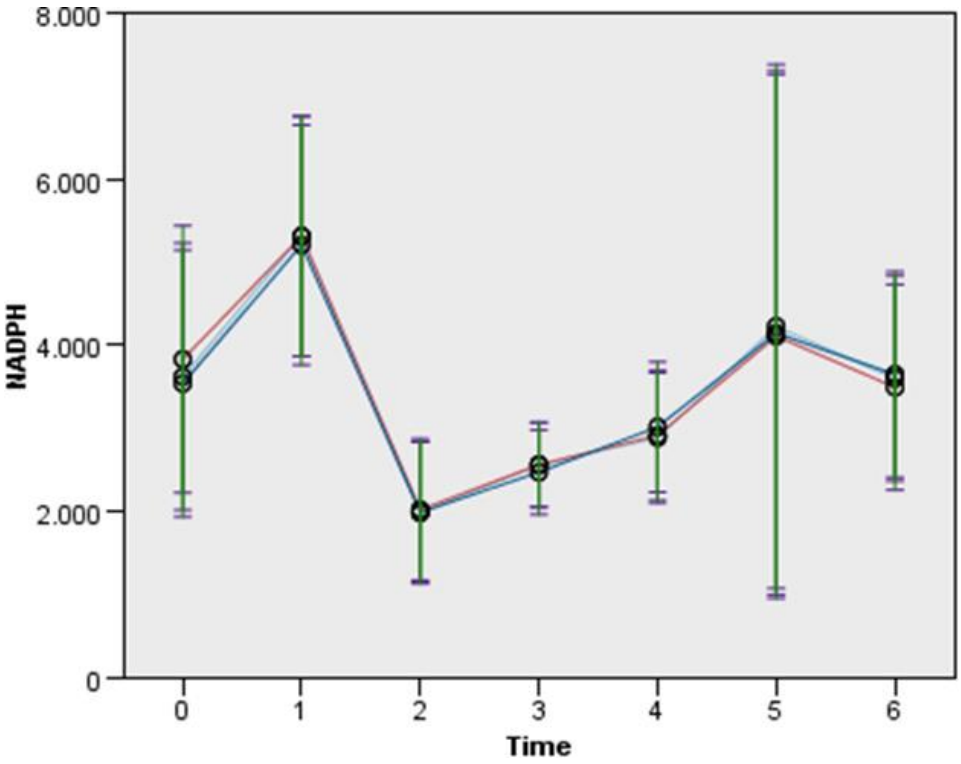


Figure 14: Dramatic decrease in 2,3-DPG level (mmol/L) in packed RBCs stored in PAGGS-M (n=9), PAGGS- MB (n=9), PAGGS-MM (n=9) for up to 42 days.

4.4. NADP/NADPH concentrations

Dynamic changes were observed in NADP and NADPH concentrations during the 6 weeks of RBCs storage. But their concentrations at the end of storage remained similar to the first day (2244.444 ±188.512 RFU day 1, 2372.259 ±307.731 RFU day 42; P=0.000 and 3667.108 ± 513.230 RFU day 1, 3582.444 ± 416.381 RFU day 42; P=0.000; NADP and NADPH, respectively). The amount of NADPH increased only in the first week in packed RBCs stored in all additive solutions. In the second week, NADP and NADPH concentrations decreased in all packed RBCs stored in PAGGS-M, PAGGS-MB and PAGGS-MM. However, none of these changes were statistically significant between three different additives (Figure 15). .



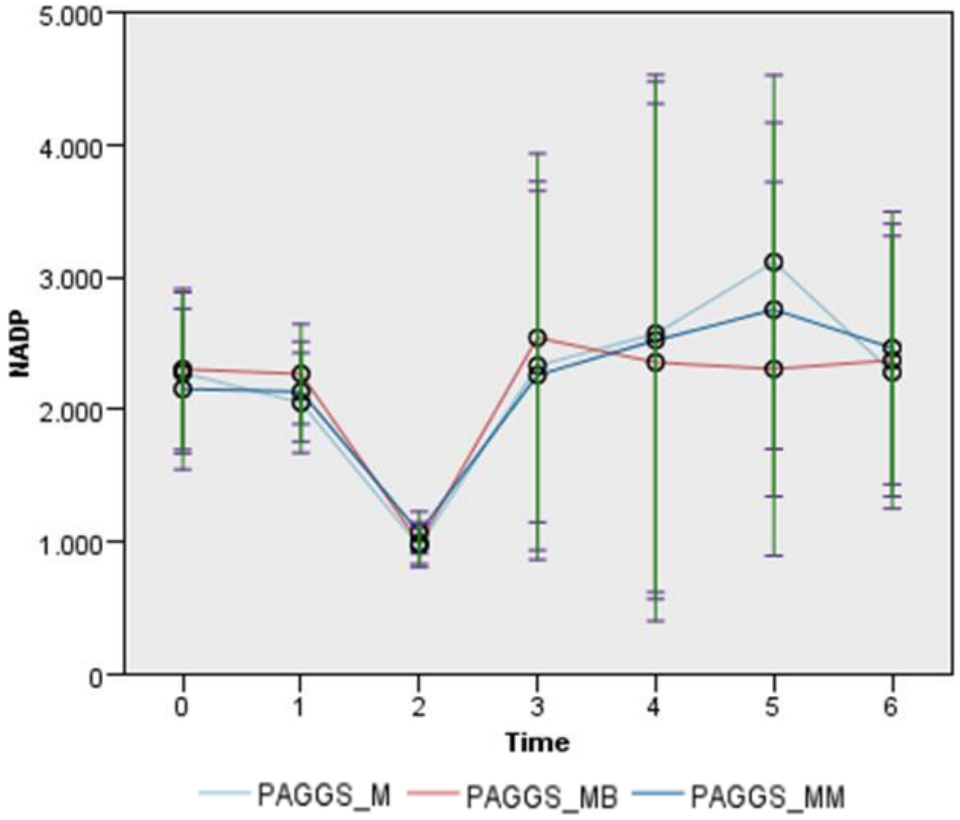


Figure 15: A dynamic changes in the level of NADP and NADPH (RFU) in packed RBCs stored in PAGGS-M (n=9), PAGGS- MB (n=9), PAGGS-MM (n=9) additive solutions for up to 42 days.

4.5. NAD/NADH concentrations

The NAD and NADH levels were dinamically changed in all tested RBCs during six week of storage. They were slightly reduced at the end of storage compared with the first day. (3.389 ± 0.042 RFU on day 1, 3.357 ±0.040 RFU on day 42; P=0.000 and 3.200 ±0.060 RFU on day 1, 2.900 ±0.040 RFU on day 42; P=0.000; NAD and NADH, respectively). The changes in NAD and NADH concentrations during the storage period were not statistically singificant among the tested additive solutions (Figure 16).

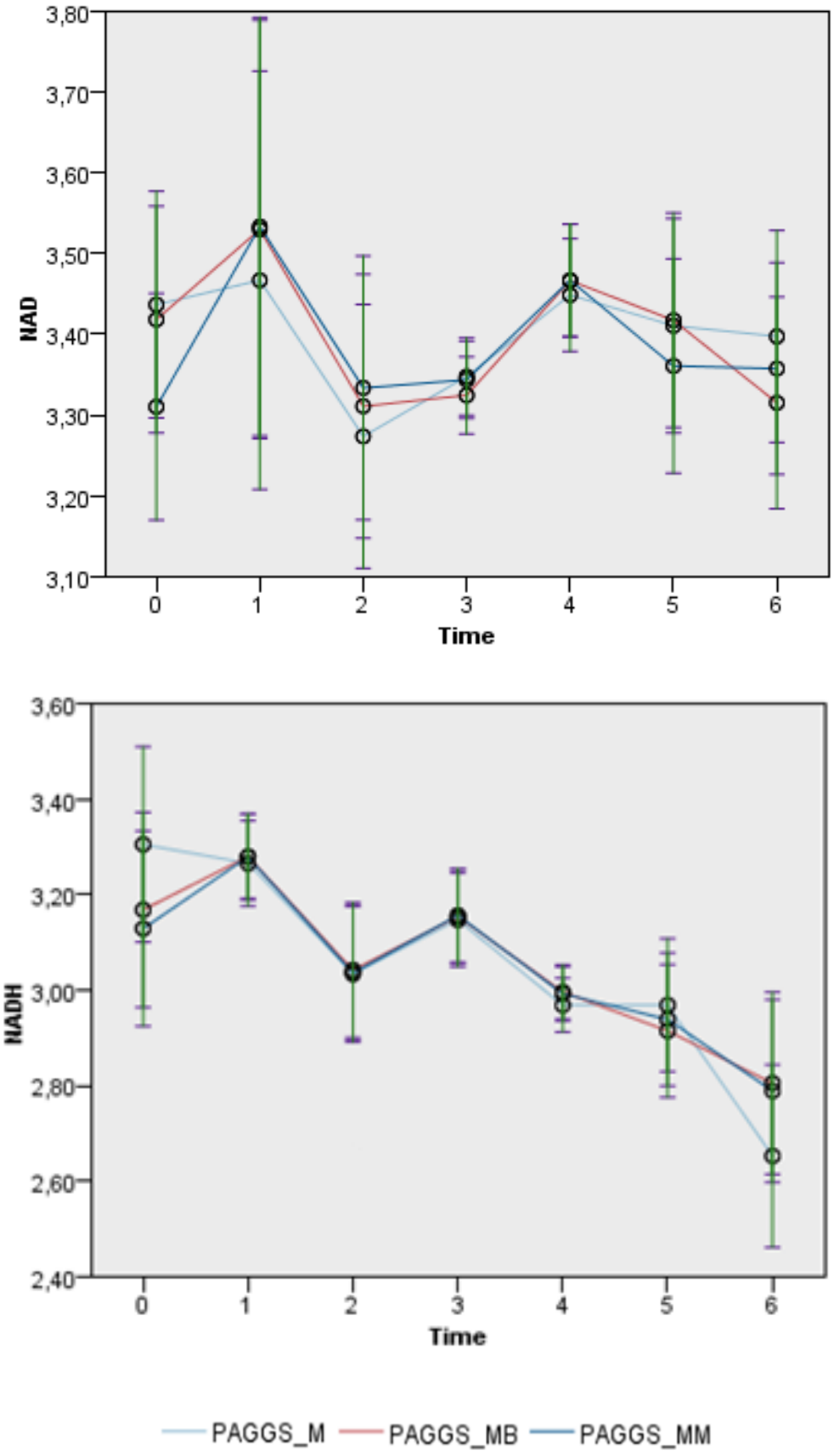
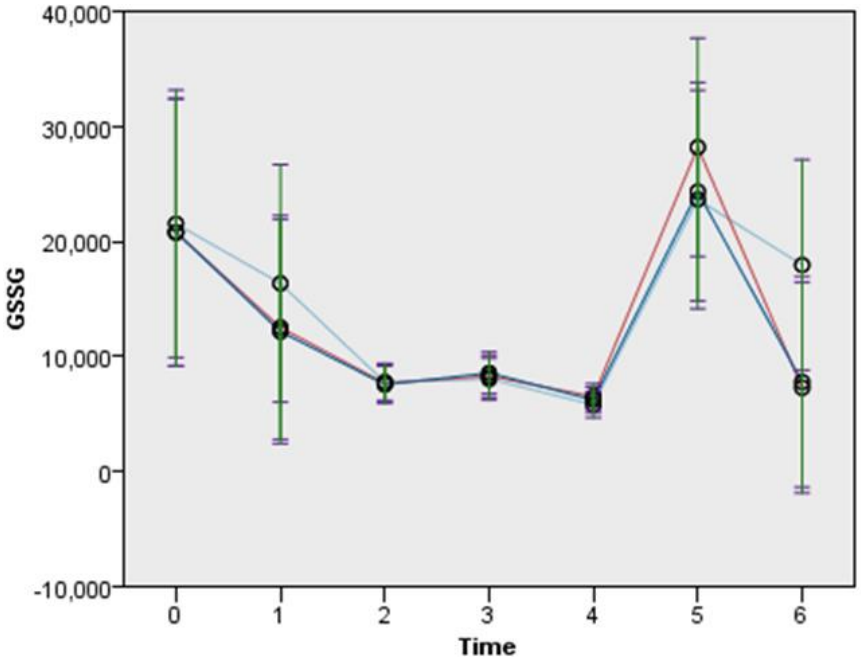


Figure 16: Dynamic changes in the concentrations of NAD and NADH (RFU) in packed RBCs stored in PAGGS-M (n=9), PAGGS- MB (n=9), PAGGS-MM (n=9) additive solutions for up to 42 days.

4.6. Total and oxidized glutathione –the redox status of stored RBCs

The redox buffer capacity of RBCs is calculated by measuring total (total GSH) and oxidized (GSSG) glutathione. In our study, these parameters were measured to assess the redox status of RBCs stored in three different additive solutions. Total and oxidized glutathione in all tested RBCs gradually decreased during the first two weeks of storage. (total GSH: 114.102 ± 13.269 RFU, 109.657 ± 13.269 RFU, 110.726 ± 13.269 RFU, P= 0.962 on day 1; 97.721 ±8.656 RFU, 97.703 ±8.656 RFU, 96.403 ± 8.656 RFU, P= 0.941on day 7; 68.415 ± 9.436 RFU, 75.854 ± 9.436 RFU, 75.191 ±9.436 RFU, P=0.716 on day 14; PAGGS-M, PAGGS-MB and PAGGS-MM, respectively). (GSSG: 21.535 ± 5.887 RFU, 20.786 ± 5.887 RFU, 20.795 ± 5.887 RFU, P= 0.995 on day 1; 25.847 ± 7.312 RFU, 12.515 ± 7.312 RFU, 12.160 ± 7.312 RFU, P=0,312 on day 7; 7.775 ± 4.822 RFU, 7.673 ± 4.822 RFU, 7.547 ± 4.822 RFU, P= 0.972 on day 14; PAGGS-M, PAGGS-MB and PAGGS-MM, respectively)(Figure 17). But, GSSG level was slightly higher in PAGGS-M during this period compared with PAGGS-MM- and PAGGS-MB-stored RBCs (Figure 17). At week 5, total glutathione level increased in all tested RBCs due to the elevation of GSSG. However, the changes either in total GSH, or in GSSG levels was not statistically significant among the RBCs stored in three different additive solutions.



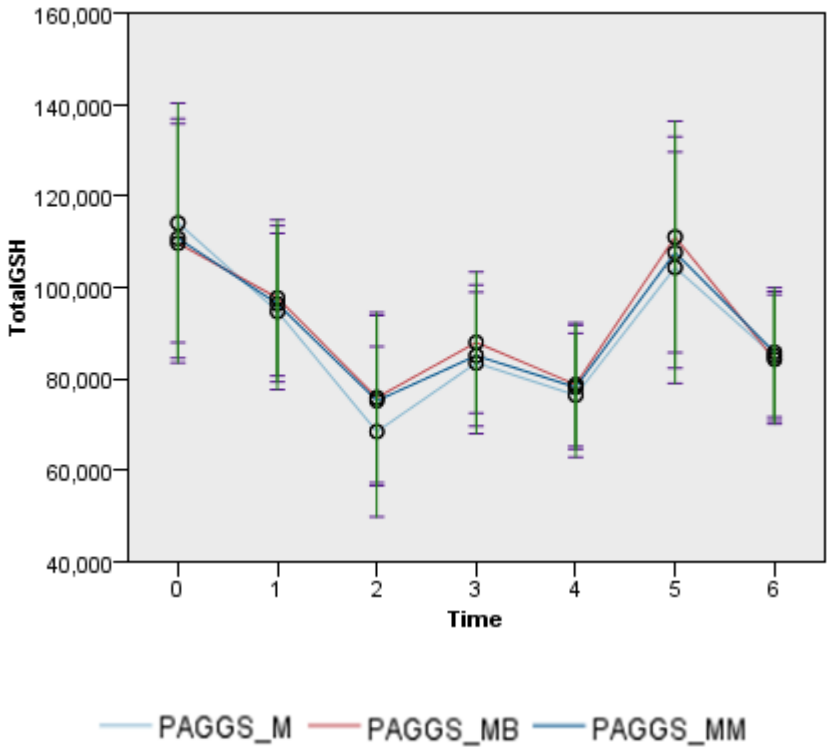


Figure 17: Alteration in total and oxidized (GSSG) glutathione levels (RFU) in packed RBCs stored in PAGGS-M (n=9), PAGGS- MB (n=9), PAGGS-MM (n=9) additive solutions for up to 42 days.

The ratio of oxidized form of glutathione to total glutathione (GSSG / Total GSH) was calculated to estimate the level of oxidative stress in RBCs stored in three different additive solutions. Interestingly, oxidative stress in packed RBCs stored in PAGGS-M additive solution was 2-fold higher in the first week compared with PAGGS-MB and PAGGS-MM (30.081 ± 8.998 % vs 13.753 ± 8.998 %, and 13,861 ± 8.998 %, P= 0.337), but it was not statistically significance due to the high standard deviation (Figure 18).

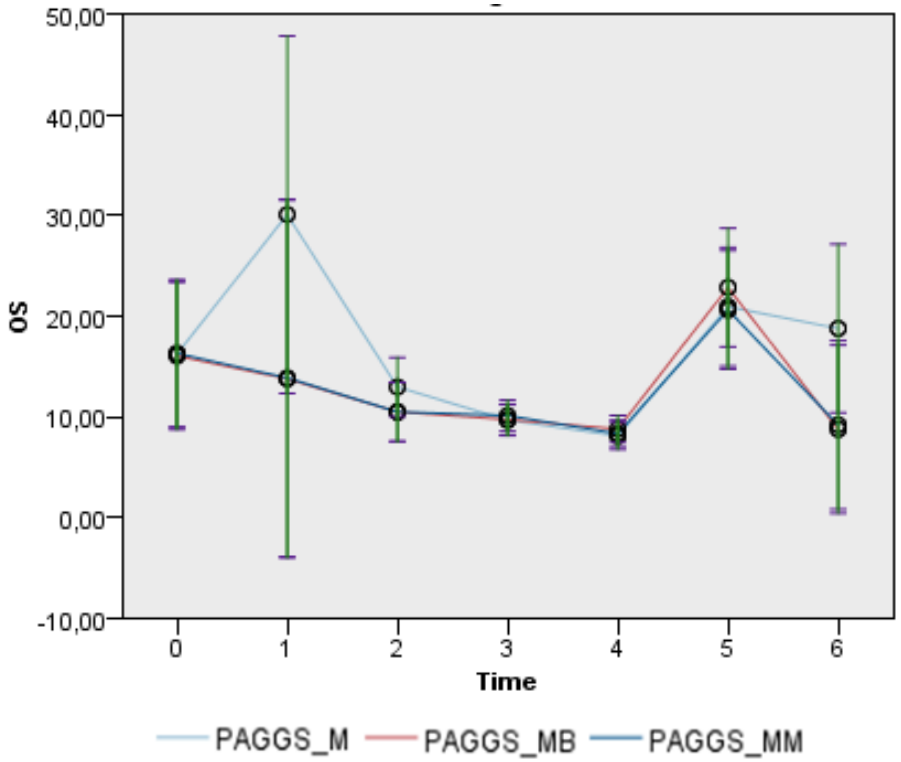


Figure 18: The state of oxidative stress (% GSSG / Total GSH) in packed RBCs stored in PAGGS-M (n=9), PAGGS- MB (n=9), PAGGS-MM (n=9) additive solutions for up to 42 days.

4.7. Extracellular hemoglobin (eHB) and hemolysis rate

Hemolysis rate is a widely accepted and well established parameter to monitor of RBC storage. We observed a significantly lower hemolysis rate in PAGGS-MM-stored RBCs in comparison to PAGGS-M- and PAGGS-MB-stored RBCs (0.215 ± 0.043 % vs. 0.410 ± 0.043 % and 0.410 ± 0.043 %, P=0.000). The visual differences among the supernatant of RBCs stored in three different additives are shown on Figure 20. The hemolysis rate increases significantly over storage time (0.332 ± 0.073 % on day 1, 0.434 ± 0.051 % on day 42; P=0.000). Though, there was no correlation between type of additive solution and storage time (P = 0.986). This indicates the slope of the increase in hemolysis rate over the storage time is not different among any of three types of additive solutions. More precisely, L-Methionine supplementation has demonstrated a protective effect only from the beginning of the storage period. However, the lowest hemolysis rate was maintained in PAGGS-MM-stored RBCs during 6 weeks of storage compared with other two additives. As shown in figure 19, changes in extracellular hemoglobin concentration over storage time has demonstrated very similar characteristics to hemolysis rate, as these two variables are correlated.

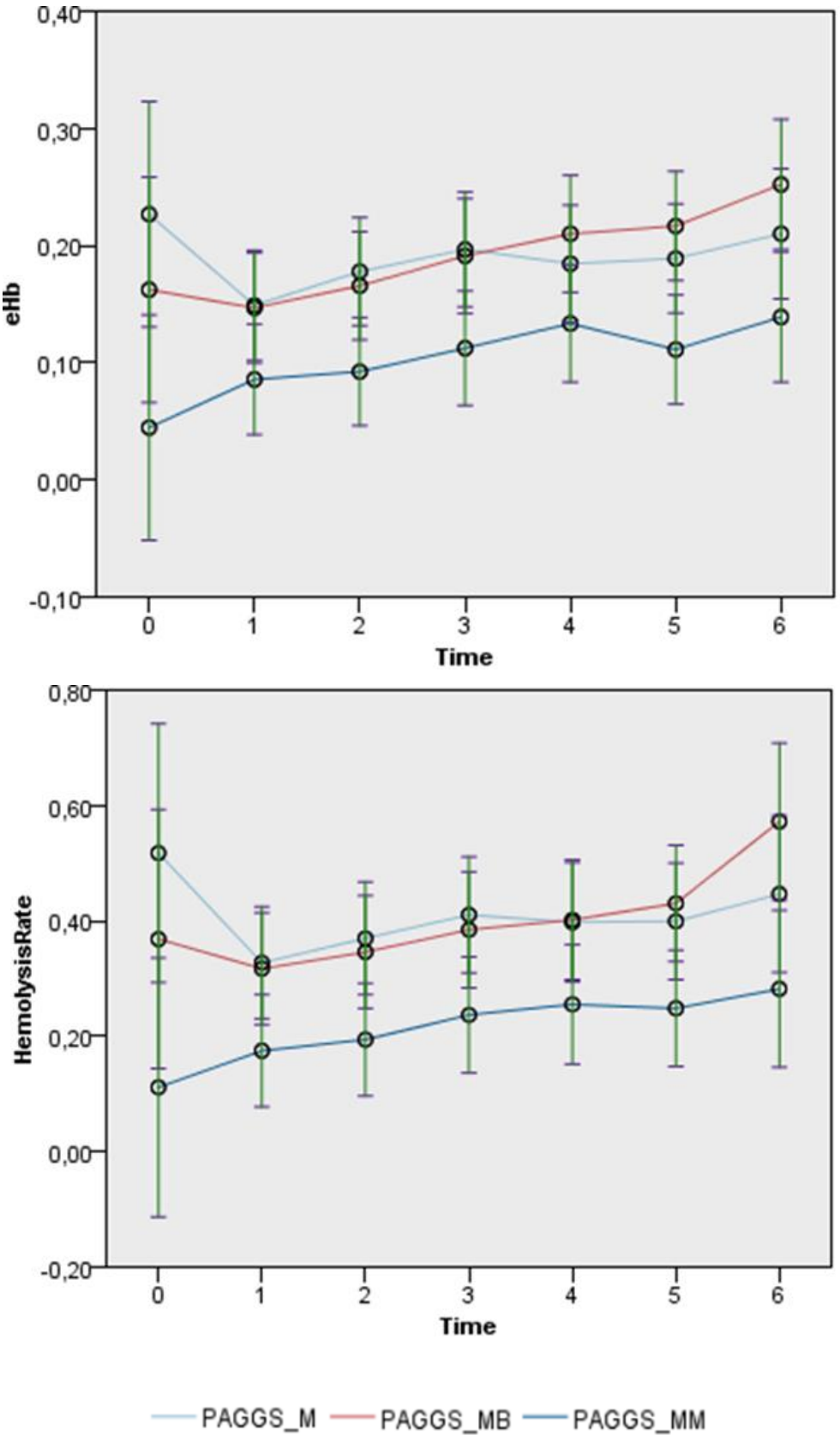


Figure 19: Gradual increase in extracellular hemoglobin level (g/dl) and percent of hemolysis rate in packed RBCs stored in PAGGS-M (n=9), PAGGS-MB (n=9), PAGGS-MM (n=9) additive solutions for up to 42 days.

Figure 20. Comparison of RBC supernatants of different pools stored in PAGGS-M, PAGGS-MB and PAGGS-MM additive solutions in the second and third weeks of RBCs storage. PAGGS-M (**C** - control, pool C, D, E, F, G, H, I, J, in the second and third weeks of RBCs storage), PAGGS-MB (**B** – supplemented with vitamin B_{12} + 5MTHF, pool C, D, E, F, G, H, I, J, in the second and third weeks of RBCs storage), PAGGS-M (**M** – supplemented with L-Methionine, pool C, D, E, F, G H, I, J, in the second and third weeks of RBCs storage).

4.8. Metabolomics

4.8.1. Comparative metabolomics analysis of stored RBCs in PAGGS-M, PAGGS-MB and PAGGS-MM

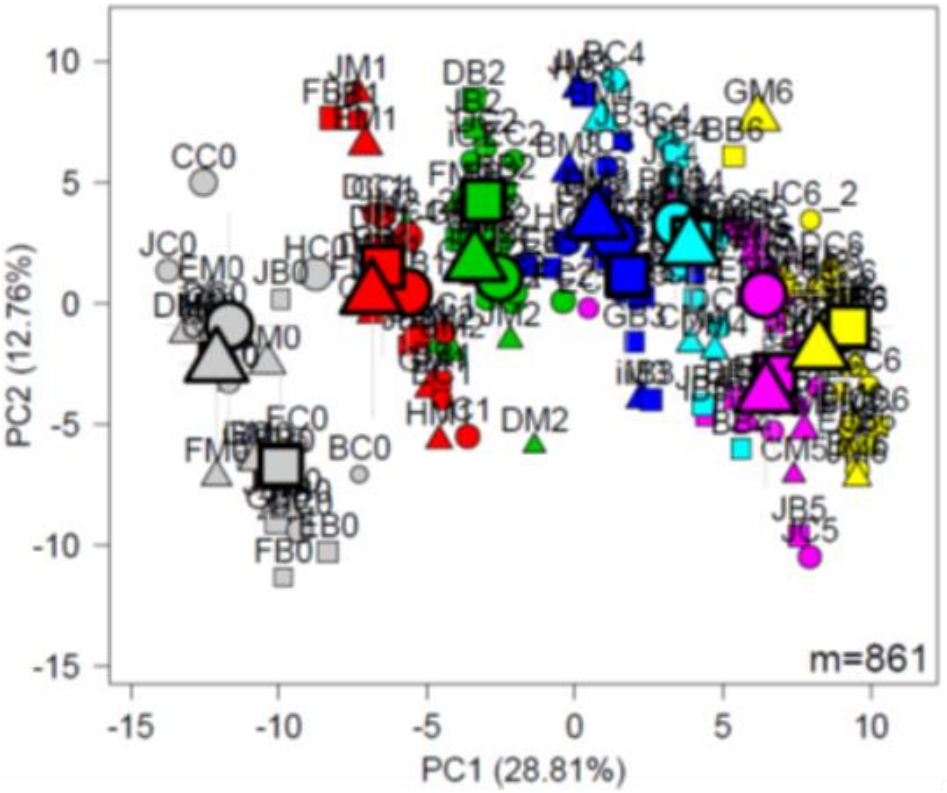
The comparative metabolomics analysis was performed from the supernatants of PAGGS-M-, PAGGS-MB- and PAGGS-MM-stored RBCs using GC-APCI/MS based metabolomics platform. A total of 861 base peak signals from MS spectra (untargeted), representing 788 non-annotated and 73 unique annotated metabolites (targeted) were detected and analyzed by two-way ANOVA approach.

Using unsupervised PCA, 166 analyzed samples were clustered according to the profiles of untargeted metabolites (861 data points) or targeted metabolites (73 data points) in the first two principal components (Figure 21). PCA of non-filtrated normalized data revealed a distinct metabolic profile of each stored RBCs, which were separated on the basis of storage time and additive solution. We found that the metabolic profile of PAGGS-MM-stored RBCs separated from the metabolic profile of PAGGS-M- and PAGGS-MB-stored RBCs (Figure 21A). Moreover, the metabolic profile of PAGSS-M- and PAGGS-MB-stored RBCs clustered together. This distinct metabolic profile of PAGGS-MM-stored RBCs is more visible through supervised PCA, when non-significantly metabolites were filtered using P-values for Treatment: TP (Figure 21B). Interestingly, supervised PCA, in which the metabolites were filtered by P-values for storage time (TP), also demonstrated metabolic changes during the storage occurred predominantly in the first three week of storage and metabolic profile from 21^{th} days of storage tended to cluster together (Figure 21C).

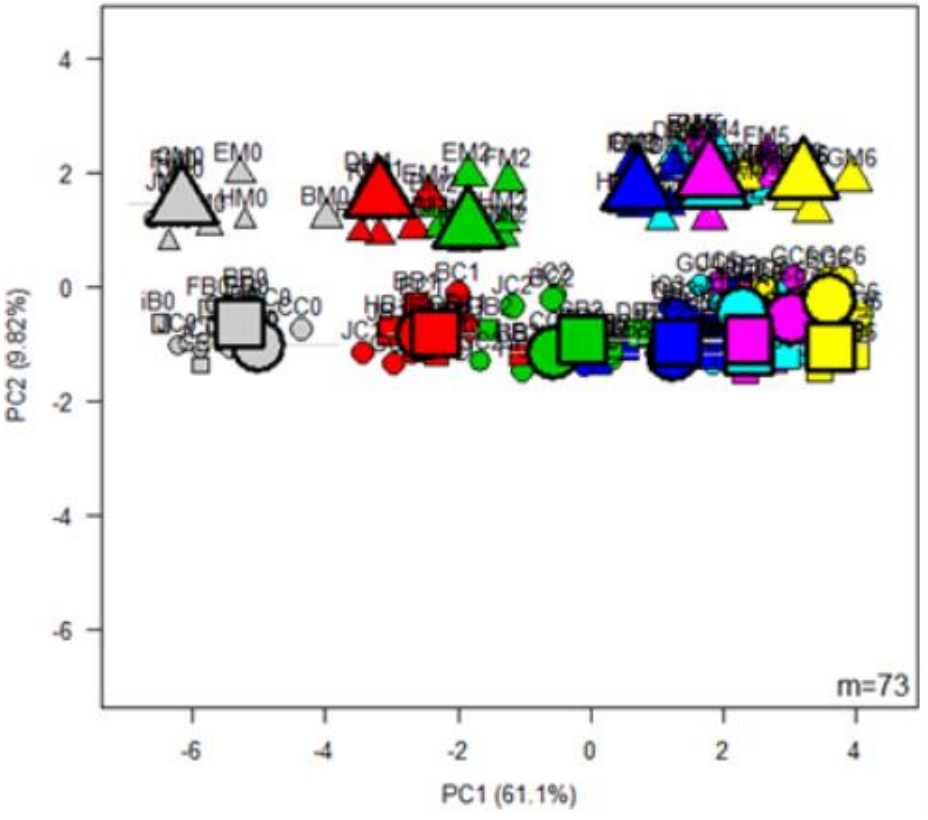
Altogether, PCA analysis of metabolomics data indicates that methionine supplementation of PAGGS-M additive solution (PAGGS-MM) resulted in pronounced changes in the metabolic profile of stored RBCs, whereas vitamin B supplementation (PAGGS-MB) is hardly different from the PAGGS-M additive solution in respect to metabolomic profile during the 6-week storage.

A

Untargeted Metabolomics

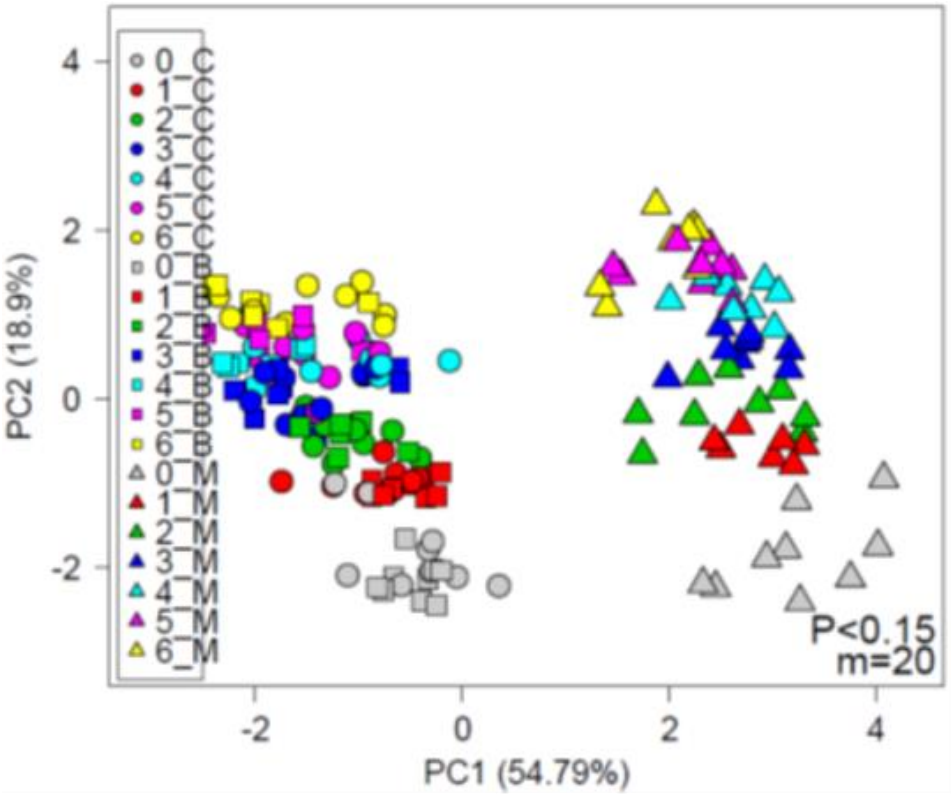


Targeted Metabolomics

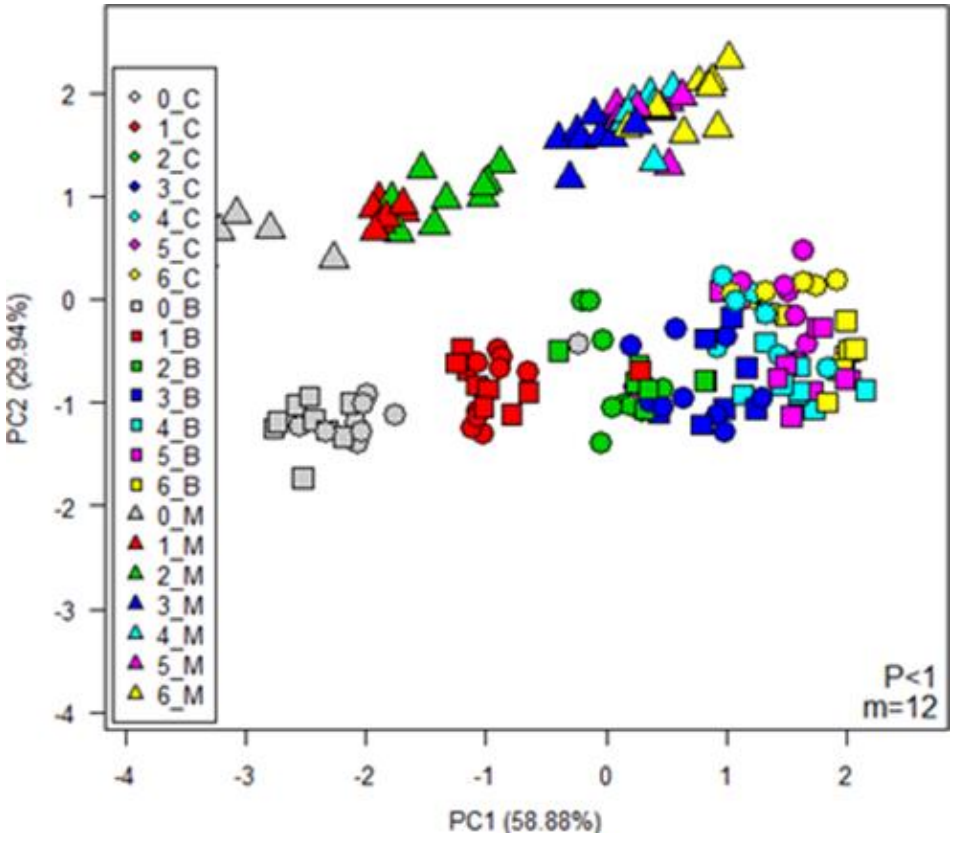


B

Untargeted Metabolomics

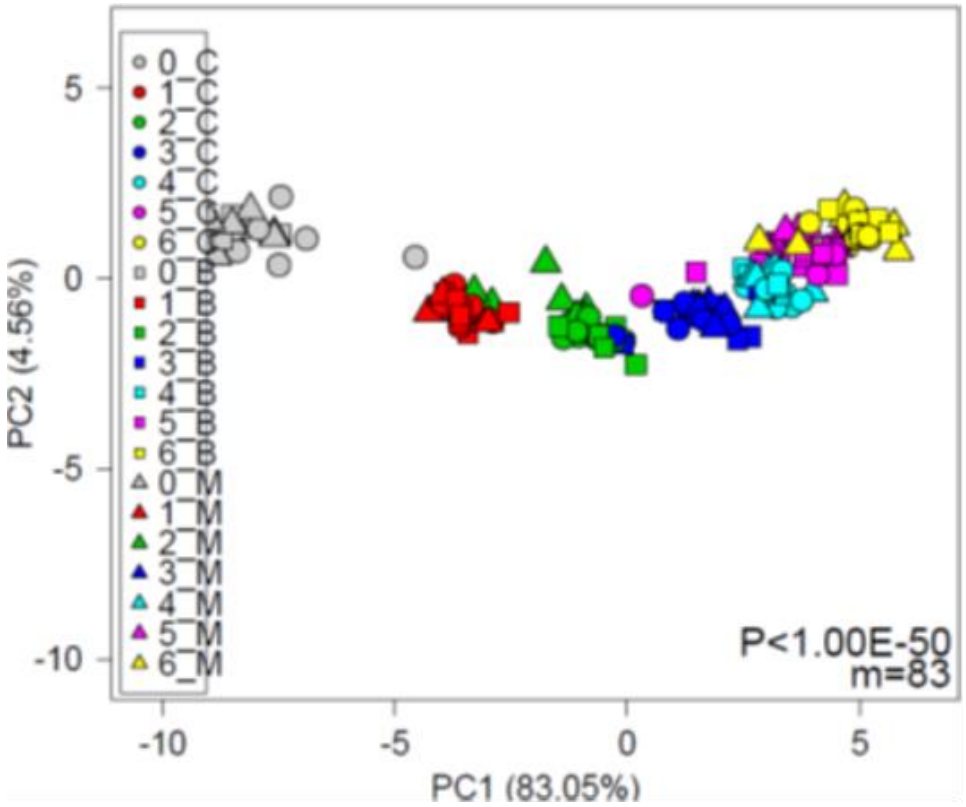


Targeted Metabolomics



C

Untargeted Metabolomics



Targeted Metabolomics

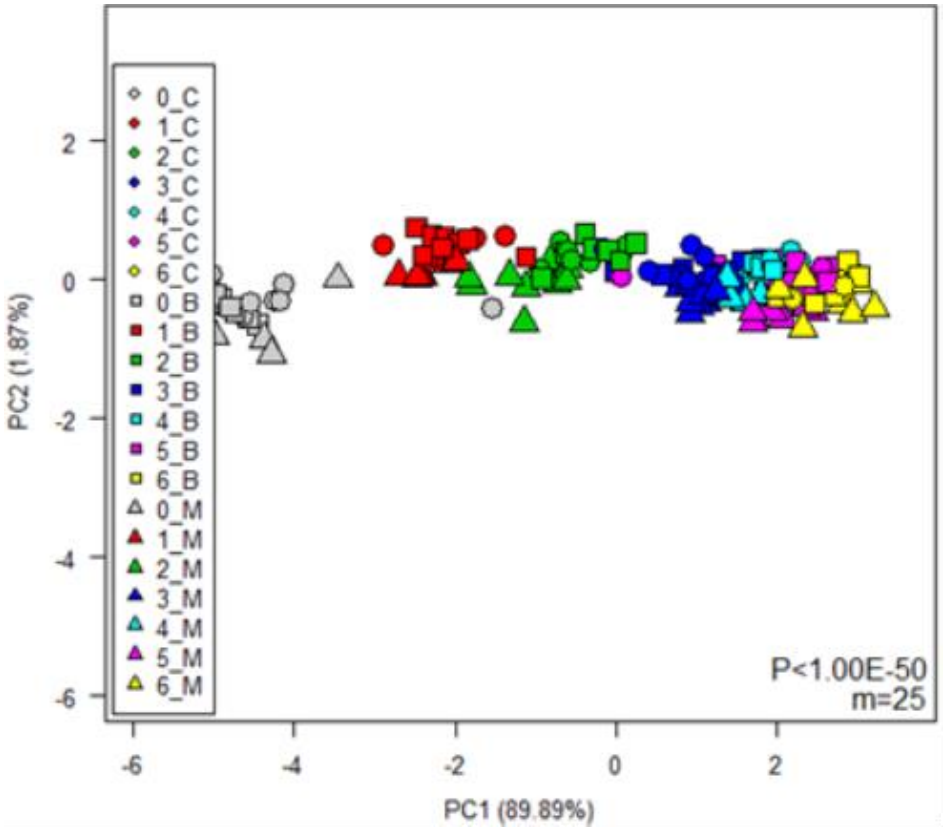


Figure 21: Comparative analysis of metabolomics profiles of PAGGS-M-, PAGGS-MB- and PAGGS-MM-stored RBCs using principal component analysis (A- unsupervised PCA, B- supervised PCA by filtration of metabolites using P-values for "Treatment:TP" C- supervised PCA by filtration of metabolites using P-values for "storage period "TP").

4.8.2. Comparison of the glycolysis and pentose phosphate pathways (PPP) in stored RBCs

PPP and glycolysis are closely linked metabolic pathways, as the intermediate of glycolysis (glucose-6-phosphate) serves as an initial substrate for the PPP. Under normal physiological conditions, up to 92% of glucose is metabolized through glycolysis to produce ATP, while 8% of glucose is utilized through PPP to form NADPH and reduce GSSG [199-201]. In contrast, under oxidative stress, RBCs diverts nearly 90% of glucose metabolism toward the PPP. The main function of PPP is to generate the majority of NADPH in RBC through reduction of $NADP^+$. This reaction is coupled with the formation of 6-phosphogluconolactone from glucose 6-phosphate (G6P) and ribulose 5-phosphate (R5P) from gluconic acid−6−phosphate. In the present study, an increase in the concentrations of PPP intermediates was not observed. Moreover, most of them significantly decreased in PAGGS-MM-stored RBCs in comparison to PAGGS-M- and PAGGS-MB-stored RBCs (Figure 22). Interestingly, the concentrations of PPP intermediates, such as ribose 5-phospahte, glucose 6-phosphate, fructose-6-phospate and glycemic acid-3-phosphate, were abruptly reduced in the first day of storage in the supernatant of RBCs stored in PAGGS-MM additive solution. However, the changes of their concentrations during the 6 weeks' storage demonstrated a similar profile among the tested additive solutions. This indicates that the effect of methionine on PPP occurred mainly at the start of the storage and afterward the metabolic shift of the glycolysis toward PPP was not negatively regulated by methionine supplementation. The reduction of PPP caused by methionine supplementation may be related to a lower hemolysis rate in PAGGS-MM-stored RBCs

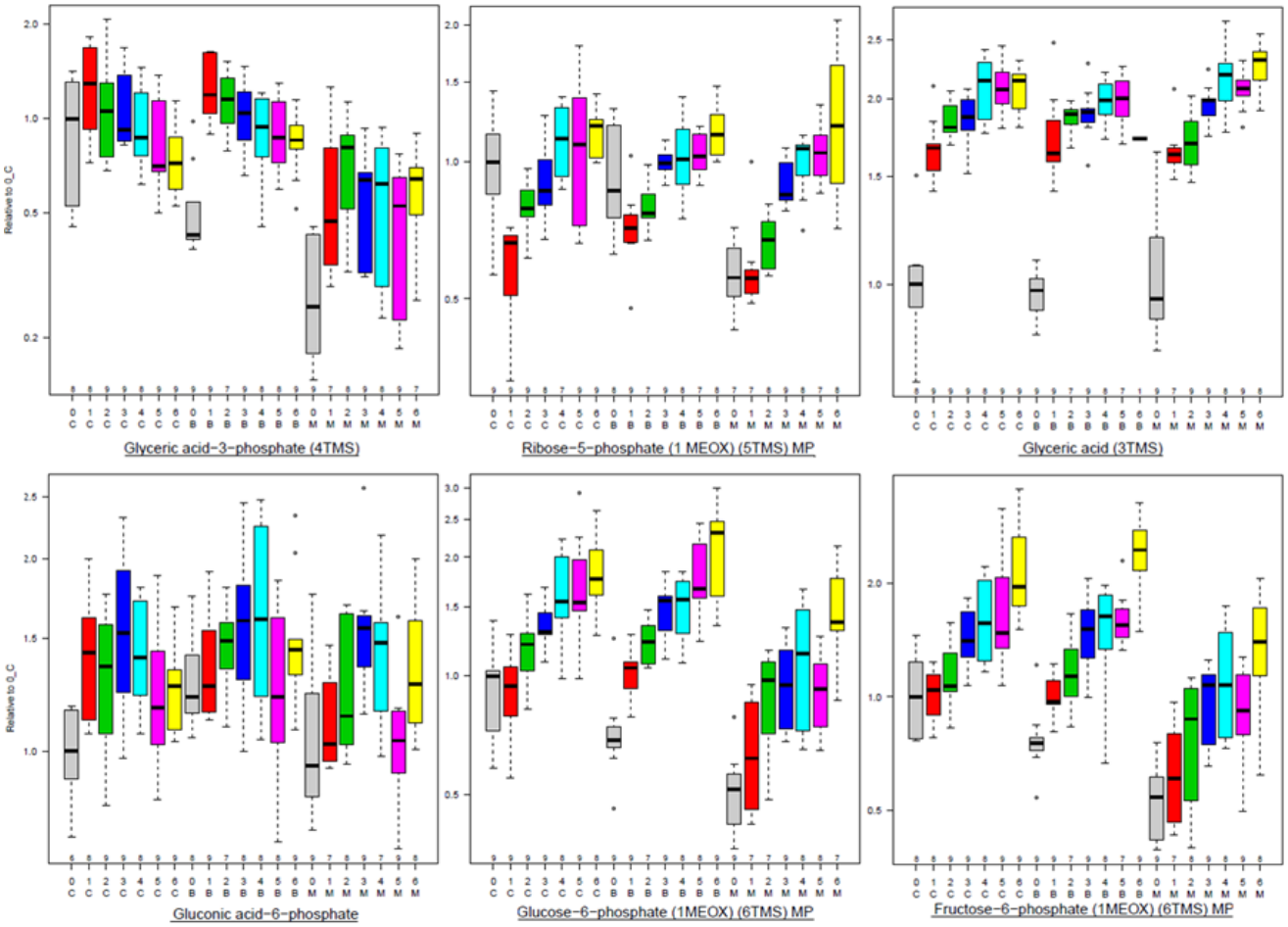


Figure 22: An overview of the trends for the pentose phosphate pathway intermediates in the course of storage in PAGGS-M, PAGGS-MB, and PAGGS-MM additive solutions. Results are plotted on a weekly basis as fold-change variations upon normalization against week 0 of PAGGS-M controls.

4.8.3. Metabolic fate of methionine in stored RBCs: transamination or transsulfuration pathway

Methylation and anti-oxidative metabolism in RBCs are linked by the transsulfuration pathway, where the methionine cycle intermediate - homocysteine is converted to cysteine, which is a precursor for glutathione biosynthesis. In the present study, we proposed to increase anti-oxidative capacity of RBCs and to replace the depleted methyl donor with the supplementation of L-Methionine. As shown in Figure 23, methionine supplementation resulted in nearly 100-fold higher methionine concentration in PAGGS-MM compared with PAGGS-M, or PAGGS-MB. Interestingly, methionine in PAGGS-MM-stored RBC supernatant was consumed only in the first week of storage, then its concentration increased again till the fourth week of storage. At the fifth and sixth weeks, the concentration of

methionine was equal to its initial level. In contrast, the level of methionine progressively decreased in RBCs supernatant stored in PAGGS-M and PAGGS-MB additives.

The concentrations of methionine transsulfuration intermediates, such as homocysteine and cysteine, were increased in PAGGS-MM-stored supernatants compared with PAGGS-M- and PAGGS-MB-stored supernatants (P=0.004 for homocysteine, and P=0.001 for cysteine). However, this remarkable change of increased intermediates did not correlate with methionine consumption in packed RBCs stored in PAGGS–MM additive solution. The results suggest that a small amount of consumed methionine was metabolized in the transsulfuration pathway. Only one non-annotated metabolite, detected in untargeted metabolomics (177.0764 @ 584 [600]) with highest P value (0.0008), has shown such high fold changes in PAGGS-MM stored RBC supernatant and this may correspond to high methionine consumption in PAGGS-MM. A corresponding spectrum was manually annotated as 2-Hydroxy-4-(methylthio) butyric acid (HMBA). Manual annotation of HMBA was confirmed using standard compounds (Sigma #55875). . The formation of HMBA we also observed in supernatant of RBCs stored in PAGGS-M and PAGGS-MB additive solutions.

This result indicates that the metabolic fate of the supplemented methionine in PAGGS-MM stored RBC is shifted particularly towards the transamination pathway. However, the formation of HMBA in PAGGS-M and PAGGS-MB additive solutions confirmed that methionine in physiological concentration also undergoes transamination.

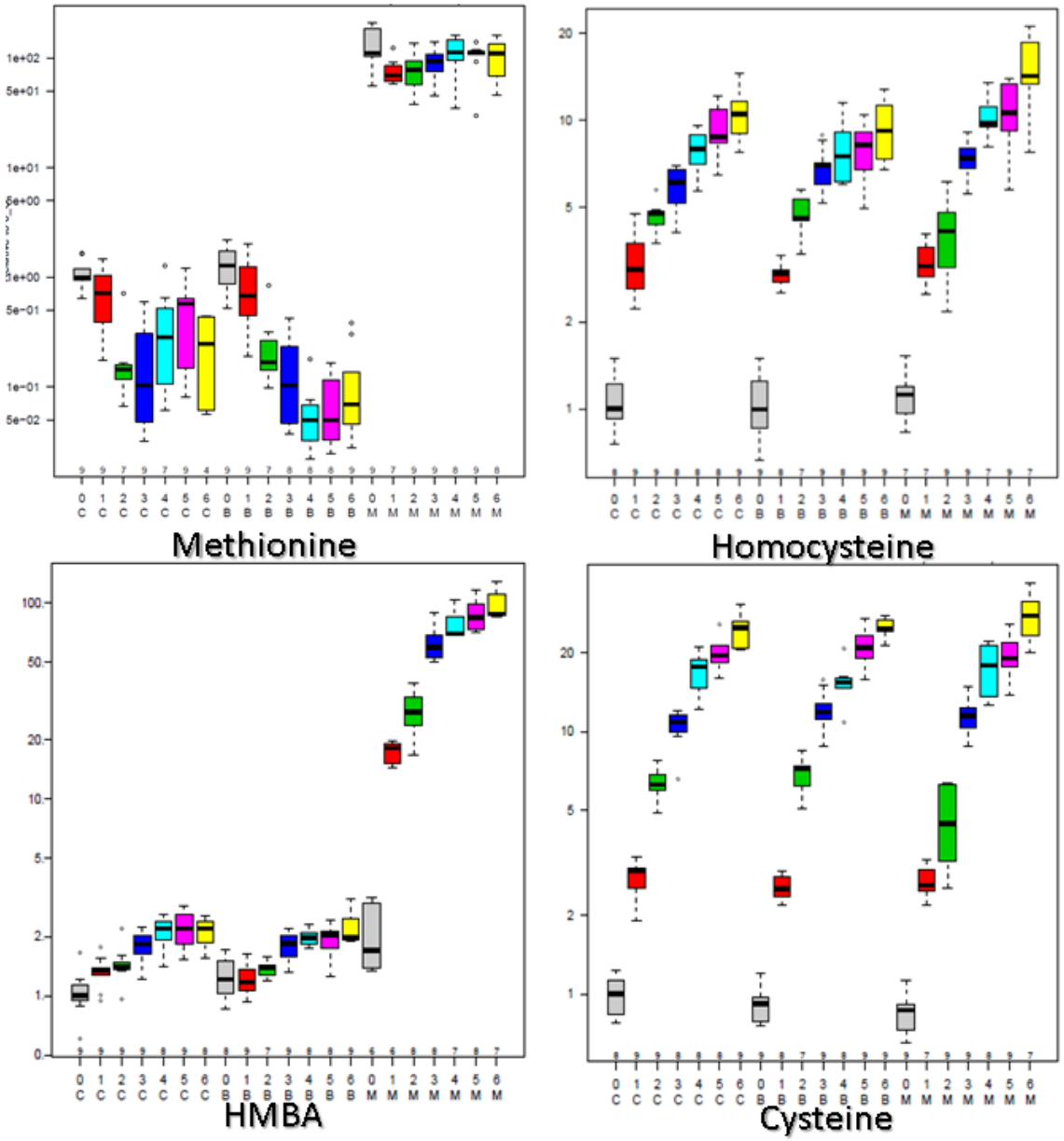
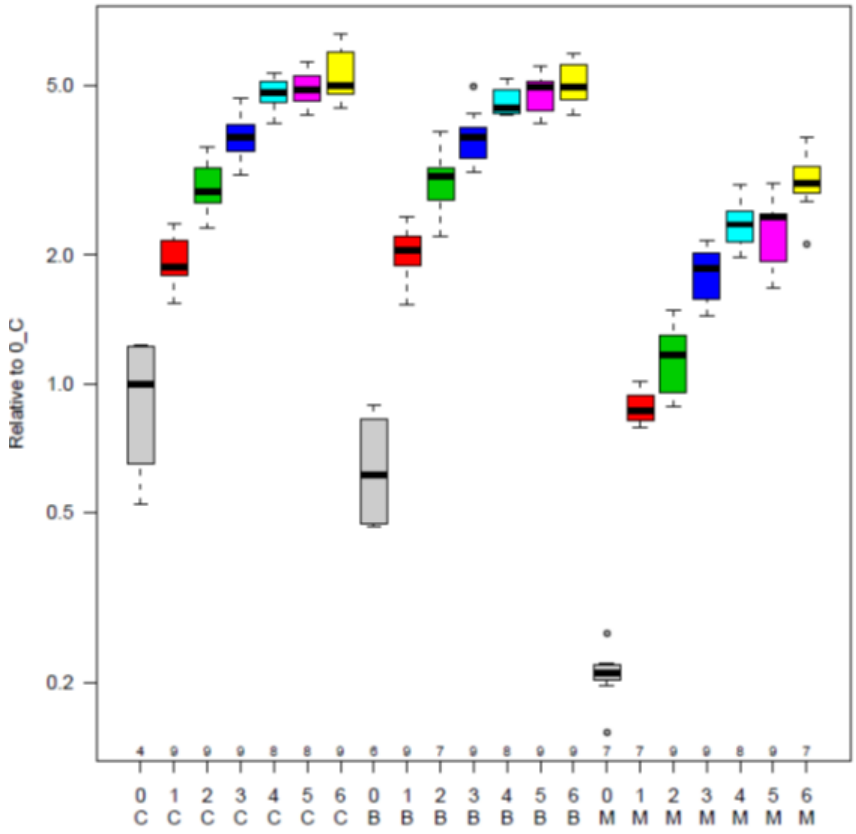


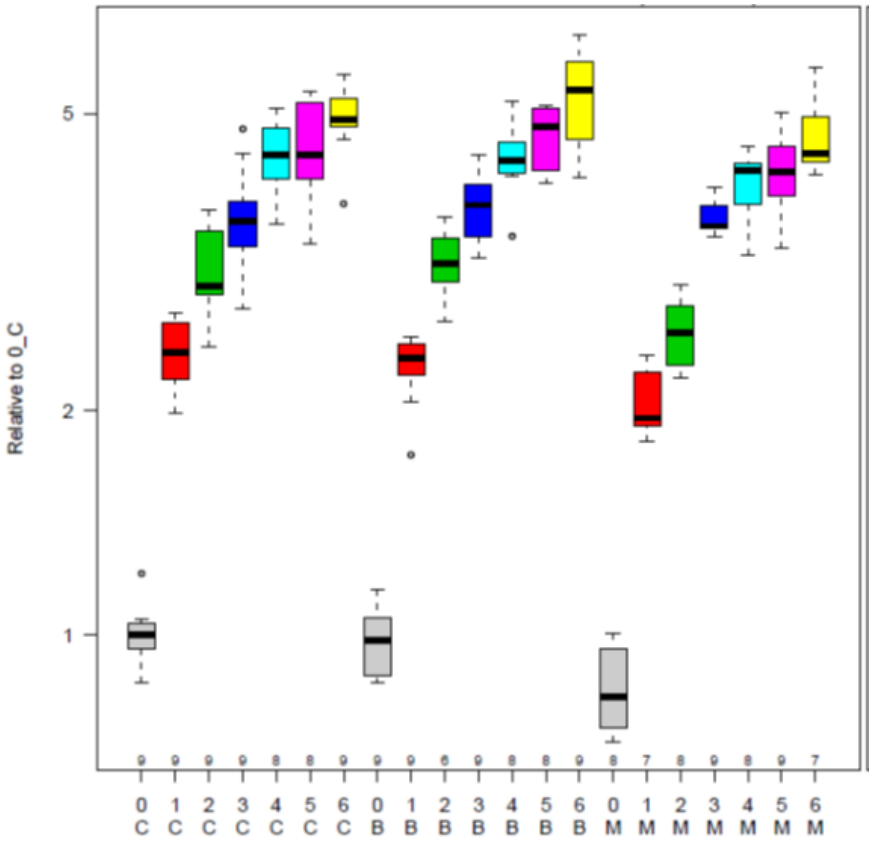
Figure 23: An overview of methionine metabolism in the course of storage in PAGGS-M, PAGGS-MB, and PAGGS-MM additive solutions. Results are plotted on a weekly basis as fold-change variations upon normalization against week 0 of PAGGS-M controls. HMBA: 2-Hydroxy-4-(methylthio) butyric acid.

Interestingly, methionine supplementation inhibited the formation of aspartic acid in RBCs stored in PAGGS-MM additive solution. Moreover, the level of aspartic acid was significantly lower in PAGGS-MM- stored RBCs compared with the RBCs stored in two other additives. This may be explained by the activation of the transamination reaction (Figure 24).

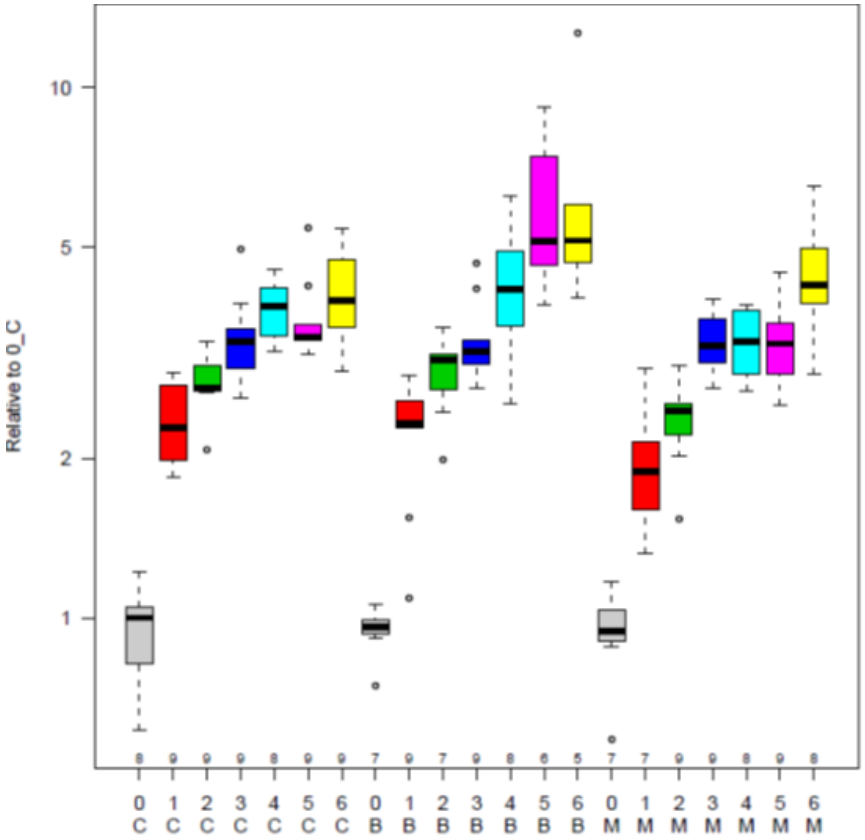
Aspartic acid (3TMS)



Glutamic acid (3TMS)



Glutaric acid, 2-oxo- (2TMS)



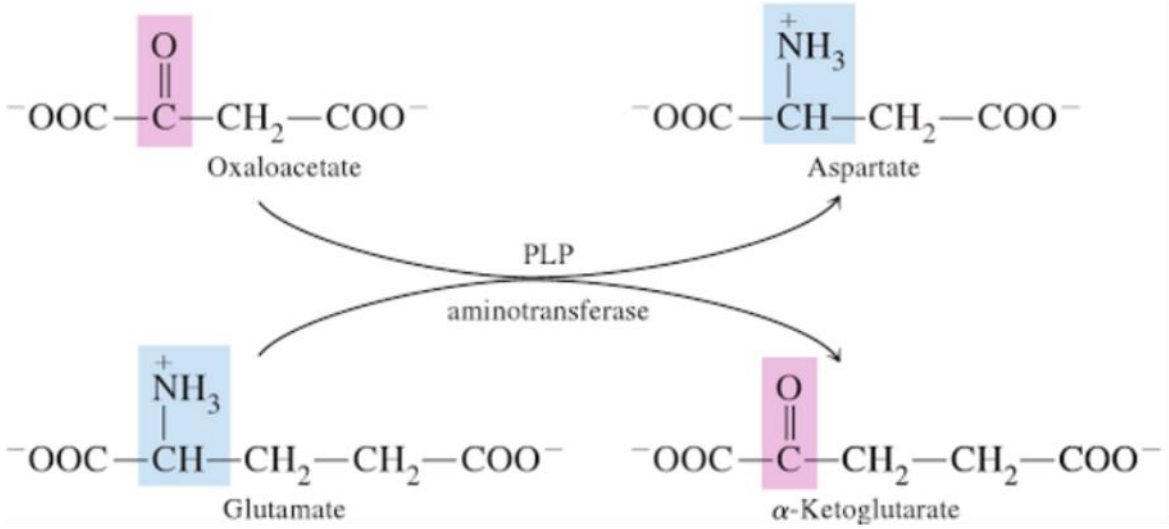
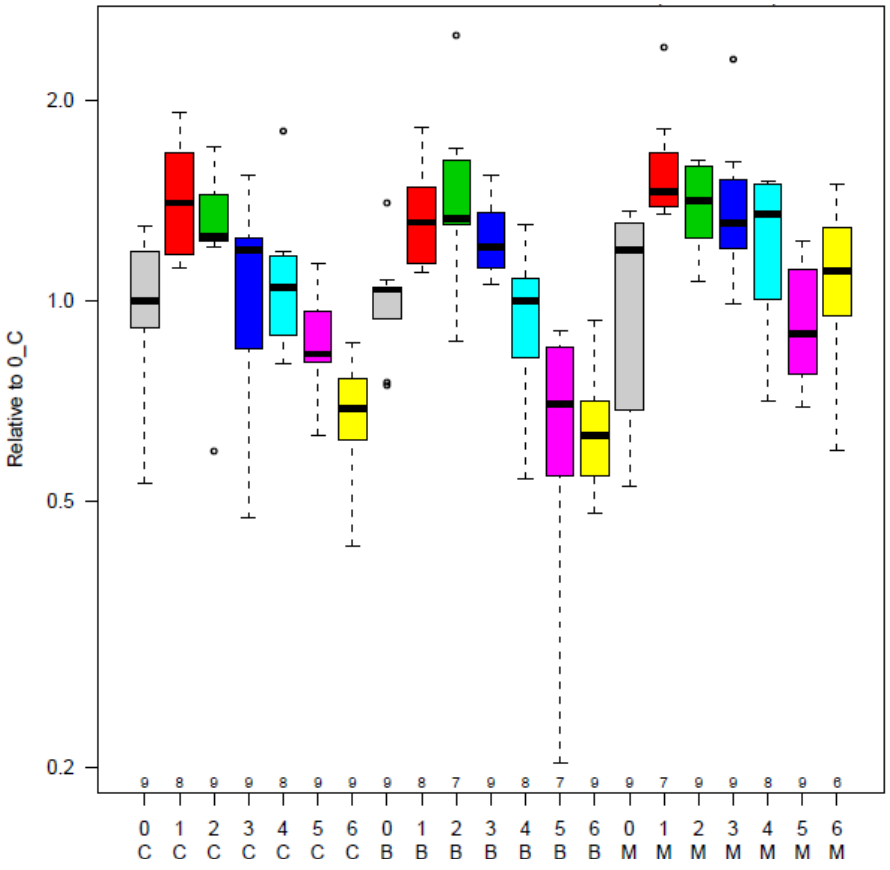


Figure 24: The formation of aspartic acid by the transamination pathway is significantly reduced in RBC supernatants stored in PAGGS-MM in comparison to the RBC supernatants stored in PAGGS-M and PAGGS-MB additive solutions.

4.8.4. The various effects of methionine supplementation on metabolic profile in packed RBCs

RBCs use extracellular glutamine to form intracellular glutamate, which is a precursor of glutathione synthesis. We observed a significantly high concentration of glutamine in the supernatants of RBCs stored in PAGGS-MM during 6 weeks of storage compared with other samples (Figure 25A). Another effect we observed on the consumption of extracellular adenine during the storage of RBCs, which was 2-fold lower in PAGGS-MM in the second week of RBCs storage compared with PAGGS- M and PAGGS-MB additives (Figure 25 B). Furthermore, citric acid concentration in PAGGS-MM stored RBCs was significantly higher in comparison to PAGGS-M and PAGGS-MB additives. The concentration of citric acid was increased only in PAGGS-MM additive solution, especially in the first week of storage, while it decreased in other additives (Figure 25 C).

Glutamine (4TMS) (A)



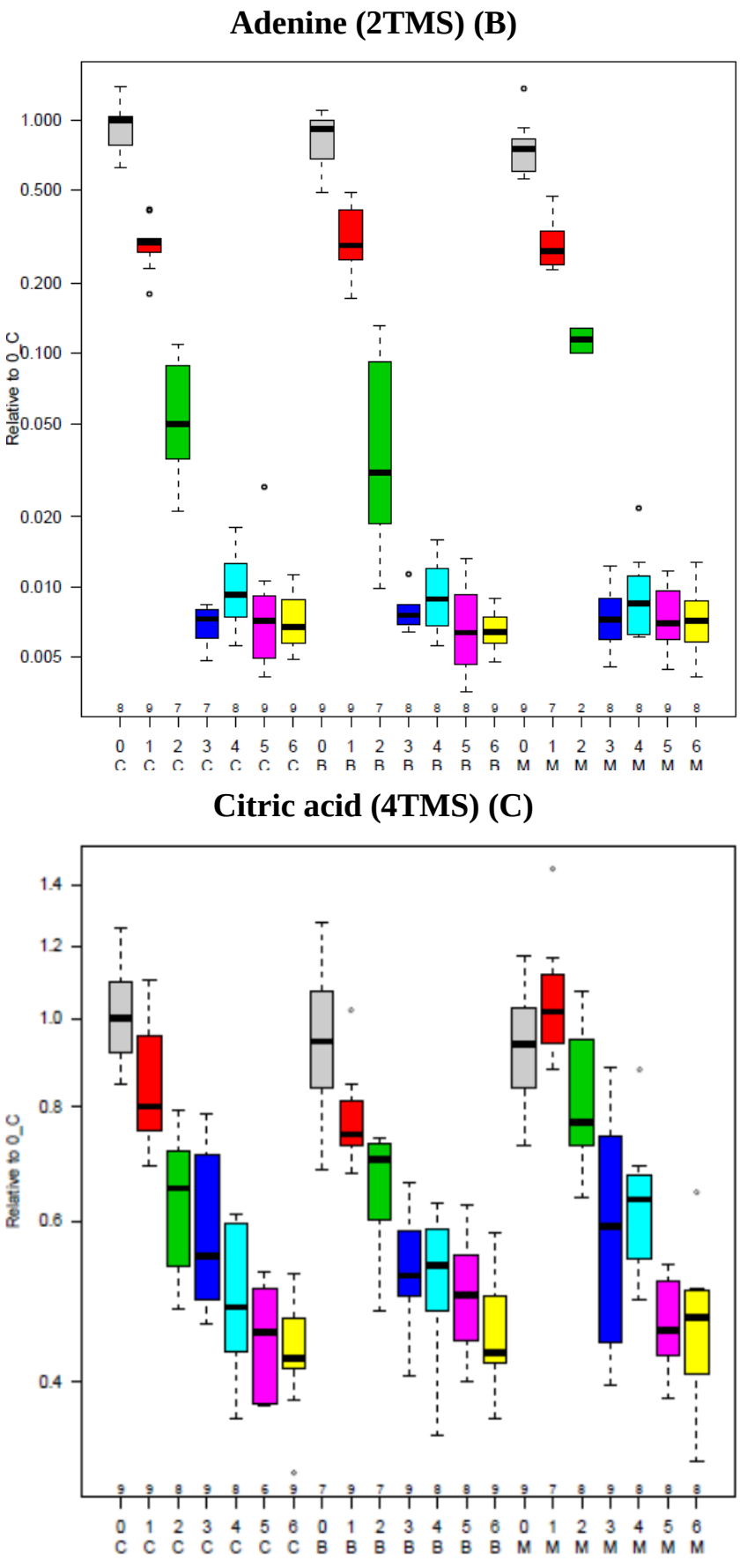


Figure 25: Effect of methionine supplementation on the glutamine, adenine and citrate concentrations during storage for up to 42 days.

In our targeted metabolomics data, the concentrations of 56 metabolites increased, while the concentrations of 12 metabolites decreased in RBC supernatants stored in PAGGS-M, PAGGS-MB and PAGGS-MM additives throughout the storage period. The levels of 5 metabolites remained relatively stable. The increased metabolites were basically amino acids, including threonine, tyrosine, cysteine, histidine and some neutral amino acids such as isoleucine, valine and phenylalanine. The elevation of these amino acids in RBC supernatants during RBCs storage has been also reported by other author [202].

Table 3. The list of statistical differentially changed metabolites in GC/APCI-MS based targeted metabolites from the supernatants of packed RBCs stored in PAGGS-M, PAGGS-MB and PAGGS-MM additive solutions during 6 weeks of storage.(RT: retention time, m/z: mass to charge ratio, RI: retention index, TP: time point)

Name	RT	m/z	RI	Formula	Treatment	TP	Treatment:TP
Pyruvic acid (1MEOX) (1TMS)	338	190.0898	215350	C7H15NO3Si	4.26e-01	1.31e-39	8.43e-01
Lactic acid (2TMS)	345	235.119	215807	C9H22O3Si2	5.05e-02	4.29e-09	5.08e-01
Alanine (2TMS)	373	234.135	243457	C9H23NO2Si2	6.96e-01	1.50e-79	6.91e-02
Butanoic acid 3-hydroxy- (2TMS)	410	249.1345	278195	C10H24O3Si2	2.38e-01	1.73e-01	6.18e-01
Isocaproic acid, 2-oxo- (1MEOX) (1TMS)	443	232.1364		C10H21NO3Si	1.66e-03	1.10e-31	9.68e-01
Valine (2TMS)	447	262.1659	312469	C11H27NO2Si2	2.39e-01	9.77e-20	6.67e-03
Leucine (2TMS)	481	276.1814	345292	C12H29NO2Si2	6.68e-01	3.40e-15	5.13e-02
Glycerol (3TMS)	481	309.1736	344018	C12H32O3Si3	8.57e-01	4.92e-67	3.69e-04
Phosphoric acid (3TMS)	482	315.1031	344280	C9H27O4PSi3	1.93e-01	6.67e-02	3.04e-01
Isoleucine (2TMS)	493	276.1814	358082	C12H29NO2Si2	1.53e-01	1.00e-10	2.94e-03
putative_Isobutanoic acid 2-amino	494	248.1495		C1H1	1.20e-01	7.53e-04	1.04e-01
Proline (2TMS)	497	260.1501	363219	C11H25NO2Si2	3.80e-01	1.58e-20	8.31e-03
Succinic acid (2TMS)	501	263.1129	371353	C10H22O4Si2	2.75e-01	5.68e-05	1.38e-02
Glycine (3TMS)	502	292.1584	366771	C11H29NO2Si3	4.90e-01	4.67e-107	5.14e-03
Glyceric acid (3TMS)	511	323.1525	377286	C12H30O4Si3	1.72e-02	4.24e-53	4.07e-01
Uracil (2TMS)	518	257.114	391877	C10H20N2O2Si2	3.64e-01	1.65e-27	3.95e-03
Fumaric acid (2TMS)	518	261.0974	385733	C10H20O4Si2	2.42e-09	3.18e-130	2.12e-02
Serine (3TMS)	530	322.1688	395486	C12H31NO3Si3	1.71e-01	5.83e-68	1.45e-01
Threonine (3TMS)	545	336.1847	408356	C13H33NO3Si3	9.61e-02	3.79e-40	1.51e-02
Glutaric acid (2TMS)	547	187.0784		C11H24O4Si2	2.72e-02	2.12e-85	4.48e-02
Alanine beta- (3TMS)	565	306.1739	433728	C12H31NO2Si3	1.90e-01	3.05e-04	4.39e-02

putative_Aminomalonic acid (2TMS)	588	336.1479		C12H29NO4Si3	2.75e-03	4.70e-101	1.50e-01
Nicotinamide (2TMS)	592	267.1343	460591	C12H22N2OSi2	5.23e-02	6.52e-71	1.17e-01
Malic acid (3TMS)	595	351.1474	463265	C13H30O5Si3	3.47e-02	2.71e-129	1.51e-01
Threitol (4TMS)	607	411.2229	466669	C16H42O4Si4	8.24e-01	1.18e-75	3.53e-01
Aspartic acid (3TMS)	611	350.1636	479623	C13H31NO4Si3	4.17e-69	9.31e-93	4.86e-07
Methionine (2TMS)	612	294.1374	482140	C11H27NO2SSi2	5.86e-98	4.23e-18	4.18e-11
Proline, 4-hydroxy-, cis- (3TMS)	614	348.1841	486124	C14H33NO3Si3	7.77e-01	1.33e-46	2.17e-01
Pyroglutamic acid (2TMS)	615	274.1291	485228	C11H23NO3Si2	1.68e-01	4.33e-116	1.73e-02
Butanoic acid, 4-amino- (3TMS)	617	320.1891	486470	C13H33NO2Si3	5.30e-01	2.06e-103	9.18e-04
Threonic acid (4TMS)	623	425.203	496443	C16H40O5Si4	7.27e-01	3.20e-20	2.64e-01
Cinnamic acid, trans- (1TMS)	628	221.099	501853	C12H16O2Si	3.42e-03	1.43e-113	2.21e-03
Cysteine (3TMS)	630	338.1453	479326	C12H31NO2SSi3	1.88e-02	1.42e-124	1.27e-03
Creatinine (3TMS)	633	330.1842	500044	C13H31N3OSi3	6.02e-02	7.15e-43	1.64e-01
Glutaric acid, 2-hydroxy- (3TMS)	636	365.163	507359	C14H32O5Si3	1.79e-01	1.37e-94	2.16e-01
Glutaric acid, 2-oxo- (1MEOX) (2TMS) MP	637	320.1341	509032	C12H25NO5Si2	8.60e-03	1.18e-58	1.46e-02
Phosphoethanolamine (3TMS)	639	358.145		C11H33NO4PSi3	5.69e-01	7.98e-01	5.92e-02
Hypotaurine (3TMS)	653	326.1456	524574	C11H31NO2SSi3	8.08e-02	6.77e-62	3.92e-01
Glutamic acid (3TMS)	658	364.1789	528501	C14H33NO4Si3	1.08e-04	3.09e-102	5.53e-01
Cytosine (3TMS)	663	328.1689	534833	C13H29N3OSi3	6.95e-02	2.02e-06	7.19e-01
Phenylalanine (2TMS)	665	310.1652	535904	C15H27NO2Si2	9.75e-01	3.07e-29	6.30e-01
Asparagine (4TMS) BP1	665	421.2182	518770	C16H40N2O3Si4	4.16e-01	1.93e-52	2.66e-01
Glutamic acid, N-methyl-	680	378.1946	560609	C15H35NO4Si3	4.85e-05	3.26e-20	6.16e-01
Ribose (1MEOX) (4TMS) MP	689	468.245	553613	C18H45NO5Si4	4.96e-01	4.22e-46	6.50e-01
Xylitol (5TMS)	701	513.2734	566179	C20H52O5Si5	4.29e-01	1.25e-33	1.17e-01
Glycerol-2-phosphate (4TMS)	708	461.1791	575215	C15H41O6PSi4	4.07e-03	4.30e-60	4.11e-01
Glutamine (4TMS)	711	435.2338	578393	C17H42N2O3Si4	8.59e-05	1.51e-18	4.22e-02
Dihydroxyacetone phosphate (1MEOX) (3TMS)	713	416.1499	585344	C13H34NO6PSi3	2.53e-02	1.18e-08	5.61e-01

Putrescine (4TMS)	714	377.2649	584887	C16H44N2Si4	6.00e-01	1.61e-31	6.52e-01
Glycerol-3-phosphate (4TMS)	724	461.1784	592350	C15H41O6PSi4	1.81e-02	1.51e-55	7.68e-01
Glyceric acid-3-phosphate (4TMS)	743	475.1576	611575	C15H39O7PSi4	5.75e-20	6.04e-09	1.72e-01
Ornithine (4TMS)	745	421.255	618272	C17H44N2O2Si4	4.69e-01	1.38e-36	6.51e-01
Citric acid (4TMS)	748	481.1916	618059	C18H40O7Si4	1.74e-03	9.61e-42	2.66e-02
Adenine (2TMS)	768	280.1402	646729	C11H21N5Si2	6.23e-01	7.27e-101	3.35e-01
Fructose (1MEOX) (5TMS) MP	771	570.294	638185	C22H55NO6Si5	2.91e-05	3.03e-16	4.87e-01
Pyridoxine (3TMS)	778	386.1998	654238	C17H35NO3Si3	2.68e-01	8.61e-03	4.84e-02
Lysine (4TMS)	787	435.2709	662689	C18H46N2O2Si4	5.48e-01	1.95e-52	5.51e-01
Histidine (3TMS)	789	372.1953	663331	C15H33N3O2Si3	4.75e-01	7.30e-06	9.25e-01
Glucose (1MEOX) (5TMS) BP	793	570.2938	657191	C22H55NO6Si5	9.61e-01	4.09e-42	1.39e-01
Tyrosine (3TMS)	796	398.1992	670927	C18H35NO3Si3	4.94e-01	3.34e-25	2.94e-01
Pantothenic acid, D- (4TMS)	801	508.2753	669058	C21H49NO5Si4	1.90e-01	6.54e-26	2.94e-01
Uric acid (4TMS)	856	457.1934	732958	C17H36N4O3Si4	4.35e-01	1.89e-52	8.56e-02
Inositol, myo- (6TMS)	860	613.307	727476	C24H60O6Si6	3.58e-02	9.90e-25	3.80e-01
Ribose-5-phosphate (1 MEOX) (5TMS) MP	862	620.2506	732012	C21H54NO8PSi5	5.34e-03	8.54e-22	4.89e-03
Octadecanoic acid (1TMS)	897	357.3182	788107	C21H44O2Si	8.55e-01	2.63e-05	4.04e-01
Gluconic acid-6-phosphate	899	603.2241	802192	C21H51O8PSi5	9.27e-03	1.01e-05	8.10e-01
Tryptophan (3TMS)	902	421.2149	779768	C20H36N2O2Si3	2.19e-01	1.96e-17	1.77e-01
Cystine (4TMS)	924	529.1892	803353	C6H12N2O4S2	8.94e-01	3.94e-75	2.45e-01
Fructose-1-phosphate (1MEOX) (6TMS) MP	934	722.3007	800471	C25H64NO9PSi6	3.56e-20	1.26e-26	7.75e-01
Fructose-6-phosphate (1MEOX) (6TMS) MP	939	722.3007	803558	C25H64NO9PSi6	3.56e-20	1.26e-26	7.75e-01
Glucose-6-phosphate (1MEOX) (6TMS) MP	946	722.3006	808382	C25H64NO9PSi6	1.47e-17	1.46e-28	4.43e-01
Inosine (4TMS)	1012	557.2454	897451	C22H44N4O5Si4	3.80e-01	2.01e-37	1.77e-01
Adenosine, alpha- (4TMS) MP	1033	556.2617	929134	C22H45N5O4Si4	1.09e-03	4.89e-70	2.69e-01

5. Discussion

RBC transfusion remains the most common therapeutic intervention, performed in hospitals. According the current protocol, RBCs can be stored at 4 ± 2 °C for up to 42 days [65]. However, refrigerated storage of RBC units is associated with several biochemical and morphological changes, so-called storage lesions[66]. These alterations affect RBC energy metabolism, the antioxidant defense system, membrane lipid asymmetry and deformability, hemoglobin solubility and oxygen-carrying capacity, thereby accelerating RBC senescence and resulting in eryptosis [38, 72, 76, 95, 100]. From a molecular point of view, storage lesions within the RBCs or storage media are not fully understood and require more investigations. The study conducted by Sparrow et al. showed that RBC storage lesions are influenced by the additive solution, in which RBC concentrates are stored [203]. In the past decade, several additives have been developed. They improved RBC storage, but could not minimize storage-associated changes. Therefore, improvement of additive manufacturing is an important issue for better RBC storage.

In a previous study, the reduction of methionine concentration along with accumulation of homocysteine during RBC storage have been already found [105]. On the basis of this fact, we tried to develop the new additive solutions. We prepared two different additive solutions: PAGGS-MM (PAGGS-M supplemented with L-methionine) and PAGGS-MB (PAGGS-M supplemented with B12 + 5-Methyltetrahydrofolic acid disodium salt) and investigated their effects on stored RBCs over a storage period of 42 days in comparison with well-known PAGGS-M.

The elevation in homocysteine level was reported to be caused either by decrease in folate, B12 or B6 concentrations, or the decreased activity of Methylene tetrahydrofolate reductase (MTHFR) [204]. This enzyme catalyzes the conversion of 5, 10-methylene-THF to 5-methyl-THF, which is the methyl group donor required for the vitamin B-12-dependent conversion of homocysteine to methionine. The latter reaction is catalyzed by methionine synthase [205]. In our study, we used B12 + 5-Methyltetrahydrofolic acid disodium salt, but the homocysteine level was not reduced in RBCs stored in PAGGS-MB compared with PAGGS-M-stored RBCs. This fact may indicate that methionine synthase, but not MTHFR is inactivated in stored RBCs.

Moreover, supplementation of B12 + 5-Methyltetrahydrofolic acid disodium salt did not show any effect on the parameters of RBCs stored in PAGGS-MB additive solution compared with PAGGS-M-stored RBCs, except for the RBC count, Hb concentration and HCT. These parameters were significantly higher in PAGGS-MB-stored RBCs compared with RBCs stored in other additives. This phenomenon may be explained by the different evaporation rate of packed RBCs in the test conditions. Nevertheless, no negative effects were observed on the RBCs stored in PAGGS-MB compared with PAGGS-M- and PAGGS-MM-stored RBCs.

Unlike B12 +5-Methyltetrahydrofolic acid disodium, supplementation of L-methionine showed several morphological and metabolic changes in RBCs stored in PAGGS-MM additive solution in comparison with PAGGS-M-stored RBCs. In particular, significantly higher MCV was found in packed RBCs stored in PAGGS-MM additive. In 2008, Zehnder et al. showed statistically higher MCV caused by erythrocyte swelling in RBC units stored in SAGM additive solution that may be clinically irrelevant as it is a reversible event upon transfusion [206]. Moreover, Flynn et al. reported that the impact of RBC swelling was probably related to the storage lesions and could be a consequence of the cation leak observed from first day of RBC storage. This leakage caused not only redistribution of monovalent cations but also an overall uptake of base and water, which swelled out the cells [207]. Although, all stored RBCs leak cations, regardless of the storage medium, affect MCV in different ways in comparison to RBCs stored in SAGM [206]. On the other hand, loss of RBC membrane caused by the blebbing of microvesicles results in reduced MCV and shape changes of stored RBCs culminating in spherocytosis [82, 208]. This is an irreversible process and causes the hemolysis of RBCs in the presence of osmotic stress. The MCV level of RBCs during storage depends on the osmolality of additive solutions. Hypertonic additive solution such as SAG-M promoted the increase in MCV level of stored RBCs [198]. In our study, we observed similar leakage of the cations between RBC units stored in three different additive solutions. This may indicate that statistically higher MCV of RBCs stored in PAGGS-MM is the result not only of cell swelling, but also of decreased vesiculation of cell membrane. This process in PAGGS-MM stored RBCs can be confirmed by the lowest hemolysis rate. Here we showed that RBCs stored in PAGGS-MM had significantly lower hemolysis rate compared with RBCs stored in two different additives. This means that RBCs stored in PAGGS-MM better maintained their membrane integrity compared with RBCs stored in PAGGS-M or PAGGS-MB additives. In any case, MCV of packed RBCs stored in PAGGS-MM was

maintained at an acceptable level throughout the storage period (85.511 ± 0.953 fL on day 1, 88.889 ± 0.938 fL on day 42).

Extracellular calcium chelation with citrate during RBCs storage is a well-known phenomenon. Calcium reacts with citrate present in RBC additive solutions that reduces its extracellular level [209, 210]. Ionized calcium readily binds to negatively charged sites of protein molecules. Calcium ions compete with hydrogen ions for the same binding sides of albumin and other calcium-binding proteins. The calcium-binding capacity is a pH dependent event. It is reduced by acidosis and results in increased free calcium levels, whereas alkalosis promotes the binding of calcium to protein and reduces free calcium levels [211]. In our study, we found an elevation of extracellular calcium concentration in all additives tested throughout the storage period. This may be a consequence of a decrease in pH level in all RBC units during storage. The reduction in pH, found in our study, is consistent with other reports [65, 212]. Low pH has been reported to reduce *in vitro* activity of hexokinase and phosphofructokinase [30, 105] and to inhibit bisphosphoglycerate mutase, which is responsible for the synthesis of 2,3 DPG [38]. Our results demonstrated a gradual reduction in the extracellular glucose level along with accumulation of lactate in all tested RBC units. However, the consumption of glucose was also reduced throughout the storage time. This fact can be explained by the inhibition of the main glycolysis enzymes: hexokinase and phosphofructokinase due to the lactate accumulation. The reduction in pH affected the 2,3-DPG level, as it sharply decreased in all RBC units after 14 days of storage. This may be related to the inhibition of bisphosphoglycerate mutase. Nevertheless, none of the experimental additives had a harmful effect on glycolysis, or on the synthesis of 2,3 DPG, compared with PAGGS-M. Furthermore, glycolysis is the main source of ATP production in stored RBCs. On the other hand, the enzymatic functions of hexokinase and phosphofructokinase are ATP-dependent [30, 105]. This means that there is a mutual negative interplay between reduction in ATP synthesis and the inhibition of hexokinase and phosphofructokinase. The continual decrease in ATP production in PAGGS-M additive solution have already been shown by Gulliksson et al. [213]. In our study, ATP concentration decreased in all tested RBCs units without any significant differences between the RBCs stored in three different additives. The ATP level gradually decreased in all RBC units during the first two weeks of storage, following an increase in the third week and then decreased again. ATP in stored RBCs is produced either by glycolysis or by utilization of extracellular adenine present in additive solution. We did not observed any difference in glucose

consumption in the third week of RBCs storage compared with other weeks. This may indicate an increased utilization of adenine in all RBCs units in the third week of storage.

Another parameter affected by glycolysis blockage is the NADH / NAD^+ balance. NADH generated via glycolysis, is used by cytochrome b5 reductase (methemoglobin reductase) for reduction of ferric ion in methemoglobin to the ferrous form [43, 214]. RBC contains cytosolic isoforms of NAD-dependent malate dehydrogenase and fumarate hydratase. They use malate and fumarate as substrates [215]. Their activations promote the generation of NADH, which is used by methemoglobin reductase [1]. Time-course investigation of RBCs stored in CPD-SAGM additive showed that the NAD^+ level increased until the 7^{th} day of storage, followed by a permanent decrease [103]. Our results demonstrated the same changes in NAD^+ level as those reported above. The NADH level also gradually decreased throughout the storage period. In our metabolomics data, we observed a permanent elevation of malate and fumarate concentrations in the supernatant of all tested samples. Moreover, their concentrations were significantly higher in the supernatant of packed RBCs, supplemented with L- Methionine. This means that none of these metabolites was utilized by NAD-dependent malate dehydrogenase and fumarate hydratase in RBCs units stored in PAGGS-M, PAGGS-MM, PAGGS-MB additives resulting in a reduction of the NADH level during storage.

Similar to NADH/NAD^+, NADPH/$NADP^+$ molecules belong to the antioxidant defense system in RBCs. Pentose phosphate pathway generates NADPH, which is used for the reduction of oxidized glutathione [105, 216]. Furthermore, storage-dependent oxidation of glyceraldehyde-3-phosphate dehydrogenase at functional residues diverts metabolic flux from glycolysis to the PPP in order to produce NADPH and to restore glutathione homeostasis [217]. Stored RBCs respond to the aggravation of oxidative stress with the over-activation of the PPP, which drives the formation of ribose-5 phosphate from G6P [104]. RBCs contains high concentration of oxygen and facilitates oxidation-induced pathological changes in RBC. The aerobic storage of RBCs is associated with increased oxidation of membrane proteins [78, 218], whereas, anaerobic storage of RBCs reduced oxidative stress and improved quality of stored RBCs and PTR [70, 219]. The oxidative phase-associated metabolites of the PPP in RBCs were found to decrease under altitude hypoxia [220]. A metabolomics study, conducted Pallotta et al. showed that supplementation of vitamin C and N-acetylcysteine significantly reduced the elevation of the PPP-related metabolites that improved the quality of stored RBCs

[43]. All findings mentioned above confirm a strong correlation between oxidative stress and elevations of PPP intermediates. In our metabolomics analysis, we found that PPP was significantly reduced by supplementation of L-methionine, as its six metabolites (gluconic acid-6-phosphate, glucose-6-phosphate, fructose-6-phosphate, glyceric acid-3-phosphate, ribose-5-phosphate and glyceric acid) were significantly decreased compared with RBCs stored in two different additive solutions. This indicates that supplementation of L-methionine reduced oxidative stress in RBCs stored in PAGGS-MM additive solution. In addition, the hemolysis rate of RBCs is directly proportional to oxidative stress [221]. As previously noted by other authors, free Hb plays an important role in inflammatory process in blood. Extracellular Hb readily reacts with superoxide and hydrogen peroxide (H_2O_2) [84]. This causes the breakdown of hemoglobin and results in formation of heme and free iron, which can have pro-oxidant and inflammatory effects [222, 223]. Extracellular Hb, whether it originates from RBCs storage, or from transfusion due to hemolysis, is a major source of oxidative stress. The harmful effect of heme on various cell types has already been reported [224, 225]. In RBCs, heme affects membrane proteins resulting in cell aging [224]. Exogenous heme also activates neutrophils and promotes the upregulation of ROS. This induces neutrophil extracellular traps (NET) [225]. Moreover, heme and free iron are involved in the pathogenesis of atherosclerosis due to lipid oxidation [226]. Hemoglobin and heme *in vivo* are removed by haptoglobin and hemopexin, respectively [227]. This mechanism does not exist *in vitro* and stored RBCs are readily affected by oxidative stress. In our study, we observed a gradual elevation of the extracellular hemoglobin level in RBCs stored in PAGGS-M, PAGGS-MB and PAGGS-MM additive solutions. However, PAGGS-MM-stored RBCs had the significantly lower concentration of extracellular hemoglobin compared with RBCs stored in two different additives. This finding can be associated with reduction of oxidative stress and decreased activation of PPP in PAGGS-MM-stored RBCs, or inversely, decreased oxidative stress and PPP respectively, prevented RBC hemolysis in PAGGS-MM additive solution. The antioxidant property of protein methionine residues have been reported by other authors. The surface-exposed methionine residues in Escherichia coli serve as endogenous antioxidants and protect the proteins and macromolecules from oxidation [181, 228]. In the presence of oxidative stress, methionine undergoes oxidation and forms methionine sulfoxide and methionine sulfone, which have less anti-inflammatory effect compared to methionine [229]. None of the oxidized forms of methionine was detected in our metabolomics data. Instead, we found 2-Hydroxy-4-(methylthio) butyric acid (HMBA). It is

an intermediate of the methionine transamination pathway. HMBA was 100-fold higher in PAGGS-MM stored RBCs compared with other additives.

Although supplementation of L-methionine reduced the activity of PPP, we could not detect any significant differences in NADPH or total glutathione concentrations between RBCs stored in three different additives. Seneviratne et al. showed that administration of exogenous L-methionine in male Sprague-Dawley rats significantly improved the activities of antioxidant enzymes such as glutathione peroxidase and superoxide dismutase, but did not change the total glutathione level [230]. Cysteine with glutamate and glycine are involved in glutathione synthesis, which requires ATP-dependent enzymes such as glutamate cysteine ligase (GCL) and GSH synthetase [52]. Storage-associated accumulation of glutathione precursors in stored RBCs has been reported by D'Alessandro et al. [1]. Consistent with this study, we found that cysteine, glutamate and glycine concentrations permanently increased in all additives tested in the time course of storage. Moreover, cysteine and glutamine concentrations were significantly higher, while glutamate (glutamic acid) level was significantly lower in PAGGS-MM stored RBCs compared to RBCs stored PAGGS-M and PAGGS-MB additives. The RBC membrane is practically impermeable to one of the glutathione precursor glutamate [231]. Therefore, it is synthetized intracellularly from glutamine or α-ketoglutarate, which readily inflow across the RBC membrane [232, 233]. Glutamate is produced through the hydrolysis of glutamine by glutamine aminohydrolase or through the conversion of α-ketoglutarate by aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the presence of aspartate and alanine, respectively [234]. The latter two reactions are readily reversible as the serum normally contains the substrates for AST and ALT reactions [235]. Up to 90% of glutamate pool, which used for glutathione synthesis is derived from alanine aminotransferase [234]. This suggest that intracellular glutamate concentration may increase at the expense of extracellular glutamine, aspartate and alanine. On the other hand, glutamine consumption during RBCs may tend to *de novo* glutathione biosynthesis [236]. In our study, the concentrations of alanine and aspartate permanently increased in all samples during storage. Similar to glutamate concentration, aspartate level was significantly lower in PAGGS-MM stored RBCs compared with RBCs stored in other additives. This indicates that glutamate may be produced from aspartate and α-ketoglutarate by aspartate aminotransferase, or conversely. Statistically higher concentration of extracellular glutamine in PAGGS-MM, suggests either lower consumption, or higher production of extracellular glutamine. In addition, Glutamine can be synthetized from

glutamate in the presence of ammonia in brain [237]. Oxidative deamination of L-methionine results in formation of ammonia and α-Keto-γ-(methylthio) butyric acid (KMBA) by bacterial and snake venom l-amino acid oxidase [238]. KMBA is an intermediate of the methionine transamination pathway and leads to the formation of HMBA, which can be converted to KMBA in a reversible reaction in lactococci [239]. In our study, a significant elevation of HMBA was detected in RBC units supplemented with L-methionine. These findings indicate that a significantly high level of extracellular glutamine in PAGGS-MM stored RBCs may be synthetized from extracellular glutamate and ammonia produced by oxidative deamination of L-methionine. It is also possible, that supplementation of L-methionine inhibited glutaminolysis. This is series of biochemical reactions, which was previously described in RBCs stored in AS3 for up to 42 days [202]. Glutathione synthesis has been reported to be an ATP-dependent event [240]. ATP depletion at the beginning of storage is one of the major factors responsible for a decrease in glutathione synthesis. Thus, the presence of glutathione precursors is not sufficient for glutathione synthesis.

Cysteine is a sulfur-containing amino acid obtained by the methionine transsulfuration pathway. A significant increase in the cysteine level was observed in the neonates fed with enterally or parenterally methionine [241]. The first step of transsulfuration is regulated by the enzyme cystathionine β-synthase to form cystathionine after the condensation of homocysteine and serine, while the second step begins with the cleavage of cystathionine by cystathione γ-lyase to produce cysteine and α-ketoglutarate [242]. The elevation of the cysteine and homocysteine levels in stored RBCs have already been found by other authors [1, 105]. Our metabolomics data showed a gradual increase in the cysteine and homocysteine concentrations in all RBC units tested throughout the storage period. However, their levels were significantly higher in RBC units stored in PAGGS-MM additive solution; this is evidence for a methionine transsulfuration pathway. Interestingly, a small amount of L-methionine was catabolized through the transsulfuration pathway. Therefore, the cysteine and homocysteine levels in our results do not correlate with methionine concentration. The large amount of supplemented L-methionine was utilized by the transamination pathway. This may indicate that a small amount of L-methionine can produce a sufficient amount of methyl donor (SAM) for the metabolism of RBCs throughout the storage period or further transamination of L-methionine protected stored RBCs from an excess amount of homocysteine. L-methionine metabolism via the transamination pathway was believed to be a

protective mechanism to excess homocysteine in patients with homocystinuria caused by cystathionine beta-synthase deficiency [243].

Methionine transmethylation and transsulfuration pathways during RBC storage have already been described [1, 105]. We are the first to report methionine transamination pathway in stored RBCs. Methionine transamination was discovered 30 years ago [244]. This alternate pathway of methionine metabolism was first proposed by Benevenga [245, 246]. Methionine in physiological concentration was found to undergo transamination. But, the levels of its metabolites is very low or difficult to detect, as this amino acid is catabolized mainly via transsulfuration pathway *in vivo* [247]. However, *In vitro* methionine is mostly degraded through the transamination pathway [248]. Methionine transamination in humans with hepatic methionine adenosyltransferase deficiency resulted in formation of several products, such as KMBA, 3-methylthiopropionate, methanethiol and its metabolites [247]. Furthermore, methionine is the substrate for various transaminases, which are divided into two groups: the first family contains glutamic acid or 2-oxoglutaric acid transaminases, whereas glutamine and asparagine transaminases belongs to the second group [249]. Studies on isolated rat hepatocytes have already shown that glutamine:2-oxoacid amino transferase is a major enzymes for methionine transamination and for the formation of L-methionine from its 2-oxoacid analogue, KMBA, which requires the amino group of glutamine [250]. Most of the tissues contain glutamine at higher concentration than methionine [251]. The formation of HMBA from KMBA has already reported [239]. In our study, packed RBCs were supplemented with 25-fold higher L-methionine compared with its normal plasma concentration. This amount of methionine contributed to the activation of the transamination pathway. Interestingly, the methionine level decreased only in the first week of storage in PAGGS-MM-stored RBCs. Afterwards, it gradually increased throughout the storage and plateaued at its initial level. This suggests that *de novo* synthesis of methionine from glutamine probably occurs in stored RBCs. Methionine transamination has been reported to be associated with formation of toxic metabolites such as methanethiol, dimethylsulfide, methanethiol-mixed disulfides. However these metabolites are generated from 3-methylthiopropionic acid which is produced from the metabolism of KMBA in mitochondria [244]. The RBC is devoid of mitochondria, and methionine transamination in our study may be arrested at KMBA level in PAGGS-MM-stored RBCs. The administration of DL-HMBA in chicks resulted in an increase of broiler growth and mitigated high temperatures-induced oxidative stress through the production of reduced glutathione and activation of non-

enzymatic antioxidant defense mechanism [252]. Moreover, glutathione precursor cysteine is formed more efficiently from DL-HMBA than from DL-methionine in chicken fed with DL-HMBA and DL-methionine [253]. Martin-Venegas et al. found that HMBA better neutralizes H_2O_2 than DL-methionine and protects the epithelial barrier function of intestine from oxidative stress [254]. According to our metabolomics data, the formation of HMBA significantly increased throughout the storage period, but we did not find any beneficial effect on the production of the reduced form of glutathione level in RBCs stored in PAGGS-MM additive solution. This can be attributed to ATP reduction during RBC storage. HMBA may serve as an antioxidant in our study and resulted in low hemolysis rate and decreased PPP in RBCs stored in PAGGS-MM additive solution.

The changes in malate levels in supernatant of stored RBCs have been previously observed [1]. RBC has no functional tricarboxylic acid (TCA) cycle due to the absence of mitochondria, but accumulation of malate in stored RBCs has been already reported [202, 236]. D'Alessandro et al. supposed that malate accumulation may be caused either by platelet and leukocyte contamination, or by activation of NADP-dependent malate dehydrogenase and fumarate dehydrogenase to generate NADH and NADPH respectively, [1]. Later, the same research group showed that malate formation was not fully dependent on glucose consumption. On the basis of this findings, they postulated the existence of a new unexplored pathway in RBCs [255]. Moreover, Malate can be produced from citrate by the enzyme malate dehydrogenase I. 90% of malate in RBCs is produced from citrate. Citrate metabolism results in formation of lactate and pyruvate. These substrates are further metabolized by TCA cycle enzymes to produce malate [255]. RBC contains TCA cycle enzymes such as isocitrate dehydrogenase I and malate dehydrogenase I. They can produce NADH and NADPH and restore redox balance [255]. In our study, we observed a gradual elevation of the malate and fumarate concentrations, while the citrate concentration decreased. Moreover, the malate and fumarate concentrations were significantly higher in PAGGS-MM-stored RBCs compared with RBCs stored in PAGGS-M and PAGGS-MB. However, their elevation had no effect on the production of NADH and NADPH in PAGGS-MM-stored RBCs. This suggests that the activities of isocitrate dehydrogenase I and malate dehydrogenase I were reduced throughout the storage period. We also found that supplementation of L-methionine significantly slowed citrate catabolism. Malate accumulation did not correlate with the consumption of citrate in RBCs stored in PAGGS-MM additives. Citrate catabolism was significantly lower, while malate accumulation was significantly higher. This fact can be explained either by a high

glycolysis rate in PAGGS-MM stored RBCs, which we did not observe throughout the storage period, or by the new unexplored pathway mentioned above.

In fact that a donates frequently must be taken into account in blood collection, as this blood contains more immature red blood cells such as reticulocytes. They have mitochondria and result in TCA activation with formation of malate. The RBC units for our study were also collected from frequent donors.

Adenine is the extracellular source for ATP synthesis. The addition of adenine to stored RBCs maintained the adenine nucleotides level and contributed to the formation of high-energy phosphate [256]. The utilization of adenine is strongly related with storage period, but not with its concentration in RBCs during storage. Adenine in packed RBCs is not fully utilized. RBCs consume adenine till the 18^{th} day of storage but, only the first phase (0-8 days) is beneficial for RBCs, when adenine is partially converted into inosine and inosine monophosphate (IMP) [257]. Adenine metabolism results in formation of hypoxanthine[256], which is associated with generation of reactive oxygen species and with damage of stored RBCs [258]. In our study, we observed a gradual consumption of adenine in all RBCs tested throughout the storage. However, in the second week of storage the consumption of adenine was relatively slower in RBCs stored in PAGGS-MM.

In summary, 1) the supplementation of L-Methionine has significantly reduced the rate of hemolysis at the beginning of RBC storage, but not during the storage. Though, relatively low hemolysis rate was maintained in PAGGS-MM stored RBCs throughout the storage period compared with other additives. Hemolysis determines quality of blood products and plays an important role for patenting new additives [65]. 2) The activity of PPP, which is a marker of oxidative stress was significantly reduced in packed RBCs supplemented with L-methionine. 3) The highest mean corpuscular volume of RBCs stored in PAGGS-MM additive solution can be explained by lower vesiculation of RBC membrane. 4) Metabolic fate of the supplemented methionine in PAGGS-MM stored RBC was mostly shifted towards the transamination pathway. Further investigations, especially *in vivo* experiments are needed in order to estimate the effect of L-methionine on stored RBCs.

6. Abbreviations

ACD	Acid citrate dextrose
ADP	Adenosine diphosphate
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AS-1	Additive solution 1
AS-2	Additive solution 2
AS-3	Additive solution 3
BHCO3-	Bicarbonate Ions
Cl-	Chlorine ions
CPD	Citrate-phosphate-dextrose
2, 3-DPG	2, 3-diphosphoglycerate
EAS	Experimental additive solution
EAS 76v6	Experimental additive solution 76 variant 6
E-Sol 5	Erythro-Sol 5,
FDA	Food and Drug Administration
GC-APCI-MS	Gas Chromatography atmospheric pressure chemical ionization- Mass spectrometry
G6P	Glucose 6-phosphate
G6PD	Glucose-6-phosphate dehydrogenase
GSH	Reduced form of glutathione
GSSG	Oxidized form of glutathione
Hb	Hemoglobin
HCT	Hematocrit
HMBA	2-Hydroxy-4-(methylthio)butyric acid
KMBA	α-Keto-γ-(methylthio)butyric acid
MCV	Mean corpuscular volume
5'-MTHF	5'-methyltetrahydrofolate
MTHFR	Methylene tetrahydrofolate reductase
NADH	Reduced form of nicotinamide adenine dinucleotide

NADP+	Oxidized form of nicotinamide adenine dinucleotide phosphate
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
PAGGG-M	Phosphate–adenine–guanosine–glucose–gluconate–mannitol
PAGGS-M	Phosphate–adenine–guanosine–glucose–saline–mannitol
PAGGS-MB	PAGGS-M supplemented with Vitamin B12 + 5' methyltetrahydrofolate
PAGGS-MM	PAGGS-M supplemented with L-methionine
PC	Phosphatidylcholine
PCA	Principal Component Analysis
PE	Phosphatidylethanolamine
PPP	Pentose phosphate pathway
PS	Phosphatidylserine
PTR	Post-transfusion Recovery
RAS2	Red-cell Additive Solution 2
RBC	Red blood cell
RES	Reticuloendothelial system
RFU	Relative Fluorescence Units
ROS	Reactive oxygen species
SAG	Saline-adenine-Glucose
SAGM	Saline-Adenine-Glucose-Mannitol
SAM	S-adenosylmethionine
TCA	Tricarboxylic acid
Total GSH	Total glutathione
UK	United Kingdom
U.S.	United States

7. References

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8. Eidesstattliche Versicherung

„Ich, Maia Dzamashvili, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: “ Effect of L-methionine on RBCs metabolism during storage” selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum 09.06.2017 Unterschrift

Anteilserklärung an etwaigen erfolgten Publikationen

Maia Dzamashvili hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: **Dzamashvili M**, Bal G, Schmieder P, Jaeger C, Lisec J, Ringel F, Salama A. Identification of metabolic changes and reaction network in packed RBCs stored in PAGGS-M, PAGGS-MM and PAGGS-MB additive solutions during storage for 7 weeks, in preparation

M. Dzamashvili has performed practical part of the research. She measured the parameters of stored RBCs and prepared the samples for metabolomics analysis. She took part in the analysis of the data using SSPS statistics and in the writing of the manuscript. M. Dzamashvili was also involved in the analysis of targeted metabolomics with the members of the group.

Publikation 2: Bal, G; Depré F, **Dzamashvili M**, Ringel F, Salama A. Effect of thrombopoietin receptor agonists on leucocyte and haematopoietic stem and progenitor cell in peripheral blood of patients with immune thrombocytopenia, submitted to Annals of Hematology

M. Dzamashvili selected the ITP patients for the study. She collected the peripheral blood from them and separated the peripheral blood mononuclear cells. She used the colony forming cell (CFC) assay for detection of progenitor cells and counted the colony forming units (CFU) and bursa forming units (BFU) under the microscope.

Unterschrift, Datum und Stempel des betreuenden Hochschullehrers/der betreuenden Hochschullehrerin

Unterschrift des Doktoranden/der Doktorandin

9. Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

10. List of publications

1: **Dzamashvili M**, Bal G, Schmieder P, Jaeger C, Lisec J, Ringel F, Salama A. Identification of metabolic changes and reaction network in packed RBCs stored in PAGGS-M, PAGGS-MM and PAGGS-MB additive solutions during storage for 7weeks, in preparation

2: Bal, G; Depré F, **Dzamashvili M,** Ringel F, Salama A. Effect of thrombopoietin receptor agonists on leucocyte and haematopoietic stem and progenitor cell in peripheral blood of patients with immune thrombocytopenia, submitted to Annals of Hematology,

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